The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

The impact of very high gravity fermentation conditions on brewing yeast health and physiology

Chris Alexander BSc.

Thesis submitted to the University of Nottingham for the degree of

Doctor of Philosophy

July 2021

Abstract

As modern industrial efforts shift towards more economic and sustainable practices, the brewing industry is no different. Namely, the application of high gravity (HG) and very high gravity (VHG) brewing practices provide the means to increase brewery efficiency and reduce energy input. However, the conditions experienced throughout VHG fermentations exert an increased degree of stress on the fermenting yeast population, resulting in poor ethanol yields, impaired yeast quality and unbalanced beer flavour. The aim of this research was to investigate the influence of stress factors experienced by yeast during fermentation on yeast physiology and key quality indicators.

The impact of osmotic stress, a key stress factor in relation to VHG practices, was highlighted to cause unwanted changes to yeast vacuolar and mitochondrial physiology, as well as plasma membrane damage and cell death, contributing to reduced fermentation performance. The alleviation of osmotic stress was found to be possible through the application of a sugar top-up regime to a VHG fermentation, preventing exposure of yeast to extreme osmolarity upon pitching. This optimisation procedure yielded positive results in terms of improving yeast quality and was confirmed to reduce the occurrence of characteristically stressed organelle morphologies and decrease stress exertion. This research not only provides a further understanding of the yeast physiological response to stress, but offers brewers a viable method to improve fermentation efficiency and harness the potential of VHG brewing.

List of abbreviations

bp	Base pairs
^{o}C	Degrees centigrade
g	Grams
nm	Nanometre
mm	Millimetre
w/v	Weight/volume
v/v	Volume/volume
µL	Microlitre
mL	Millilitre
L	Litre
^{o}P	Degrees Plato
Rpm	Revolutions per minute
µM	Micromolar
mM	Millimolar
ppb	Parts per billion
ppm	Parts per million
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
BOX	Bis-oxonol
PI	Propidium iodide
R123	Rhodamine 123
HOG	High osmolarity glycerol
RDF	Relative degree of fermentation
HTseq	High throughput sequence analysis

Acknowledgements

Firstly, I would like to thanks Dr Chris Powell for his guidance and support through the entirety of this project, for which I am truly grateful.

Thank you to my industrial partner AB InBev for sponsorship of the project and the invaluable input from members of the Yeast & Fermentation team that contributed to the impact of this work. Also to Jack Cavanagh, Sue Takaira and Taylor Carral for the help and provision of materials from the RPB.

To the departments with whom I collaborated with, thank you for your technical advice. Particular thanks to Robert Markus, Tim Self, David Onion, Nicola Croxall, Johnny Debebe, Victoria Wright and all of the members of SLIM, the Flow Cytometry facility & Deepseq.

Thank you to the technical team in BABS for always being at hand to help. A huge thanks to Emily Fong, Khatija Nawaz, Mel Stuart, Dave Greening and Sue Clegg. Thank you also to Prof. David Quain for the guidance, support, and most importantly the discussions about music in the lab.

Thank you to all of the members of BABS for not only being great colleagues, but also thank you for your friendship and support throughout my time at Nottingham. A huge thank you to Eoin Moynihan, James Mallett, Marit Nijman, Neil Doherty, Celina Dugulin, Marcus Awoniyi, Joanna Yorke, Saskia Hofmann, Stephanie Brindley, Toby Pendlebury, Alex Jevons and everyone else that made the whole experience so rewarding.

A massive thank you to my parents and my Nan for always being there for support and perspective, without which I wouldn't have made it to this point. A big thank you also to my friends at home in Northern Ireland for the craic and support throughout.

Last but certainly not least, the biggest thank you to Imogen Ramsey. Thank you for making this entire journey so enjoyable, for being there through all the highs and lows and for helping me every step of the way. I truly could not have done this without you. P.s. you're welcome for all the sandwiches!

Table of Contents

CHAPTER 1: INTRODUCTION

1.1 Applications of Yeast

1.1.1 Yeast and biotechnology

Harnessing the fermentative capability of yeast represents one of the oldest applications of biotechnology, and the first example of domestication of a microbial agent (Steensels et al., 2019). Arguably the most important product of yeast metabolism is ethanol, which has applications and associations within the food and beverage, and biofuel industries (Boulton and Quain, 2001; Mohd Azhar et al., 2017). However, yeast are able to produce additional high-value end products and can be found utilised within the cosmetic and agriculture industries for the production of carboxymethylglucan (Kanlayavattanakul and Lourith, 2009), mannosylerythritol (Morita et al., 2013) and fertilizers (Orts et al., 2008; Mukherjee et al., 2020). Furthermore, the use of 'non-conventional' yeasts (belonging to species other than the commonly used *Saccharomyces cerevisiae; Section 1.3.1*) have traditionally been used to produce bio-products such as riboflavin and astaxanthin, which are common health supplements (Roya et al., 2013; Lin et al., 2017) as well as a range of other proteins with biomedical functions (Cregg and Higgins, 1995). Related to this, with the advancement of molecular genetics, it has become possible to alter the nature of yeast-derived compounds in order to produce useful metabolites or specific by-products at higher yields than could normally be obtained without genetic or metabolic engineering (Nielsen, 2013). This application is of particular interest to the pharmaceutical industry, with yeast being used as cell-factories

to produce a range of biopharmaceutical proteins including insulin (Danielsen, 1992; Kjeldsen, 2000).

In order for biotechnological processes to be viable, the organism being employed must meet certain requirements to fulfil the needs of the procedure. These include the ability of the organism to produce not only the desired product (either naturally or through genetic modification) but that product in sufficient yields (Nielsen, 2013). As a biological agent, yeast are well suited for such applications due to their high substrate uptake capability and because they are relatively robust when subjected to environmental extremes (Attfield, 1997; Mattanovich et al., 2014). For example, *S. cerevisiae* yeast strains have been employed for the production of lactic acid, due to their ability to withstand low pH (Dequin and Barre, 1994; Pacheco et al., 2012).

1.1.2 Yeast as a model organism

As a model eukaryotic organism, the study of yeast has been of great importance in the fields of genetics, cell physiology and understanding the metabolome. In 1996, yeast became the first eukaryotic organism to be fully sequenced and have its annotated genome fully published online (Goffeau et al., 1996). This work was undertaken through collaboration between multiple research groups across the globe and was integral to understanding gene function in higher organisms such as humans, due to analysis of genetic homologues shared between human and yeast cells (Kachroo et al., 2015; Liu et al., 2017). Although humans and yeast may appear to differ vastly in complexity as organisms, key similarities in cellular structure and function have

allowed for the study of yeast to elucidate the role of specific genes and proteins in processes such as cell proliferation, cell death, oncogene occurrence and cell aging (Toda et al., 1986; Botstein et al., 1997; Denoth Lippuner et al., 2014). Following the disclosure of the full yeast genome, the understanding of gene functions began to increase exponentially, due to collaborative input by over 1000 researcher groups involved in characterizing the physiological traits of knock-out gene mutants (Goffeau, 2000).

The importance of yeast in the discovery of cellular processes is such that several Nobel Prizes in the field of Physiology or Medicine have been awarded to researchers for their work with yeast (Hohmann, 2016). Most recently, the Nobel Prize was awarded to Yoshinori Ohsumi in 2016 for research conducted into autophagy mechanisms in yeast; the mechanism by which living cells degrade intracellular components such as proteins, structures and organelles (Thumm, 2000; Farré et al., 2009; Zimmermann et al., 2016). The work of Paul Nurse in understanding the cell cycle (Nurse et al., 1976; Hohmann, 2016; Nurse, 2017) has also been recognised and continues to be a valuable tool to understand processes such as cellular ageing and cancer generation in human cells.

The benefits of using yeast as a model eukaryote lie in the simplicity of the organism and its genetic tractability. Laboratory strains of baker's yeast are easily manipulated whether in haploid or diploid form (Section 1.3.1), meaning that genetic modifications can be efficiently applied in order to study the physiological implications of gene mutations (Mohammadi et al., 2015).

Furthermore, these effects can be rapidly assessed due to the increased cellular growth rate of yeast when compared to mammalian cell lines (Botstein and Fink, 2011). Finally, the ethical implications of using higher eukaryotes (such as mice) in mutation and disease studies must also be considered, a hurdle that is not present in yeast-based studies.

1.1.3 Yeast in food and beverages

The use of yeast in the production of food and beverages long predates the discovery of the organism. Utilised for its ability to ferment sugars and impart positive sensory properties, evidence that yeast has been used for production of beer-type beverages dates back as far as ancient Egypt (Malek, 2001; Farag et al., 2019), although it should be noted many different historic eras have claimed to be the first. Despite some dispute over precise origins of products, it is broadly accepted that the involvement of yeast in the manufacture of food and drink was not understood, as changes occurring to a substance were often believed to be supernatural (Lodolo et al., 2008). With the invention of the microscope came an understanding of the presence and impact of yeast and other microbes, however it was not until the 1850's that the role of yeast in alcoholic fermentation was demonstrated by French chemist Louis Pasteur (Barnett, 2000). This finally provided an answer to the long-standing question of where the characteristics of fermented products were derived.

In parallel to the discovery of the role of yeast in fermented beverages, it became apparent that the same biological agent was also involved in bread-making (Boulton and Quain, 2001). Since this time, the use of yeast has evolved

and it was quickly realised that a huge variety of end-products could be created. Indeed, waste or 'spent' yeast can even be used in the preparation of supplements. For example, yeast extract, a common ingredient in food preparation is produced from dead yeast cells and can be added to enhance flavours or be consumed directly by itself. Thus, yeast has played and continues to perform an important role in the day-to-day lives of humans for the production of both food and beverages.

The relationship between food and yeast is not always positive; yeasts and moulds are often associated with food spoilage. During the spoilage of food and beverages, yeast utilise available substrates resulting in the production of metabolites which can be perceived by the consumer as 'off flavours' (van der Aa Kühle and Jespersen, 1998; Fleet, 2007; Fleet, 2011). In addition, the growth of yeast and the production of insoluble particles can lead to undesirable physical properties such as beer haze (Quain, 2015; Mallett et al., 2018). In some instances, however, final products can display some resistance to spoilage from microbes (including yeast and bacteria), due to the nature of the yeast-derived metabolites. For example, the high ethanol concentrations and low pH in beer resulting from yeast fermentation can restrict the growth of many other microorganisms (Boulton and Quain, 2001).

1.2 The Brewing Process

1.2.1 Evolution of the brewing process

As a worldwide industry, a diverse portfolio of beer styles exists in the modern marketplace (Papazian, 2006). The creation of these products typically involves the use of a range of raw materials and different brewing techniques that give each beer its unique characteristics (Briggs et al., 2004). Although brewing technology has evolved significantly since its conception, the general principles remain the same and a broadly similar procedure is applied irrespective of the final product type. The brewing process involves the liberation of sugars from grains to create a nutritious medium known as 'wort'. The subsequent fermentation of sugars, combined with the utilisation of other wort components by yeast, creates ethanol and a range of flavour compounds desirable in the final product (Briggs et al., 2004). A summary of the brewing process can be found in Figure 1.1.

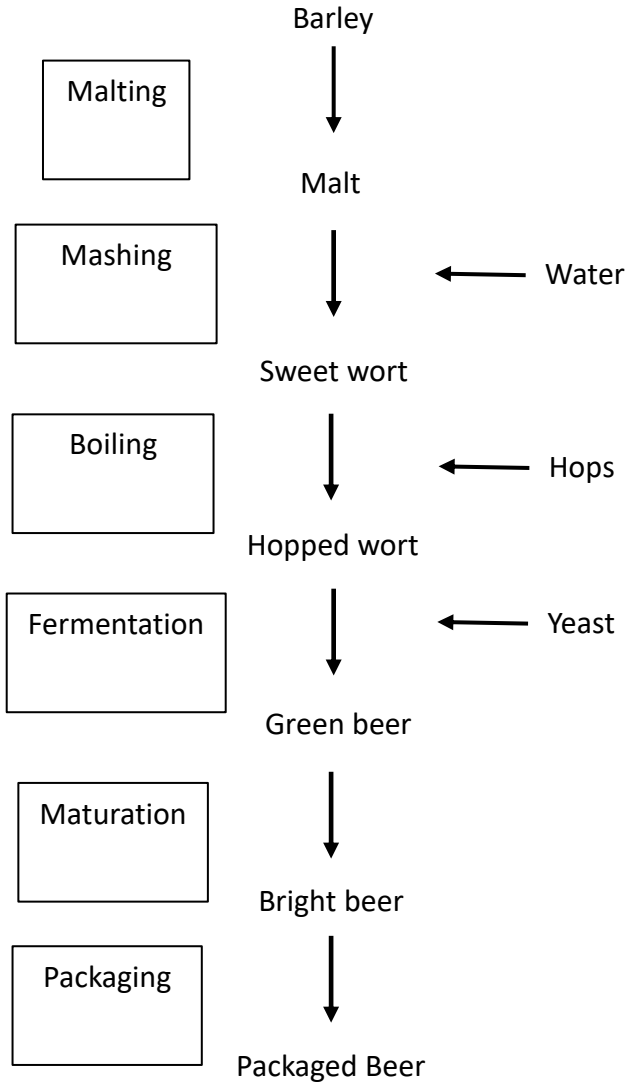


Figure 1.1. The brewing process.

Typically barley grains and hops are used to produce wort, an aqueous solution which is fermented by yeast to produce beer. This can sometimes undergo a period of maturation, before being packaged and moved to the point of sale/consumption. The fermentable sugars in wort can be derived from a range of different cereal grains, which are often used in conjunction with one another. Generally, malted barley is the most common cereal used for brewing

and is found in the greatest proportion (Briggs et al., 2004). However beer recipes can include the use of other grains, especially if they can be sourced more cheaply based on geographical location or availability (Lloyd, 1986; Ogbonna, 1992). The use of brewing adjuncts (additional or supplementary sugar sources) is also common, and includes materials derived from sources such as rice, wheat, corn or rye. In some instance these are used to impart specific properties to the final product, for example to enhance or reduce flavour and colour, or to impact other qualities such as foam/head retention and altered sensory properties (Poreda et al., 2014; Yorke et al., 2021). In addition, the use of adjuncts can also be used to 'intensify' the brewing process leading to a more cost-efficient process (Section 1.4.1). However, on occasion the use of excessive amounts of adjunct can also lead to issues during and post fermentation (Section 1.4.2) (Pollock and Weir, 1973).

During the brewing process, wort can also be supplemented with additional ingredients to enhance or provide specific characteristics. One major example is the addition of hops. Although historically ale-style beers were originally produced without hops, these beers typically had a very short shelf life (Briggs et al., 2004). The anti-microbial and preservative qualities of hop constituents were found to produce a beer less susceptible to spoilage, as well as imparting desirable bitterness and aroma attributes (Sakamoto and Konings, 2003; Schönberger and Kostelecky, 2011), leading to their increase in popularity and almost universal use at the current time. Correspondingly, modern brewing practices employ a vast array of different hopping techniques, and different

varieties of hops are used in variable ratios based on their individual characteristics and the desired beer style.

1.2.2 Wort production: Malting

The brewing process begins when barley (or other cereal) grains are steeped in water, allowing the grain to germinate under controlled conditions. During this stage, cellular metabolism is activated and the synthesis of hydrolytic enzymes occurs (Briggs et al., 2004). These are ultimately responsible for the degradation of macromolecules leading to germination of the embryo. Germination is halted by kilning, in which the malt is heated, dried and cured. This allows for optimisation of the product and the stage is characterised by the accumulation of hydrolytic enzymes, as well as modification of the endosperm, while also preventing starch and protein utilisation by the embryo (Bamforth, 2006). At this stage, the level of heat treatment that the malt is subjected to during kilning is determined by the desired beer type, as this can influence the colour of the final product and effect flavour compound degradation as a result of Maillard reaction products (Coghe et al., 2006) during roasting at high temperatures.

1.2.3 Wort production: Milling and Mashing

Following the malting process, malt is subjected to milling and mashing (Briggs et al., 2004), processes that are often grouped together as they are both involved in the release and enzymatic digestion of the starch housed within the malt (Bamforth, 2006). The malted barley is first milled, by which the grains are crushed to release the starch. This is done to increase the contact surface

area of the malt; the finer the particles are, the more fermentable sugars and nitrogen compounds are released (Mousia et al., 2004; Kok et al., 2019). However, this must be tightly regulated since the size of the particles produced during milling directly influence the rate of wort separation. Milling is followed by mashing, in which the crushed grains are mixed with hot water. This allows the enzymatic digestion of starch into sugars, providing the nutritional source for fermentation by the yeast later in the process (Boulton and Quain, 2001). The non-soluble components are removed from the mixture, leaving soluble sugars, lipids and proteins, as well as other flavour compounds, forming a substance known as 'sweet wort' (MacWilliam, 1968).

The sweet wort is boiled to sterilize the mixture and simultaneously hops are added for flavouring of the wort (Skinner, 1927; Lewis and Young, 2001). The boiling step has the additional effect of removing unwanted volatiles and allowing precipitation of wort protein components (Willaert and Baron, 2001). The solid particles, including hop and grain residues, lipids, and the precipitated proteins are then removed via filtration or through the use of a whirlpool (Briggs et al., 2004). Finally, the wort is cooled and aerated in preparation for the addition of yeast (Ohno and Takahashi, 1986).

1.2.4 Fermentation

Yeast is inoculated, or 'pitched' into the wort to initiate the fermentation process, usually in the form of a wet slurry, although active dried yeast (ADY) may be preferred by smaller brewing companies. Since the fermentation stage is subject to an array of variables, the process parameters can differ according

to the type of yeast used and the individual product (Boulton and Quain, 2001). For example, if the desired product is an ale or lager beer, the appropriate type of yeast (correspondingly an 'ale' or 'lager' strain) must be employed (see Section 1.3.1). Similarly, parameters such as fermentation temperature must be adjusted to reflect the nature of the yeast strain. Additionally, process parameters such as pitching rate, wort gravity, nutrient additions and wort dissolved oxygen can also vary based on the optimisation of individual practices (Casey et al., 1983; Ohno and Takahashi, 1986; Bafrncová et al., 1999; Erten et al., 2007).

Once the yeast is introduced to the wort, it begins to utilise the sugars present to produce energy in the form of ATP, and as a source of carbon for anabolic pathways (D'Amore et al., 1989). During the initial stages of fermentation, a period of yeast reproduction occurs which is necessary to generate biomass and ensure activity for an effective fermentation (Gilliland, 1962). Brewing yeast cells divide via an asexual mitotic process known as budding, whereby an individual cell develops a daughter cell which grows out of the mother before being released (Herskowitz, 1988; Duina et al., 2014) to facilitate yeast growth, adequate levels of wort oxygen must be provided to support lipid synthesis during cell proliferation. As a result, dissolved oxygen levels in wort have a direct influence on fermentation progression through their impact on cell division (David and Kirsop, 1973; Ohno and Takahashi, 1986). Yeast growth is largely limited to the initial stages of fermentation; as wort oxygen becomes depleted the equilibrium of carbon assimilation shifts to the production of ethanol rather than the formation of biomass (Boulton and Quain, 2001).

The major products of fermentation are ethanol and carbon dioxide, which result from the breakdown of wort sugars during glycolysis (Boulton and Quain, 2001) as outlined in Section 1.3.3., esters, higher alcohols and other secondary metabolites are also produced by yeast during fermentation, contributing to the organoleptic properties of the end-product (Gilliland and Harrison, 1966; Stewart, 2017). Depending on the type of yeast and the desired product specifications, the raw materials used may vary, which consequently effects the synthesis of flavour compounds. Furthermore, the fermentation variables can be also adjusted, especially related to the type of yeast used: 'top fermenting' ale yeasts are usually employed at a higher temperature (16-22^{o}C) than 'bottom fermenting' lager yeasts (12-16^{o}C) (Boulton and Quain, 2001).

In modern beer production, fermentations of all types are typically conducted in stainless steel cylindroconical vessels. This shape of vessel has a range of advantages, although the main driver behind their utilisation is to aid the collection of yeast that settles at the cone of the fermentation vessel (FV). This stems from a practice unique to the brewing industry, whereby yeast biomass from a fermentation is collected and reused in subsequent fermentations in a process known as 'serial repitching'. Yeast can theoretically be recycled as many times as the brewer requires, although as the number of 'repitchings' increases, so does the risk of contamination or of variation within the yeast population, potentially leading to inconsistent fermentations (Smart and Whisker, 1996; Powell and Diacetis, 2007).

1.2.5 Post-fermentation processing

The endpoint of fermentation is typically determined when no further reduction in gravity (representative of fermentable sugar content) is achieved. However, due to the production of diacetyl by yeast during the fermentation process (Section 1.3.4), a 'warm maturation' period is often needed to reduce the concentration of this compound to acceptable levels in beer. This process is halted by separation, or 'cropping' of the yeast, aided by yeast flocculation (Section 1.3.1). The product at this point is referred to as 'green beer', and in the case of lager production is subsequently transferred to a conditioning vessel maintained at cold temperatures. The 'cold conditioning' process allows for further clarification and maturation of beer flavour. Fining agents such as isinglass can be added at this stage in order to enhance the removal of other particulates and solid matter from the green beer (Briggs et al., 2004).

In modern brewing processes, beer is often fermented to a higher ABV than the desired end-product, through the use of high-gravity brewing (Section 1.4.1). As such, the fermentation product must be diluted to achieve a beer with the desired ethanol concentration (Hackstaff, 1978; Puligundla et al., 2011). Once the beer has been processed according to the desired specifications, it is then packaged, typically into bottles, cans or kegs (Briggs et al., 2004).

1.3 Brewing Yeast

1.3.1 Genetics, taxonomy and species/strain identification

Brewing yeasts are single-celled microscopic fungi that can be broadly described as being oval or spherical in shape. The key components of the yeast cell are identical to all other eukaryotic organisms, with some variations related to key cell structures (for example the presence of chitin in the cell wall) and the organism's genetic make-up. Broadly speaking, the yeast genome is organised into 16 chromosomes; the percentage of genes contributing to different categories of cellular activity can be found in Table 1.1. It should be emphasised that much of the basic yeast genome data was generated using 'laboratory' yeast strains, which tend to be haploid and differ from polyploid/aneuploid industrial strains (Gallone et al., 2018). Ploidy can result in significant phenotypic differences between laboratory and industrial strains, with the latter being more robust, more efficient at fermentation, and with more consistent qualities when used in an industrial environment (Boulton and Quain, 2001).

Table 1.1. Functional gene assignment of yeast genome. Adapted from Briggs et al. (2004).

Gene function	Proportion of identified genome (%)
Cellular organisation and biogenesis	28
Intracellular transport	5
Transport facilitation	5
Protein trafficking	7
Protein synthesis	5
Transcription	10
Cellular growth (division and DNA synthesis)	14
Energy transduction	3
Cellular metabolism	17
Cell rescue	4
Signal transduction	2

The majority of brewing yeasts belong to the genus *Saccharomyces*, a word derived from Latin and meaning 'sugar-fungus' (Boulton and Quain, 2001). Traditionally, *Saccharomyces cerevisiae* was the species of choice for brewing, described as a budding yeast with the capability to convert sugars into ethanol (Boulton and Quain, 2001). These yeasts were also historically referred to as 'top fermenting', due to their tendency to rise to the top of the vessel towards the end of fermentation (Dengis et al., 1995). Currently, *S. cerevisiae* strains are exclusively used in the production of ale-type beers, prompting the generic term 'ale yeast'. However, with the evolution and the diversification of beer types, alternative species of yeast are increasingly employed, including non-*Saccharomyces* yeast strains for the production of 'ale-type' products. These

beers represent a small market share when compared to more traditional beer styles, although the application of such yeasts has increased in recent years (Callejo et al., 2019).

With the popularisation of lager-style beers, the landscape of the brewing industry shifted towards the use of a compatible yeast species for lager production. Yeast strains with desirable properties for lager production are not found in nature (Turakainen et al., 1994), but are believed to be hybrid organisms derived from *S. cerevisiae* and *S. eubayanus* (Libkind et al., 2011) strains, presumably originating within an industrial location. Lager yeasts have undergone several name changes based on the evolving understanding of their genetic origins but are currently referred to as *S. pastorianus* (Barnett, 1992; Baker et al., 2015). These strains, contrary to ale yeasts, have a tendency to sink to the bottom of the fermentation vessel, and are therefore classified as 'bottom fermenting'. This characteristic is owed to the relatively higher hydrophobicity of lager yeast cell walls (Smit et al., 1992; Dengis et al., 1995). Ale and lager yeast can be distinguished by a number of different behavioural, metabolic and genetic characteristics and as such can be characterised using a variety of different experimental techniques. By analysing a strain's metabolic capabilities with regards to carbohydrate sources, it is possible to distinguish between lager yeast based on its ability to grow on melibiose as a sole carbon source (Tubb and Liljestrom, 1986; Box et al., 2012) as detailed further in Section 3.2.1.

Modern lager-brewing yeasts can be divided into two distinct lineages, dubbed Saaz/Group I and Frohberg/Group II yeasts, assumed to be dependent on their original geographical origins (Dunn and Sherlock, 2008). Although both yeast types are believed to be interspecies hybrids of *S. cerevisiae* and *S. eubayanus*, they differ in their physiology and fermentation properties. Possibly the difference that is most relevant to brewing is the inability of Saaz type yeasts to utilise maltotriose, which contributes 16-26% of the fermentable sugars in wort (Boulton and Quain, 2001; Gibson et al., 2013). Saaz yeast are also better-adapted to colder growth and fermentation temperatures than Frohberg strains (Gibson et al., 2013).

The universal method for identifying a yeast species is by analysis of the ITS (internal transcribed spacer) region of the genome (Schoch et al., 2012). This can be achieved using PCR-RFLP in conjunction with specific primers and restriction enzymes, and brewing used can be identified as either an ale/type II lager or type I lager (Pham et al., 2011). This method provides a more sophisticated approach to strain identification than growth/metabolic analysis. However, a combined approach is needed due to limitations of differentiation, for example ITS analysis alone cannot distinguish between S*. cerevisiae* ale and *S. pastorianus* Frohberg yeasts.

Individual yeast strains can be differentiated using genome sequencing or a variety of PCR-based methods. One method which is widely applied is through amplification of interdelta regions of the genome, giving rise to a unique genetic fingerprint by which yeast strains can be identified (Ness et al., 1993; Legras and Karst, 2003). This method is recommended for analysis of brewing

strains by the American Society of Brewing Chemists (Van Zandycke et al., 2008) and can also be used to evaluate the presence of mutations within a yeast strain genome, which can occur when yeast are exposed to environmental stresses and when a single yeast population is utilised for long periods of time (Powell and Diacetis, 2007) .

1.3.2 Yeast cell ultrastructure

As a eukaryotic organism, yeast cells contain membrane-bound organelles that carry out the functions necessary for the metabolism, homeostasis and functioning of the host cell (Cavalier-Smith, 1975; Vellai and Vida, 1999). The outermost layer of the yeast cell is responsible for providing structure and rigidity to the cell and is known as the cell wall (Lipke and Ovalle, 1998). The yeast cell wall is mainly comprised of β1,6-glucan, β1,6-glucan, mannoprotein and chitin which form an insoluble fibrous network (Klis et al., 2002). The cell wall is usually around 150-200nm thick and accounts for roughly 30% of cellular dry weight depending on growth conditions (Lipke and Ovalle, 1998).

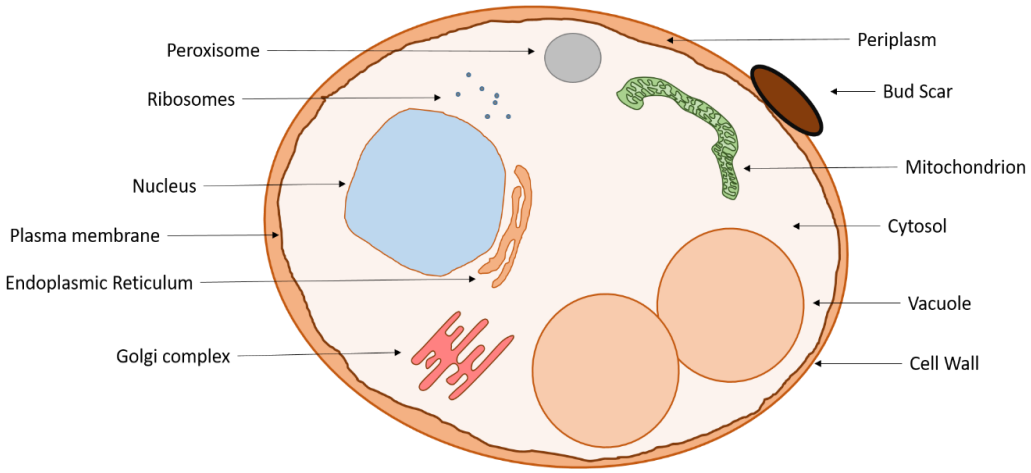


Figure 1.2. Ultrastructure of a yeast cell. A typical cell exhibits an ellipsoidal shape measuring 5-10 µm in diameter under normal conditions.

The yeast plasma membrane is a phospholipid bilayer interspersed with a range of membrane proteins and other lipids acting as the main barrier between the inside of the cell and the surrounding environment (Singer and Nicolson, 1972; van der Rest et al., 1995). One of the major roles of the plasma membrane is to control the movement of substances into and out of the cell (van der Rest et al., 1995). This function is carried out by a number of different mechanisms, depending on the type of molecule being transported and/or their quantity. The lipid constituents of the plasma membrane are mainly phospholipids, namely phosphatodyinositol, phosphatidyserine, phosphatidylcholine and phosphatidylethanolamine (van der Rest et al., 1995). Smaller quantities of sterols are also present, responsible for regulating the fluidity of the plasma membrane (Alexandre et al., 1994; Harris et al., 2002; Cournia et al., 2007). The

most abundant sterol is ergosterol, which plays a similar role to cholesterol in mammalian cells (Thomas et al., 1978; Cournia et al., 2007).

The yeast cytosol, or cytoplasm, is the portion of the cell enclosed by the plasma membrane and is responsible for the housing and support of cellular organelles and sub-cellular cytosolic components (Boulton and Quain, 2001; Feldmann, 2012). This is the primary site of fermentative metabolism and contains the enzymes responsible for glycolysis and fermentation (Moseley and Goode, 2006; Malakar et al., 2020). Other cytosolic bodies include peroxisomes, lipid particles and ribosomes (Osumi, 1998). Glycolytic metabolism and transport of cellular components are facilitated by the cytoskeleton, a structural network of actin polymers which also serves to maintain cellular shape and co-ordinate cell division (Moseley and Goode, 2006).

The nucleus is responsible for housing the genomic DNA, necessary for cellular proliferation and reproduction, within a porous nuclear envelope. The nuclear envelope facilitates the trafficking of small molecules and genetic material between the cytoplasm and the nucleus (Hurt et al., 1992; Boulton and Quain, 2001; Taddei and Gasser, 2012). The endoplasmic reticulum (ER) is closely associated with the nuclear envelope and consists of a network of interconnected tubules responsible for protein folding and translocation while also playing a role in cell lipid synthesis (Austriaco, 2012). The ER forms part of an intracellular membrane system involved in endocytic and secretory pathways (Riezman, 1985). Proteins are trafficked from the ER to the Golgi complex, another organelle that consists of a network of membrane structures

(Wooding and Pelham, 1998; West et al., 2011; Flis and Daum, 2013). Within the Golgi complex, proteins are sorted and trafficked into vesicles where they are either transported within the cell (for example, to the vacuole for storage) or exocytosed (Wooding and Pelham, 1998; Suda and Nakano, 2012).

The yeast vacuole is frequently referred to as a storage organelle in eukaryotes (Klionsky et al., 1990), but in yeast it adopts a number of additional roles including cell homeostasis (pH, water and ion regulation) (Diakov et al., 2013), proteolysis (Hecht et al., 2014), macroautophagy (Thumm, 2000) and cellular detoxification (Klionsky et al., 1990; Li and Kane, 2009). The yeast single-lipid vacuole membrane, or tonoplast, has a distinct lipid and protein composition differentiating it from the plasma membrane, with α-mannosidase typically used as a determinant marker of the vacuolar membrane (Klionsky et al., 1990). This membrane surrounds an acidic vacuolar lumen, responsible for housing the enzymes responsible for the degradation of cellular components (Thumm, 2000; Li and Kane, 2009). The structure and morphology of the yeast vacuole is highly dynamic and can adopt different conformations in response to environmental conditions and cellular growth phases (Vida and Emr, 1995; Zieger and Mayer, 2012).

The yeast mitochondria is comprised of two lipid membrane structures: the outer and the inner mitochondrial membrane. The former defines the mitochondria itself, while the latter surrounds the internal mitochondrial matrix and comprises a series of membrane invaginations known as cristae (Boulton and Quain, 2001; Feldmann, 2012; Kühlbrandt, 2015). Mitochondrial membranes contain cardiolipin, a phospholipid exclusive to mitochondria in

yeast which plays a major role in mitochondrial functionality including respiration, protein translocation and cell aging (Joshi et al., 2009; Miyata et al., 2017). The mitochondrial matrix encompasses ATP synthases and cytochromes and this is the main site of oxidative phosphorylation and ATP synthesis via the Krebs cycle and the electron transport chain during aerobic respiration (O'Connor-Cox et al., 1996; Foury et al., 1998). However, due to the Crabtree effect, this functionality is repressed in brewing yeast (Van Urk et al., 1990). Consequently, the role of mitochondria during fermentation is not fully understood. It is known that cells deficient in mitochondrial activity, termed petites, function poorly (Ernandes et al., 1993). This is believed to be due to the role of the mitochondria in nuclear DNA signalling (Rinaldi et al., 2010) lipid/sterol production (Feldmann, 2012) and flavour development through the Ehrlich pathway (Hazelwood et al., 2008), diacetyl reduction (Ryan and Kohlhaw, 1974; Krogerus and Gibson, 2013) and sulphur metabolism (Blank et al., 2009). Ultimately, the presence of petite mutants can cause slow and ineffective fermentations with poor final product quality and flavour imbalance (Josey et al., 2019). While the mitochondria are frequently referred to as discrete oblong structures, it is known that the yeast mitochondrion adopts different morphologies depending on the physiological state of the yeast (Berman et al., 2008; Gomes and Scorrano, 2013; Knorre et al., 2013a), this is further discussed in Section 1.5.3.

1.3.3 Yeast fermentative metabolism

The central dogma of fermentation revolves around the breakdown of wort sugars to ethanol and carbon dioxide. Brewer's wort provides yeast with all of

the nutrients necessary to sustain yeast growth and fermentative metabolism, the by-product of which is the conversion of wort to beer (Boulton and Quain, 2001). Thus, the fermentation process involves the assimilation and utilisation of wort constituents and the associated pathways resulting not only in the production of ethanol, but the production of flavour and aroma compounds contributing to the final beer product (Gilliland and Harrison, 1966; Boulton and Quain, 2001).

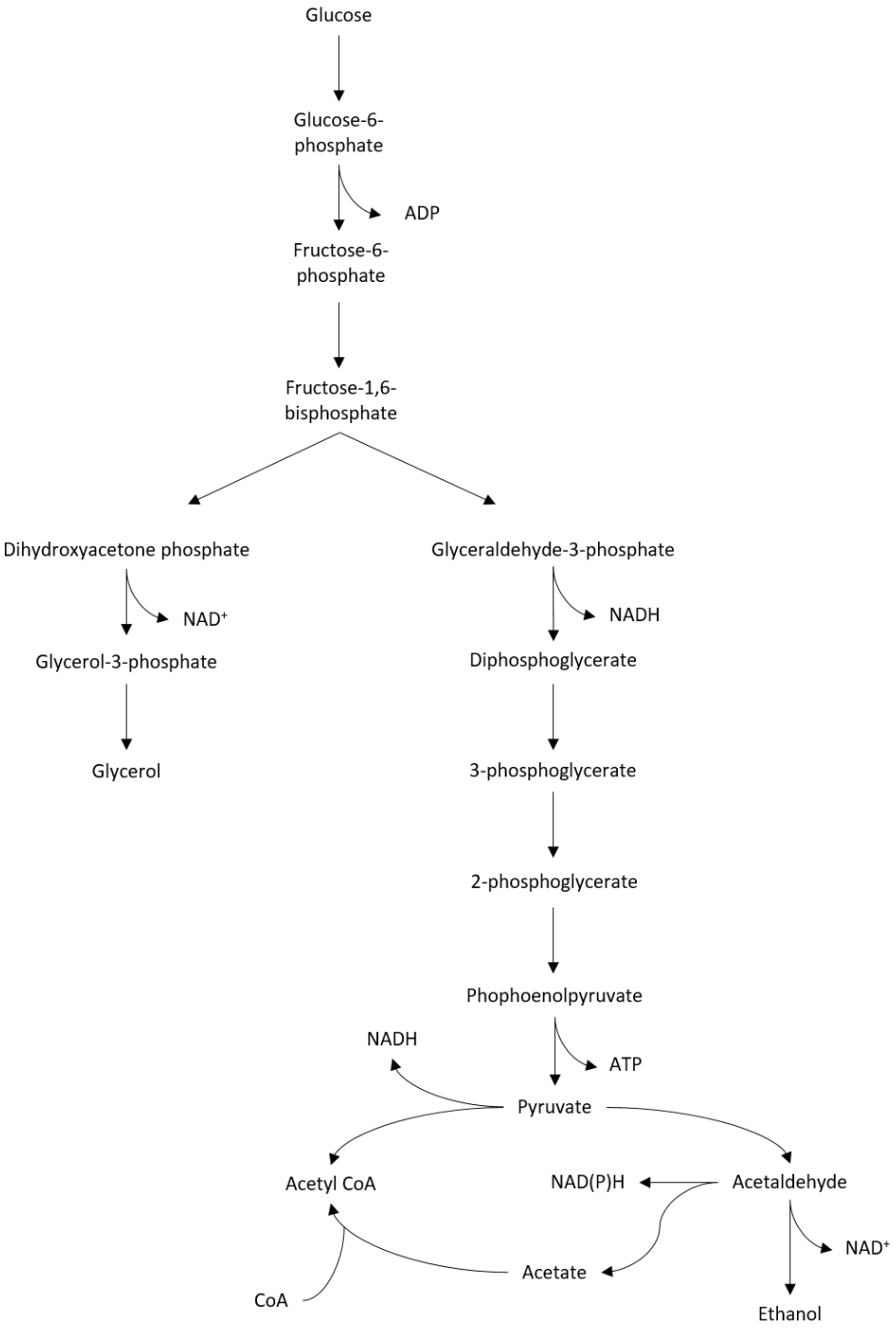


Figure 1.3. Glycolysis and the fermentation pathway resulting in the production of ethanol.

During the fermentation process, wort-derived carbon sources (maltose, glucose, fructose, sucrose and maltotriose) are taken up sequentially by the

yeast cell (D'Amore et al., 1989). Glucose, fructose and sucrose are the first sugars to be assimilated and tend to be completely removed from wort within the first 24 hours after yeast pitching. The uptake of maltose and maltotriose are subject to glucose repression and these are only assimilated once glucose has been removed, with a preference for maltose over maltotriose (D'Amore et al., 1989). Complex sugars are initially broken down to mono-saccharides: sucrose to glucose and fructose; maltose and maltotriose to glucose, before utilisation. Irrespective of their derivative source, glucose and fructose both enter glycolysis and are converted into pyruvate, which is then decarboxylated to acetaldehyde before reduction by alcohol dehydrogenase to ethanol (Ganzhorn and Plapp, 1988; Raj et al., 2014).

It should be noted that brewing yeast strains are 'Crabtree positive', meaning that they prefer to ferment sugars to ethanol even in the presence of oxygen, through repression of oxidative phosphorylation pathways (Van Urk et al., 1990). This phenomenon means that the production of ATP throughout fermentation is carried out solely through substrate level phosphorylation of ADP. Although the Crabtree effect acts a dominant regulatory switch, fermentation conditions are largely anaerobic. However, a certain amount of oxygen is desirable at the initiation of the process for the insurance of yeast quality (David and Kirsop, 1973; Kirsop, 1974; O'Connor-Cox and Ingledew, 1990). Molecular oxygen provided at the start of fermentation is required for sterol biosynthesis, which is in turn essential to ensure yeast cell division under both aerobic and anaerobic conditions (Boll et al 1975). This is widely accepted to be due to the regulatory role of 3-hydroxy-3-methylglutaryl-CoA reductase

in sterol biosynthesis; the same enzyme responsible for cholesterol synthesis regulation in mammalian cells (Thomas et al., 1978; Cournia et al., 2007). The main sterol present in yeast cells is ergosterol, contributing to 2-10% of the dry weight of cells grown aerobically, and providing flexibility and fluidity to the cell membrane (Alexandre et al., 1994; Harris et al., 2002).

Aside from sugars, nitrogen is a key nutrient required for yeast growth; nitrogenous compounds are rapidly assimilated upon yeast pitching (Lekkas et al., 2007). Assimilated nitrogen is utilised by yeast for the production of proteins, enzymes and other cellular components (Manginot et al., 1998; Hill and Stewart, 2019). Wort also contains mineral salts, vitamins, trace elements and metal ions that are essential for yeast functionality (MacWilliam, 1968; Bamforth, 2003). For example, one of the key enzymes in the conversion of sugars to ethanol is alcohol dehydrogenase, a metalloenzyme that requires zinc as a cofactor, rendering zinc vital to the fermentation process (Ganzhorn and Plapp, 1988; Walker, 2004; Auld and Bergman, 2008; Plapp et al., 2017). Zinc is often present at sub-optimal levels in wort, prompting the addition of zinc-containing salts during the wort boiling stage (De Nicola and Walker, 2011). Magnesium is also a key cofactor for many enzymes involved in yeast metabolism including phosphatases, synthases and kinases and is involved in the regulation of cell division and stress tolerance (Walker and Duffus, 1980; Walker, 1998; Udeh, 2014). Other key ions required by yeast are K^+, Mn^{2+}, Ca^{2+}, Cl^- and Fe^{2+} (Walker et al., 2006). Additionally, vitamins and cofactors such as biotin, pyridoxine, thiamine, choline, riboflavin and folic acid are required for

regulation of yeast metabolism and are typically found in wort at concentrations of 0.6 µg/100 mL (MacWilliam, 1968; Briggs et al., 2004).

1.3.4 Yeast contribution to beer flavour and aroma

Yeast are major contributors to beer flavour and aroma, with the production of volatile secondary metabolites during fermentation resulting in key sensory characteristics of the final product (Stewart, 2017). The two main compounds associated with yeast-derived beer flavour and aroma are higher alcohols and esters. Higher alcohols are synthesised either as by-products of amino acid synthesis or via the fermentation of wort carbohydrates (Boulton and Quain, 2001; Hazelwood et al., 2008). Assimilated amino acids are transaminated by branched chain amino acid aminotransferases (BCAATases), encoded by the BAT1 and BAT2 genes, to form the intermediate α-keto acid. This α-keto acid is then decarboxylated to an aldehyde and subsequently reduced to a higher alcohol via the Ehrlich pathway (Olaniran et al., 2017). The levels of higher alcohols are highly dependent on fermentation parameters such a free amino nitrogen levels, fermentation temperature or top pressure in the FV (Anderson and Kirsop, 1974; Erten et al., 2007).

As well as being flavour- and aroma-active constituents of beer, higher alcohols are also precursors to ester synthesis pathways (Anderson and Kirsop, 1974). Esters are generally present at low concentrations in beer, however this means that they can be extremely important in terms of the flavour of the final product; small changes in concentration can have a large impact on beer character (Suomalainen and Lehtonen, 1979; Pires et al., 2014). At the

approximate mid-point of fermentation, as FAN and molecular oxygen are depleted in the fermenting wort, higher alcohol synthesis ceases and ester production dramatically increases (Boulton and Quain, 2001; Pires et al., 2014). Esterification of a higher alcohol is facilitated by two enzymes, acetyl CoA synthase and alcohol acyl transferase (AAT) for the production of acetate and ethyl esters respectively (Mason and Dufour, 2000). The production of esters is inhibited by the presence of oxygen due to its inhibitory effect on the induction of AAT (Malcorps et al., 1991). The concentration of both higher alcohol and esters in the beer is not only highly dependent on brewing and fermentation parameters (raw materials, fermentation temperature, pitching rate), but also varies by yeast strain (Saerens et al., 2008; He et al., 2014b; Olaniran et al., 2017).

Table 1.2. Common yeast-derived higher alcohols, esters and carbonyl compounds and their contribution to beer sensory properties (Meilgaard, 1975; Verstrepen et al., 2003a; Olaniran et al., 2017).

Compound	Organoleptic threshold (mg/L)	Perceived flavour
Esters		
Ethyl acetate	21-30	Fruity, solvent
Isoamyl acetate	0.6-1.2	Banana, pear
Ethyl hexanoate	0.21	Sour apple
Ethyl octanoate	0.9	Sour apple
Higher alcohols		
1-propanol	800	Alcohol
Isobutanol	200	Alcohol
Isoamyl alcohol	70	Alcohol, banana
Carbonyl compounds		
Acetaldehyde	25	Green leaves, fruity
Diacetyl	0.15	Butterscotch

In some cases, brewing yeast can contribute to what may be perceived as unpleasant, or 'off' flavours. The most commonly associated group of flavour compounds that are considered to be negative are vicinal diketones (VDKs), primarily 2,3-butanedione (diacetyl) but also the less flavour-active 2,3-

pentanedione (Krogerus and Gibson, 2013). VDKs are of significance to beer due to their 'buttery' or toffee flavour and aroma, coupled with their low flavour threshold which can be especially detrimental to lager beer (Meilgaard, 1975). VDKs are produced as a result of yeast metabolism during the initial stages of fermentation (Boulton and Quain, 2001; Krogerus and Gibson, 2013). Yeast excrete acetohydroxy acids into the fermenting wort, where they are decarboxylated to form either diacetyl or 2,3-pentanedione (Chuang and Collins, 1968). In the latter stages of fermentation or during warm maturation, yeast re-assimilate the VDKs from the fermenting wort, effectively lowering their levels in the final beer (Krogerus and Gibson, 2013). This process is sometimes referred to as a diacetyl 'rest' or 'stand' and the rate at which this occurs can prompt the implementation of longer maturation periods to ensure the VDK levels in the beer fall below the flavour threshold, before cropping the yeast.

1.4 High Gravity and Very High Gravity Brewing

1.4.1 Principles of high gravity and very high gravity brewing

The gravity of a wort is defined by the amount of dissolved solids, or fermentable sugars that are present. The presence of more fermentable sugars theoretically leads to a concurrent increase in ethanol yield (Casey et al., 1983; Puligundla et al., 2011). This forms the basis for the development of high and very high gravity fermentations with the potential to increase the wort-to-beer production ratio by creation of high alcohol products. The high ABV green beer is then diluted to the desired ethanol yield before packaging, usually in-line

during transfer to bright beer tanks or immediately prior to packaging (Boulton and Quain, 2001). The process presents a strategy to increase plant output without alteration to brewery infrastructure and therefore requires limited capital expenditure (Thomas et al., 1995; Puligundla et al., 2011). Furthermore, the practice aims to decrease production costs, especially those associated with vessel cooling, required at a significant cost to the brewer, in order to maintain fermentation and maturation temperatures. In recent years, many brewing companies have experimented with altering the fermentation process by employing 'high gravity' worts, and in turn have succeeded in achieving a higher ethanolic output, resulting in increased beer production per litre of wort (Hackstaff, 1978; Puligundla et al., 2011).

The term 'high gravity brewing' is vague and can mean different things to different brewers. However, general consensus is that a wort with a gravity of 13-18^{o}P can be considered to be of 'high' gravity (Hackstaff, 1978; Younis and Stewart, 1999; Boulton and Quain, 2001). However, with modern brewing practices there has been an emergence in the use of very high gravity (VHG) worts with a sugar content of >18^{o}P in order to achieve ethanol yields exceeding 10% w/v (Casey et al., 1983; Puligundla et al., 2011). The implementation of VHG brewing can further decrease the cost of beer production, as well as reducing the amount of water and energy employed throughout the process, making the process both more cost effective and sustainable (Casey et al., 1983; Puligundla et al., 2011).

High gravity worts can be achieved by altering the grist to water ratio resulting in a higher concentration of dissolved solids, typically exceeding 160g of dissolved solids per kilogram of wort for a high-gravity brew (Boulton and Quain, 2001; Puligundla et al., 2011). However, potential barriers can arise depending on the mash efficiency or capabilities of the brewing equipment, meaning that an upper limit of wort gravity can be achieved via this method alone (Boulton and Quain, 2001; Briggs et al., 2004). A common, cost effective method of increasing wort gravity is through the addition of sugar-rich brewing adjuncts to the starting wort (Lloyd, 1986; D'Amore et al., 1989; Younis and Stewart, 1999). These can be derived from a variety of cereal plants, most commonly corn or rice, but typically comprise high concentrations of either glucose (or sometimes maltose) in a thick syrup (Younis and Stewart, 1999; Piddocke et al., 2009).

The implementation of VHG as a practice is not solely limited to the brewing industry. The potential benefits of fermenting with a high concentration of sugars are also of particular interest in the production of bioethanol and also distilled spirits (Puligundla et al., 2011). Although the objective of VHG fermentation in these practices is broadly the same, the process by which these fermentations are conducted can differ vastly from the typical serial repitched 'batch fermentation' style regime traditionally associated with brewing fermentations. In such instances innovative HG and VHG practices have often been implemented such as the use of continuous fermentation systems in conjunction with immobilised yeast to achieve high ethanol concentrations, with varying degrees of success (Thomas et al., 1995; Dragone et al., 2007).

1.4.2 Complications associated with HG and VHG brewing

The use of HG brewing techniques is increasingly accepted as being normal within the modern brewery, particularly in commercial mass lager production (Linko et al., 1998; Stewart, 2010). Although VHG brewing offers a further enhancement to the benefits of HG brewing practices, reports of its implementation within the brewing industry convey a shortfall in performance resulting in these potential benefits being negated (Panchal and Stewart, 1980; Puligundla et al., 2011). The main issue associated with VHG fermentations are the adverse effects of the fermentation conditions on yeast health and performance, resulting in sluggish or incomplete fermentations and elongated time to attenuation (Patkova et al., 2000; Puligundla et al., 2011). The impact of fermentation related stress factors on yeast physiology becomes more severe as gravity increases, such that viability can become severely compromised and the yeast culture is not able to be repitched (Pratt et al., 2003; Devantier et al., 2005b; Gibson et al., 2007).

As the fermentation environment is intrinsically stressful for a yeast population, high gravity conditions inevitably increase the amount of stress that a population is subjected to (D'Amore et al., 1988; Gibson et al., 2007). The physiological and genetic response of yeast to fermentation-derived stress factors is discussed in Section 1.5.2 and Section 1.5.3. Osmotic stress levels increase with wort gravity, intensifying the adverse effects of this challenge on the fermenting yeast population. Further to this, a corresponding increase in alcohol also leads to greater ethanol toxicity as fermentation progresses

(D'Amore et al., 1988; Patkova et al., 2000; Pratt et al., 2003; Stanley et al., 2010; Zhuang et al., 2017). It has previously been shown that the effects of both osmotic and ethanol stress are linked to reduced yeast health, lower population viability and impaired fermentation performance in VHG environments (Pratt et al., 2003; Gibson et al., 2007; Smart, 2008; White et al., 2008; Puligundla et al., 2011).

Although fermentation-related stress factors are one of the main contributors to poor yeast performance during VHG fermentations, other influences must also be considered. For example, issues may arise when adjuncts are used to increase wort gravity, since they essentially dilute essential yeast nutrients and assimilable nitrogen derived from malted barley (Casey et al., 1983; Gibson, 2011). Wort nitrogen limitation can contribute to excessively long or stuck fermentations, as yeast growth and fermentation progression are negatively affected (O'Connor-Cox and Ingledew, 1989; Hill and Stewart, 2019). Similarly, metal ions such as zinc and magnesium are often deficient in very high gravity worts, resulting in poor ethanol yields and reduced yeast viability (Rees and Stewart, 1997; De Nicola and Walker, 2011; Udeh, 2014).

For the implementation of a higher-gravity fermentation process to be viable, the flavour of the resulting beer must resemble that of a beer produced by standard gravity. This represents a major challenge since wort composition is inevitably not the same, as described above. Furthermore, the increased stress associated with VHG fermentations, along with extended fermentation times and altered yeast growth patterns can cause the concentration of yeast-

derived flavour compounds to differ from standard gravity beer (Puligundla et al., 2011). The result of this is an imbalance of 'fruity' and 'solvent-like' flavours, due to the impact on ester and higher alcohol production respectively (Anderson and Kirsop, 1974; Saerens et al., 2008). To a certain extent this can be managed through manipulation of wort composition and fermentation parameters, and through blending of the final product (Harrison, 1970). However, it remains a key issue within the industry, providing a hurdle which some brewers cannot overcome and, at the very least, it also represents an obstacle against introducing the practice, especially if there is spare plant capacity.

1.5 Yeast health, stress and fermentation performance

1.5.1 Assessment of brewing yeast quality

In order to assess whether or not a yeast population is sufficiently healthy prior to pitching, many different quantification methods can be employed. The most popular method within the industry is the assessment of viability using a brightfield stain in conjunction with a microscope and haemocytometer (Boulton and Quain, 2001). Such methods can provide a rapid assessment of the percentage of living cells within a population and are often employed in parallel to total yeast counts prior to pitching in order to calculate the volume of yeast slurry needed to supply sufficient living yeast biomass. The standard method for measuring brewing yeast viability is the methylene blue staining procedure. This relies on the ability of living (viable) yeast to reduce the blue stain as it enters the cell (Borzani and Vairo, 1958; Pierce, 1970) such that dead

yeast are stained blue, with living cells remaining colourless (Pierce, 1970). This method allows the percentage of viable cells to be expressed and has a number of benefits, primarily linked to cost, speed and simplicity (Boulton and Quain, 2001).

Although methylene blue is the brewing standard, its efficacy is often challenged as reports suggest that the test is not accurate at lower levels of viability (Pierce, 1970). Other methods of viability testing are available that are considered more accurate, but these often require microscopes with fluorescent capabilities, or a flow cytometer, both of which are expensive and require advanced expertise to use. Such fluorescent viability stains include MgANS, propidium iodide (PI), rhodamine 123 or oxonol dyes (Deere et al., 1998; Zandycke et al., 2003), all of which differ in the mechanism by which they enter the cell and their subcellular targets. For example, PI actively stains the DNA of dead/non-viable cells, however it is excluded from viable cells with intact plasma membranes (Deere et al., 1998).

1.5.2 Genetic response to yeast stress

Ethanol-induced stress and hyperosmolarity represent two key brewing-related stress factors (Gibson et al., 2007). The yeast plasma membrane is integral to the orchestrated stress response to these factors. For example, the membrane proteins Sln1p and Sho1p are responsible for sensing changes in extracellular osmolarity, allowing the cell to respond to extreme conditions and causing a signalling cascade pathway, triggering a range of cellular stress response mechanisms (Hohmann, 2002). This is initiated at high osmolarity via conformational changes to Sho1p transmembrane domains (Tatebayashi et al.,

2015), while changes in cell turgor pressure activate Sln1p (Reiser et al., 2003). Both pathways act independently and are essential for survival during osmotic stress. In order to protect the plasma membrane against osmotic challenges, yeast can also respond by upregulating ergosterol synthesis genes. Krantz et al. (2004) reported that a range of genes involved in ergosterol synthesis, including *ERG9*, *ERG10*, *ERG13* and *ERG26* showed enhanced over-representation during osmotic shock, implicating their role in cell survival under stressful environments. Furthermore, a study by Dupont et al. (2011) showed that an *erg6Δ* mutant of *S. cerevisiae* laboratory strain BY4742 showed a significantly increased sensitivity to osmotic stress when compared to the wild type strain. It is believed that loss of ergosterol biosynthesis activity directly influences the yeast cell's ability to resist a decrease in cell volume as a result of water efflux from hyperosmotic stress (Rupcic et al., 2010).

Upon sensing extracellular stress, the yeast cell responds by activating a number of pro-survival pathways, the most commonly associated being the high osmolarity glycerol (HOG) pathway and heat shock response (HSR) pathway (Hohmann, 2002; Saito and Tatebayashi, 2004). The HOG pathway is a mitogen activated protein kinase (MAPK) signalling cascade that is stimulated under osmotic stress in yeast, and is one of the best-understood eukaryotic stress response pathways (Hersen et al., 2008). The synthesis of glycerol is initiated through sequential phosphorylation of MAPK kinases (MAPK, MAPKK, MAPKKK), activating Hog1 by dual phosphorylation of threonine and tyrosine residues (Hohmann, 2002). Hog1 accumulates in the nuclear component upon activation (Saito and Tatebayashi, 2004; Krantz et al., 2004) and causes

upregulation of a wide array of osmotic response genes, leading to increased glycerol biosynthesis. Genes including Hot1, Sko1 and Smp1 also cause histone deacetylation at promoter sites of osmoresponsive genes (De Nadal et al., 2004). The HSR pathway has also been shown to activate under conditions of ethanol stress, the effects of which resemble those of exposure to elevated temperatures (35^{o}C) (Piper, 1995). This involves activation of heat shock proteins encoded by genes HSP104, HSP70 and HSP26 in response to ethanol stress (Bienz and Pelham, 1986; Piper, 1995). The plasma membrane is a primary target of ethanol toxicity and the effects of ethanol on membrane fluidity have been reported to trigger the unfolded protein response (UPR) pathway (Navarro-Tapia et al., 2018). This is a signal transduction pathway that aids in protecting the cell against ethanol stress by altering the membrane sterol content in order to preserve plasma membrane fluidity promoting survival under ethanol stress (Thomas et al., 1978; Alexandre et al., 1994; Kimata et al., 2006).

One of the major outcomes from activation of stress response pathways in yeast is the production of compounds that serve to protect the cell from the impacts of stress in general (Saito and Posas, 2012). For example, trehalose biosynthesis pathways are upregulated in response to a variety of stress factors. Although formerly believed to be a storage carbohydrate, trehalose (a disaccharide of glucose molecules), is strongly correlated with stress resistance and plays a key role in the stability and functioning of intracellular proteins (Wiemken, 1990; Gancedo and Flores, 2004). Synthesis of trehalose is upregulated upon a vast array of conditions including ethanol toxicity, osmotic

stress, oxidative stress, dehydration and heat shock (Crowe et al., 1984; Wiemken, 1990; Gounalaki and Thireos, 1994; Hohmann, 2002) all of which are relevant to brewing. It has been established that the expression of trehalose in yeast is induced by a variety of cellular stresses, including ethanol toxicity and hyperosmolarity, meaning that high levels of trehalose are broadly indicative of yeast stress (Majara et al., 1996; Gimeno-alcañiz et al., 1999). The trehalose biosynthetic pathway utilises products of glycolysis as precursors; glucose-6-phosphate and UDP-glucose are catalysed to trehalose-6-phosphate by trehalose-6-phosphate synthase (Tps1p) (Gibson et al., 2002; Avonce et al., 2006). Although precise pathways differ between organisms, it is interesting to note that the trehalose biosynthesis pathway is present in eubacteria, archaea, fungi, insects, and plants (Avonce et al., 2006). The TPS1 gene, which encodes the trehalose-6-phosphate synthase enzyme in yeast (complexes with Tps2, Tps3 and Tsl1), is classified as a stress response gene due to the presence of STRE at the promoter site for this gene, causing upregulation of the gene upon exposure to stress. Increased TSP1 expression has been linked with HG and VHG brewing conditions (Blieck et al., 2007), while also playing a role in growth and metabolism on fermentable carbon sources under standard conditions (Van Aelst et al., 1993; Walther et al., 2013). Synthesis of trehalose is upregulated upon a vast array of conditions, due to its role in various stressful conditions including ethanol toxicity, osmotic stress, oxidative stress, dehydration and heat shock (Crowe et al., 1984; Wiemken, 1990; Gounalaki and Thireos, 1994; Hohmann, 2002). As high levels of stress can be detrimental to the structural integrity of the yeast plasma membrane, accumulation of

trehalose acts to stabilize the membrane preventing leakage of components and loss of structural integrity (Mansure et al., 1994; Iwahashi et al., 1995).

1.5.3 Physiological effects of stress on the yeast cell

As yeast are non-motile, they lack the ability to physically remove themselves from a stressful environment. Consequently, as we have seen above (Section 1.5.1), in their natural environment, yeast cells are able to adapt their cell physiology through manipulation of gene expression in order to equilibrate with their surrounding conditions (Mager and De Kruijff, 1995). Similarly, although the effects of fermentation-derived stress factors can cause cell death in many instances, yeast cells are able to orchestrate a complex, pro-survival physiological response to allow the individual to adapt to an environmental stress (Saito and Tatebayashi, 2004; Zakrzewska et al., 2011; Gonzalez et al., 2016; Saini et al., 2018). It should be noted that stress factors can also induce passive effects on yeast cell physiology that are not coordinated by the cellular stress response. For example, the combined effects of osmotic and ethanol induced stress have been reported to cause a general decrease in mean cell size and cell shrinkage in a variety of industrial yeast strains, including those used in brewing (Prat et al., 2003).

One key brewing yeast related stress factor is osmotic stress. It is believed that the result of heightened environmental osmolarity (or osmotic shock) results in water efflux from the cell and a decrease in cell turgor pressure causing a decrease in cell volume (Gervais and Beney, 2001; Pratt et al., 2003). This passive change in cell volume can, in some cases, result in cell death and a

decrease in population viability (Simonin et al., 2007). However, as yeast have adapted to survive in rapidly changing environments, this can be highly dependent on both yeast strain and the extent of hyperosmolarity in which the population is exposed to (Bubnová et al., 2014; Zhuang et al., 2017). As a result of changes in osmolarity and cell volume, physical and conformational changes can also occur within the plasma membrane which can affect membrane functionality and integrity (Simonin et al., 2007; Dupont et al., 2011; Capusoni et al., 2019). Changes in osmotic pressure have been shown to damage the plasma membrane, resulting in leakage of cellular components, membrane depolarisation and impaired permeability to solutes (Simonin et al., 2007). It is believed that the cause of this is because the plasma membrane undergoes a phase transition from solid to liquid-crystalline, which negatively alters the fluidity of the membrane, resulting in damage (Parasassi et al., 1990; Learmonth and Gratton, 2002).

Ethanol is an osmotically charged molecule that it is also directly related to brewing. High concentrations of ethanol can affect plasma membrane fluidity in a similar way to osmotic stress, the extent of which is also highly strain dependant. However, an ethanol-induced increase in fluidity has also been linked to elevated production of ergosterol and UFA content (Jones and Greenfield, 1987; Mizoguchi and Hara, 1998; Capusoni et al., 2019). It has been proposed that strains with enhanced ethanol tolerance may show increased membrane fluidity at higher ethanol concentrations than less tolerant strains (Alexandre et al., 1994; You et al., 2003).

Irrespective of the form of the stress present, intracellular changes in response to the environment are of particular importance in order to elucidate the mechanism by which yeast are able to survive adverse conditions. One method by which yeast are able to adapt to a rapidly changing environment is through modification of their organelle morphology (Mager and De Kruijff, 1995; Ruis and Schüller, 1995; Li and Kane, 2009; Müller and Reichert, 2011; Li et al., 2012; Walker et al., 2014). The extent to which this occurs and the nature of the change reflects the functionality of the organelle in question. Yeast vacuoles are of particular importance in terms of the cellular adaptation to stress; upon exposure to a medium with a high osmotic potential, yeast vacuole fragmentation occurs in order to maintain cell turgor pressure (Martinez de Maranon et al., 1996; Gervais and Beney, 2001; Michaillat and Mayer, 2013). Conversely, Pratt et al. (2003) describe a scenario whereby high-gravity brewing conditions induce vacuole swelling, a physiological phenomenon associated with hypo-osmolarity. This is consistent with similar findings reported by Meaden et al. (1999). However the dynamic nature of vacuoles, and the strain/condition dependant nature of yeast physiology, suggests that changes in vacuole morphology throughout fermentation can be highly dependent on yeast strain and conditions as demonstrated in a study by Izawa et al. (2010) which addresses the conflicting morphologies observed regarding vacuole fragmentation/swelling that occurs in wine making and sake yeast strains during fermentation .

Another highly dynamic yeast organelle that is increasingly implicated in the stress response is the yeast mitochondrion (Knorre et al., 2013a). For example,

a recent study by Baker et al. (2019) highlighted the importance of mitochondrial DNA in the strain-specific cold tolerance response of lager brewing yeast (Walther et al., 2014; Baker et al., 2019). The link between yeast mitochondrial morphology and stress adaptation may therefore suggest an important role of the organelle in fermentative metabolism beyond that of an energetic capacity (O'Connor-Cox et al., 1996; Smart, 2007). This lends support to suggestions that mitochondrial dynamics are essential for brewing, and to ensure fermentation performance (O'Connor-Cox et al., 1996). Mitochondrial physiology itself is determined by the dynamic equilibrium between fusion and fission events, with mitochondrial fission playing a key role in ethanol-induced apoptosis (Kitagaki et al., 2007; Youle and van der Bliek, 2012). However, the exact relationship between mitochondrial morphology and environmental stress has yet to be fully elucidated.

1.5.4 Aims and objectives

The aim of this thesis is to elucidate the impact of very high gravity brewing and fermentation parameters on brewing yeast physiology by investigating the sub-cellular occurrences that are observed when a yeast cell is exposed to stress. This understanding will then be combined with innovative efforts to improve fermentation efficiency at VHG and negate the issues associated with the practice that render it less attractive to the industry. Furthermore, this understanding will be built using conditions that closely resemble those utilised within modern brewing practices, so as to ensure its relevancy for the industry.

CHAPTER 2: MATERIALS AND METHODS

2.1 Yeast strains

Throughout this body of work, five yeast strains held the University of Nottingham Culture Collection were employed (Table 2.1). These yeast strains were sourced from a variety of yeast collections and include one proprietary industrial yeast strain. Although each yeast strains was supplied with a designated ale/lager classification, this was confirmed experimentally in Chapter 3.

Table 2.1. Yeast strains

Strain name	Source	Strain information	Species
M2	University of Nottingham Culture Collection	Commonly used ale-production strain	*S. cervevisiae*
NCYC 1332	National collection of yeast cultures, UK	Commercially available ale-strain	*S. cervevisiae*
SMCC 100	AB InBev, Belgium	Industrial lager-strain	*S. pastorianus*
CBS 1260	Westerdijk institute, Netherlands	Frohberg lager-strain	*S. pastorianus*
CBS 1174	Westerdijk institute, Netherlands	Saaz lager-strain	*S. pastorianus*

2.2 Yeast growth media and storage

2.2.1 YPD standard growth media

All yeast strains were grown and maintained in YPD media, consisting of 1% (w/v) yeast extract (Oxoid, UK), 2% (w/v) bacteriological peptone (Oxoid, UK)

and D-glucose (Merck, UK) in RO (reverse-osmosis treated) water. For longer-term storage, yeast was maintained on YPD agar slopes, prepared by adding 2% (w/v) agarose (Fisher Scientific, UK) to liquid YPD media. All media were sterilised by autoclaving at 121^{o}C and 15 psi for 15 minutes and stored at 4^{o}C prior to use.

2.2.2 Cryogenic storage of yeast

Yeast cells from a culture grown overnight in YPD were transferred to a cryovial (Sarstedt, UK) and mixed with 50% (w/v) glycerol (Fisher Scientific, UK) at a 50:50 ratio (final glycerol concentration of 25%). Yeast stocks were then stored in a freezer at -80 ^{o}C until required.

2.2.3 Preparation of brewer's wort

Wort was supplied by the AB InBev Research Pilot Brewery and precise specifications were altered according to individual experimental requirements, as described in the corresponding Section below and relevant results Chapters. For initial experimentation, an all-malt hopped wort was brewed to a prescribed gravity of 16 ^{o}P and supplemented with glucose syrup (Murphy and Sons, UK) for the production of 24 ^{o}P very high gravity wort. A similar process was used for the preparation of high gravity and very high gravity worts used in later studies. In these instances, an all-malt hopped wort of 15 ^{o}P was prepared and a corn syrup adjunct was added to create a high gravity wort of 17.5 ^{o}P. Further supplementation with high-maltose syrup (Sedamyl, UK) was then used for the production of 22 ^{o}P very high gravity wort. Irrespective of final type, base wort was collected immediately post-boil and aseptically

transferred to pre-sterilised containers. Wort was stored at -20 °C until required.

2.2.4 Determination of wort magnesium and zinc content by ICP-MS

Concentrations of zinc and magnesium were determined by inductively coupled plasma mass spectrometry (ICP-MS). Analysis was performed on an iCAP-Q mass spectrometer (Fisher Scientific, UK) coupled with an ASx-520 autosampler (Teledyne Cetac, USA). Samples were filtered using a 0.2 µm syringe filter (Minisart, UK) to remove solid particulates and subsequently prepared by diluting ten-fold in 2% PrimarPlus nitric acid (Fisher Scientific, UK). Data was processed using Qtegra software (Fisher Scientific UK).

2.2.5 Determination of wort free amino nitrogen content

Free amino nitrogen (FAN) was measured using the Primary Amino Nitrogen Assay Kit (Megazyme, Ireland). Reactions were performed in 96-well plates (Corning, USA) and measured colorimetrically at 340 nm by an Infinite ® 200 PRO series automated plate reader (TECAN, UK). The analysis is based on the o-phthaldialdehyde method recognised by the European Brewing Convention (Hill and Stewart, 2019). Wells of the plate were filled with 5 µL of sample with 300 µL of NAC/buffer, with blank or standard reads replacing the sample with distilled water or a glycerol standard of known concentration respectively. The absorbance of the samples were taken (ΔA1), after which 10 µL of OPA solution was added and incubated at room temperature for 15 minutes to allow for a colour change to occur, after which the absorbance of the samples were taken

again (ΔA2). FAN levels were then determined according to the following equation:

$$\Delta A = \frac{(\Delta A2\ of\ sample - \Delta A1\ of\ sample)}{(\Delta A2\ of\ standard - \Delta A1\ of\ standard)}$$

$$FAN\ (\mathrm{mg/L}) = \Delta A\ X\ concentration\ of\ standard\ (mg/L)$$

2.3 Yeast characterisation and stress tolerance

2.3.1 Yeast cell count and viability assessment

Yeast viability was assessed by the methylene blue staining method as outlined by Pierce (1970). A final concentration of 0.01% (w/v) of methylene blue (Merck, UK) was prepared by dissolving in 2% (w/v) sodium citrate (ThermoFisher Scientific, UK). Yeast cell suspensions were diluted ten-fold in RO water to produce an approximate working concentration of 1 x 10^7 cells/mL. This diluted cell suspension was then mixed with methylene blue at a 50:50 ratio and incubated at room temperature for 5 minutes to allow the staining of dead cells to occur. Cell concentration and the percentage of viable cells was determined using a haemocytometer (Weber Scientific International Ltd, UK). For the former, the total number of yeast cells within a 1 mm^2 ruled area (25 squares) x 0.1 mm thickness were calculated following a standard protocol: cells touching or resting on the top and right boundary lines were not counted; budding yeast cells were counted as one cell if the bud was less than half the size of the mother cell. If the bud was equal to or greater than half the size of the mother cells both cells were counted. At least 200 cells were counted to

ensure statistical validity. The concentration of yeast cells in the initial yeast suspension was calculated using the following equation:

$$Cell\ concentration\ \left(\frac{cells}{mL}\right)$$

$$= total\ counted\ cells\ x\ dilution\ factor\ x\ area\ of\ counting\ chamber$$

Yeast cell viability was determined using the same basic procedure, with the number of live cells (colourless) and dead cells (blue) enumerated. Population viability was calculated using the following equation:

$$Viability\ (\%) = \frac{(total\ yeast\ cells) - (dead\ cells)}{total\ cells}\ X\ 100$$

For large workflow analysis of yeast concentration and viability, yeast populations were evaluated a Countstar automatic yeast counter (Aber Instruments Ltd, UK) using methylene blue prepared and applied as described above.

2.3.2 Analysis of temperature tolerance in yeast strains

A sterile loop was used to transfer a small amount of yeast from a liquid overnight YPD culture and streaked onto a YPD agar plate, prepared as described above (Section 2.2.1). Plates were then incubated at either 25 ^{o}C or 37 ^{o}C and monitored visually for growth daily. After 5 days, colonies were examined using a GelDoc XR+ imaging system (Bio-Rad, UK). This differentiation method is based on results by Walsh and Martin (1977).

2.3.3 Rapid X-α-gal test for brewing yeast classification analysis based on melibiase activity

Yeast strains were tested for melibiase activity using the protocol described by Box *et al*. (2012). This protocol tests the ability of a yeast to cleave the melibiose homolog 5-bromo-4-chloro-3-indolyl-α-D-galactopyrandoside (X-α-gal) and release a blue colour indicative of melibiase activity (Tubb and Liljestrom, 1986). A stock solution of X-α-gal (Fisher Scientific, UK) was prepared by dissolving 62.5 mg X-α-gal in 10 mL sterile RO water containing 25 % (v/v) 1, 2-propanediol (Fisher Scientific, UK). Yeast was prepared by harvesting cells from an overnight YPD media culture and centrifuging at 3000 rpm for 5 minutes. Cells were then resuspended in RO water to prevent colour from the growth media affecting the test.

An aliquot of 100 µL of yeast suspension was placed in an Eppendorf tube to which 10 µL of X-α-gal stock solution was added. Mixtures were incubated at 25 ^{o}C for 30 minutes and samples were examined for a change in colour. Each analysis was carried out in triplicate and mixtures were incubated for a further 24 hours beyond the recommended time frame to provide assurance of colour change. RO water was used as a negative control and the proprietary lager yeast W34/70 (Weihenstephan, Germany) was used to generate a positive result. Visual data was captured using a Galaxy S9 camera (Samsung, South Korea).

2.3.4 Analysis of yeast growth kinetics

In order to investigate the effect of environmental conditions on yeast growth, cultures were monitored for growth by measuring optical density at 600 nm over time. Growth reactions were carried out in 96 well plates (Corning, USA) and monitored using an Infinite ® 200 PRO series automated plate reader (TECAN, UK). Reactions were carried out in 200 µL volumes of media specified in the corresponding section with yeast cells inoculated at a concentration of 1 x 10^6 cells/mL and yeast cultures were prepared according to Sections 2.2.1 and Section 2.3.1. The reactions were incubated at 25 ^oC and shaken at 100 rpm. Optical density was measured every 15 minutes until stationary phase had been reached. Data was collected and analysed using Magellan™ software (TECAN, UK).

2.3.5 Isolation of brewing yeast nuclear DNA

In order to isolate yeast nuclear DNA, the protocol outlined by Legras and Karst (2003) was used. Yeast cells from an overnight YPD media culture were harvested and placed in a cryovial. Yeast was collected by centrifuging at 3000 rpm for 5 minutes and the growth media was discarded. The yeast pellet was resuspended in 400 µL lysis buffer (10 mM Tris pH 7.6, 1 mM EDTA, 100 mM NaCl, 2 % (w/v) Triton X-100, 1 % (w/v) sodium dodecyl sulphate (SDS). Following resuspension, 400 µL of phenol/chloroform/isoamyl alcohol 25/24/1 (v/v) (Sigma-Aldrich, UK) was added along with 500 mg glass beads (0.45 – 0.55 mm diameter, acid washed) (Sigma-Aldrich, UK). The cell suspension was then vortexed for 4 minutes to facilitate cell lysis. The beads were allowed to settle, then 200 µL of Tris EDTA (10 mM Tris, 1 mM EDTA) pH 7.6 buffer (TE buffer)

was added before centrifuging for 5 minutes at 6,000 rpm. The resulting aqueous phase was transferred to a fresh microcentrifuge tube, and 500 µL of chloroform/isoamyl alcohol 98/2 (v/v) was added. The samples were then inverted gently, and the mixture was centrifuged at 13,000 rpm for 2 min. The aqueous phase was removed and transferred to a fresh microcentrifuge tube and placed on ice. A two volumes aliquot of ice-cold ethanol was added to precipitate DNA, followed by centrifugation at 13,000 rpm for 4 minutes. The supernatant was discarded leaving a DNA pellet, which was air-dried for 15 minutes to allow excess ethanol to evaporate. The pellet was dissolved in 50 µL 10 mM TE buffer pH 8.0 (Fisher Scientific, UK) and stored at -20 °C until required.

2.3.6 Identification of yeast species by ITS PCR-RFLP

Each yeast was identified to the species level by PCR amplification of the internal transcribed spacer (ITS) region of the genome, followed by restriction fragment length polymorphism analysis of the PCR product. The PCR protocol was performed as outlined by White et al. (1990). Each 50 µL PCR reaction included 0.5 µL (10 µL) ITS1 primer (5'-TCCGTAGGTGAACCTGCGG-3'), 0.5µl (10 µM) ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') (Sigma-Aldrich, UK), 25 µL 2X PCRBIO Taq mix red (PCR Biosystems, UK), 2 µL sample genomic DNA (obtained from Section 2.3.5) and 22µL Ultrapure™ distilled water (Invitrogen, UK). Reaction mixtures were placed into a Techne TC-512 thermocycler (Cole-Parmer, UK) and subjected to the following conditions: Initial denaturation 95 °C for 15 minutes, 35 cycles of denaturing at 95 °C for 1 minute, annealing at

55 °C for 2 minutes, elongation at 72 °C for 2 minutes, and a final elongation step at 72 °C for 10 minutes. Samples were then stored at 4 °C until required.

The PCR product was subjected to digestion using three restriction enzymes in individual reactions: *HaeIII*, *HinfI* (New England Biolabs, UK) and *CfoI* (Promega, UK). These methods were applied based on results from Guillamon et al. (1998) and Pham et al. (2011). Reaction mixtures included 10 µL PCR product, 1.5 µL restriction enzyme, 1.5 µL 10X enzyme buffer and 2 µL of Ultrapure™ distilled water. All digestions were performed at 37 °C in a water bath for 1 hour.

2.3.7 Brewing yeast fingerprinting using Interdelta PCR

Fingerprinting of yeast DNA was conducted by PCR amplification of interdelta regions of the yeast genome according to Legras and Karst (2003). Each 50 µL PCR reaction consisted of 0.5 µL delta12 primer (5'-TCAACAATGGAATCCCAAC-3'), 0.5 µL delta 21 primer (5'-CATCTTAACACCGTATATGA-3'), 10 µL 5X Phusion HF buffer (NEB, UK), 1.5 µL 50 mM magnesium chloride, 1 µL of 10 µM dNTP mix (PCR Biosystems, UK) 2 µL of sample genomic DNA (Section 2.3.5), 34 µL Ultrapure™ distilled water and 0.5 µL Phusion DNA polymerase (New England Biolabs, UK). Reaction mixtures were placed into a Techne TC-512 thermocycler (Cole-Parmer, UK) and subjected to the following conditions: Initial denaturation 95 °C for 4 minutes, 35 cycles of denaturing at 95 °C for 1 minute, annealing at 55 °C for 30 seconds, elongation at 72 °C for 2 minutes, and a final elongation step at 72 °C for 10 minutes. Samples were stored at 4 °C until required.

2.3.8 Separation and visualisation of DNA fragments using agarose gel electrophoresis

All PCR products and DNA fragments were isolated and analysed using gel electrophoresis. Irrespective of the DNA preparation mechanism and the size of amplicons/DNA fragments, all gels were made by dissolving 2% w/v agarose (Sigma Aldrich, UK) in 100 mL 1X TAE buffer consisting of 5.84 g/L TRIS HCl, 1.14 ml/L, glacial acetic acid and 0.37 g EDTA (Fisher Scientific, UK). Once the mixture had cooled slightly, ethidium bromide (Fisher Scientific, UK) was added to a concentration of 0.2 µg/L before pouring into a die cast complete with comb-insert to form the wells in the gel. Once solidified, the formed gel was removed from the die cast and placed into an electrophoresis tank. DNA samples and appropriate reference DNA ladder(s) were mixed with 6X loading buffer (New England Biolabs, UK) and loaded into the wells. An electric current was passed through the gel to separate the DNA fragments at 100 mV for approximately 1 hour. Resolved DNA fragments were analysed under ultra-violet light using a GelDoc XR+ imaging system (Bio-Rad, UK).

2.4 Fermentation analysis

2.4.1 Yeast propagation and pitching

Yeast biomass for fermentation was attained through aerobic growth in incrementally increasing volumes of wort. Initially, a portion of yeast was taken from an agar slope and inoculated into a 30 mL universal bottle containing 10 mL of wort. This was then incubated at 25 ^{o}C, shaking at 150 rpm for 48 hours. The entire contents of the universal bottle were then transferred to a 250 mL

conical flask containing 100 mL or wort and incubated under the same conditions. The contents were then transferred to a larger 2 L conical flask containing 1 L of wort and incubated under the same conditions. Yeast cells were harvested by centrifugation at 3000 rpm for 5 minutes, and yeast was washed with sterile RO water. The yeast suspension was centrifuged again and the supernatant discarded. The washed yeast pellet was then resuspended in sterile RO water to form a 50/50 (w/w) slurry based on the weight of the yeast pellet. The yeast population was then analysed for cell concentration and viability (Section 2.3.1) in order to calculate the volume of slurry required to achieve the appropriate pitching rate. Yeast pitching rate was adjusted based on individual experimental parameters as described in each corresponding section, however a ratio of 1 x 10^6 cells/mL per degree Plato was typically applied unless stated. Once the pitching rate had been calculated, the appropriate volume of yeast was then transferred aseptically to the fermentation vessel.

2.4.2 Small–scale 100 mL 'mini' fermentations

Mini fermentations were performed as outlined by Quain *et al*. (1989). Hypovials (150 mL) containing a magnetic stirrer bar were sterilised by autoclaving at 125 ^oC and 16psi prior to use. Once sterilised, 100 mL of wort was transferred aseptically to the hypovial. Vessels were then closed with a foam bung and placed in an incubator set to the appropriate fermentation temperature to allow the wort to acclimatise prior to yeast pitching. Wort was aerated by stirring at 250 rpm for three hours prior to pitching. Yeast was then

pitched at the desired concentration and vessels were sealed with a rubber septum and sealed with a metallic crimp cap.

In order to allow carbon dioxide to escape, the rubber septum was pierced with a Bunsen valve consisting of syringe needle connected to a Durham tube via silicon tubing containing a small incision. This was done not only to prevent the build-up of pressure within the vessel, but also to allow the amount of CO_2 evolved to be monitored as an indication of fermentation progression. Once assembled, the vessels were weighed and placed on a stirrer plates within an incubator set to 15 ^oC and stirred at 150 rpm.

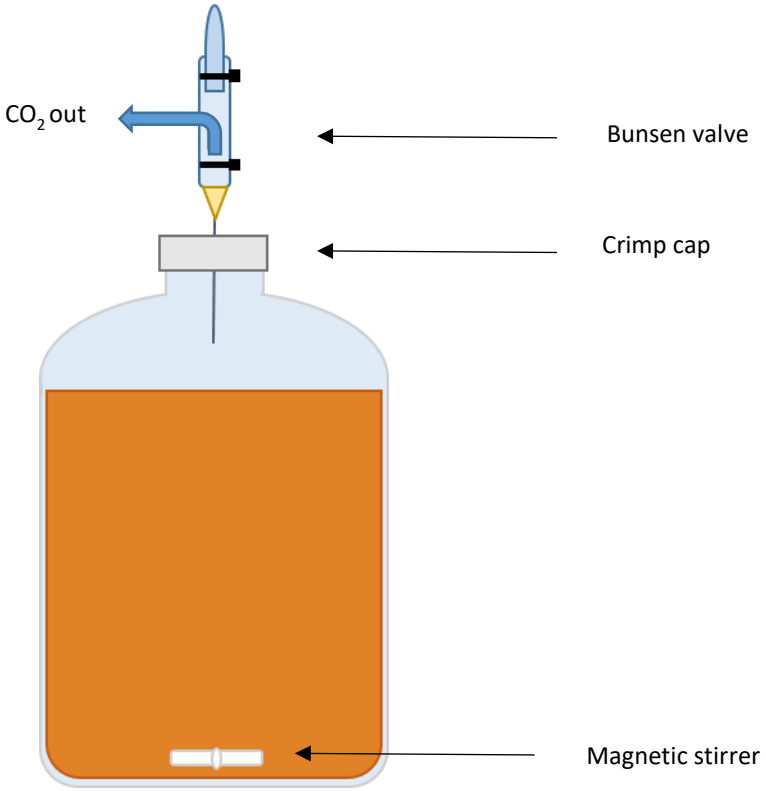


Figure 2.1. Mini fermentation (100 mL) vessel design.

Fermentation progression was monitored by weighing each vessel daily to determine weight loss caused by CO_2 release and used as an indication of both sugar assimilation and ethanol production. Fermentations were deemed to have reached completion (attenuation) when the total weight loss over 24 hours did not exceed 0.05 g.

2.4.3 Quick-fit 2 L bio-reactor fermentations

Where samples were required to be taken during fermentation, or where increased volumes of end products were needed for analysis, larger laboratory-scale fermentation vessels were utilised. Quick-fit vessels were used due to their larger working volume, as well as sample ports allowing for samples to be removed aseptically.

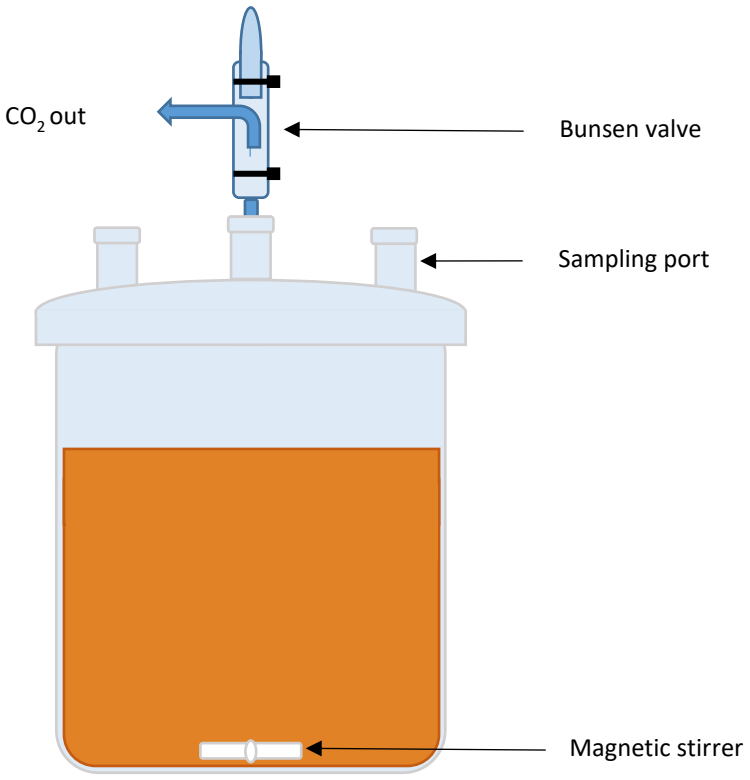


Figure 2.2. Quickfit bioreactor vessel assembly.

Vessels were sterilised by autoclaving at 125 ^{o}C and 16 psi and subsequently filled with 1800 mL of wort using a pre-sterilised funnel attached to the sample port. The sample port was then sealed with a foam bung and the vessel was placed in an incubator set to the appropriate fermentation temperature to allow the wort to acclimatise prior to yeast pitching. Yeast was then pitched at the desired concentration and the vessels were sealed with a glass stopper. A Bunsen valve (Section 2.4.2) was connected to one of the sample ports to prevent the build-up of CO_2 during fermentation.

In order to monitor fermentation progression, 15 mL of the fermentation medium was removed aseptically as required. This suspension was placed into a sterile centrifuge tube and centrifuged at 3000 rpm for 5 minutes to isolate yeast biomass. The supernatant was decanted into a fresh tube and gravity was measured using a DMA 35 handheld density meter (Anton Paar, Austria).

2.4.4 Determination of endpoint gravity, ABV and RDF

The gravity and alcohol by volume (ABV) of fermentation end-products were determined using a DMA 4500 density meter and Alcolyzer Plus Beer system (Anton Paar, Austria). Relative degree of fermentation (RDF) was also provided by the instrument. Prior to analysis, 30 mL of beer sample was centrifuged at 3000 rpm for 5 minutes to remove suspended yeast. The supernatant was then filtered using a 0.45 µm syringe-filter (Minisart, UK) to ensure any further particulate matter was removed.

2.4.5 Quantification of fermentation-derived volatile compounds

Fermentation samples were de-gassed in a sonicator bath (Fisher Scientific, UK) and subsequently filtered using a 0.45 µm syringe filter. Once filtered, 10 mL of sample was transferred to a GC vial (Agilent, UK). An internal standard of 1-butanol was added to a concentration of 1.6 mg/L as well as 3.5 g of sodium chloride (Fisher Scientific, UK) to aid volatile compound release. The vials were immediately sealed with a GC vial crimp cap (Sigma Aldrich, UK) to prevent the loss of volatile compounds from the vial headspace.

Analysis of beer volatile compounds was analysed by GC-MS according to the method described by the Analytica-European Brewing Convention (EBC) (9.39) (2000). Lower boiling volatiles were analysed using a Scion 456-Gas Chromatograph (Scion Instruments, UK): samples (500 µL) were injected in splitless mode using a PAL Combi-XT autosampler (PAL System, Switzerland) onto a Zebron ZBWax column (Phenomenex Inc, UK). The GC carrier gas was helium, which was supplied at a constant pressure of 15 psi. The initial oven temperature was 85 °C for 10 minutes, which was then increased to 110 °C for 13 minutes at a rate of 25 °C/minute, before finally being increased to 200 °C for 13.25 minute at a rate of 8 °C/minute.

Volatile compounds were identified by their *m*/*z*, and quantified using a six-point calibration curve based on individual standards. Standards were prepared with acetaldehyde (≥99.5%), ethyl acetate, (≥97%), propan-1-ol (≥99%), isoamyl acetate (3-methylbutyl acetate) (≥97%), isoamyl alcohol (3-methyl-1-

butanol) (≥99%), ethyl octanoate (≥98%), (≥98%), 1-butanol (≥99.5%), and ethyl hexanoate (≥99%), all of which were procured from Sigma Aldrich (UK).

Data was expressed as normalised concentrations corresponding to that of a 5% ABV beer as they would following dilution according to the equation below unless otherwise stated.

$$\frac{Concentration\ of\ flavour\ compound}{ABV\ of\ green\ beer}\ X\ ABV\ of\ desired\ beer\ product$$

2.4.6 Statistical analysis of data

Statistical analysis was performed using GraphPad Prism software version 8.4.0 (GraphPad, USA). A combination of one-way, two-way ANOVA tests and t-tests were performed as required depending on the analysis being performed and the number of variables in the experiment. If the p-value generated by the analysis was less than, or equal to p=0.05, the difference was deemed to be significant and referred to as such when describing results. These statistical analysis methods were applied when referring to statistical differences in all experiments in all chapters.

2.5 Physiological analysis of yeast

2.5.1 Assessment of plasma membrane damage

Plasma membrane damage was assessed using a combination of the fluorescent stains $DiSBAC_2(3)$ (bis-oxonol, or BOX) (Fisher Scientific, UK) and propidium iodide (PI) (SigmaAldrich, UK). Yeast was harvested and washed

three times in flow cytometry grade 1X PBS buffer (Invitrogen, UK) in a microcentrifuge tube. The washing procedure consisted of centrifuging at 3000 rpm for 5 minutes to collect the yeast and resuspension of the pellet in 1X PBS (Life Technologies, UK) and vortexing to disperse cells. Washed yeast were then added to 1 mL of Live Cell Imaging Solution (Gibco, UK) at a concentration of 1 x 10^6 cells/mL.

Table 2.2. Fluorescent stains for viability and membrane depolarisation assessment.

Stain	Cellular target	Assessment	Working concentration (mM)	Dissolving agent
PI	DNA	Viability	10 µM	Water
BOX	Intracellular membranes	Plasma membrane depolarisation	6 µM	DMSO

Staining solutions were prepared according to the working concentrations outlined in Table 2.2. Once the fluorescent stain had been added, the microcentrifuge tubes were covered in aluminium foil to prevent loss of fluorescence and incubated at room temperature for 30 minutes. In the case of BOX staining, the staining procedure included the addition of 10 µL of 4 mM EDTA to facilitate binding. Following the incubation period, stained yeast cells were washed once more and resuspended in 1X PBS. The excitation/emission spectra of PI and BOX is 533/617 nm and 530/560 nm respectively.

2.5.2 Analysis of yeast mitochondrial mass and mitochondrial membrane potential

Yeast mitochondrial dynamics were assessed by fluorescent staining using rhodamine 123 (Sigma Aldrich, UK) and MitoTracker Green FM (Fisher Scientific, UK) according to the specifications outlined in Table 2.3 and the methods stated by Dinsdale et al. (1995) and Chazotte (2011). Cells were washed three times in 1X PBS and resuspended in Live Cell Staining Solution (Life Technologies, UK). Cells were stained for 30 minutes, at room temperature and in the dark to avoid bleaching of the dye, using the fluorescent dyes and the related concentrations detailed in Table 2.3. After the staining period, cells were centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. Stained yeast cells were resuspended in fresh Live Cell Imaging Solution and analysed by either flow cytometry (Section 2.5.5) or confocal microscopy (Section 2.5.3) as required. Excitation/emission spectra for Rhodamine 123 and MitoTracker Green FM were 505/534 nm and 490/516 nm respectively.

Table 2.3. Mitochondrial stains

Stain	Cellular target	Assessment	Working concentration (mM)	Dissolving agent
Rhodamine 123	Mitochondria	Mitochondrial membrane potential	50 µM	DMSO
MitoTracker Green FM	Mitochondria	Mitochondrial mass	100 nM	DMSO

2.5.3 Visualisation of yeast mitochondria

For qualitative analysis of mitochondrial dynamics and morphology, yeast mitochondria were visualised using confocal microscopy. Mitochondria were stained with rhodamine 123 according to the procedure outlined in Section 2.5.2. Following staining, 8 µL of the staining solution was placed onto a 75 X 25 mm glass slide (Corning, US) and covered with a no. 1.5 glass cover slip (VWR, UK). Once assembled, the slide was then visualised with an LSM880 confocal microscope (Zeiss, Switzerland) and image analysis was performed using ZenBlack software (Zeiss, Switzerland). Live cells were visualised using a 63X magnification water objective. Approximately 50-100 cells, or groups of cells, were analysed and images were selected based on representative physiologies.

2.5.4 Visualisation of yeast vacuoles

Fluorescent staining of yeast vacuoles was performed using the stain FM 4-64™ (ThermoFisher Scientific, UK) as outlined by Vida et al. (1995). Yeast cells were harvested and placed in a microcentrifuge tube at a concentration of 1 x 10^6 cells/mL and washed three times in 1X PBS. Cells were then resuspended in Live

Cell Imaging Solution and the FM 4-64 stain was added to a final concentration of 1 mM. Finally, reaction tubes were covered in aluminium foil and incubated at 25 ^{o}C for 30 minutes. Following staining, cells were centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded, removing any of the stain that has not been taken up by the cells. Yeast cells were resuspended in fresh Live Cell Imaging Solution and incubated in the same fashion for a further 30 minutes. Stained yeast samples were then visualised using the same procedure as outlined in Section 2.5.3. The excitation/emission spectrum for FM 4-64™ is 515/640 nm.

2.5.5 Flow cytometry and data analysis

All flow cytometry analysis was performed using an Astrios EQ cell sorter (Beckman Coulter, UK) equipped with seven configurable wavelengths at 355 nm, 405 nm, 488 nm, 532 nm, 560 nm, 592 nm, and 645 nm, set to the appropriate laser/pinhole for the fluorescent stain being employed. For each experiment, a sample size of 20,000 cells was selected for analysis of populations. All data analysis was carried out using Kaluza software (Beckman Coulter, UK). Gating was used to select for single cells or sub-groups using data obtained from the forward and side scatter data (Figure 2.3). Additionally, in some instances a live/dead (or depolarised) sample set was used in order to allow for selection and analysis of different factions within a yeast population (Figure 2.4) for the appropriate fluorescent stain being employed.

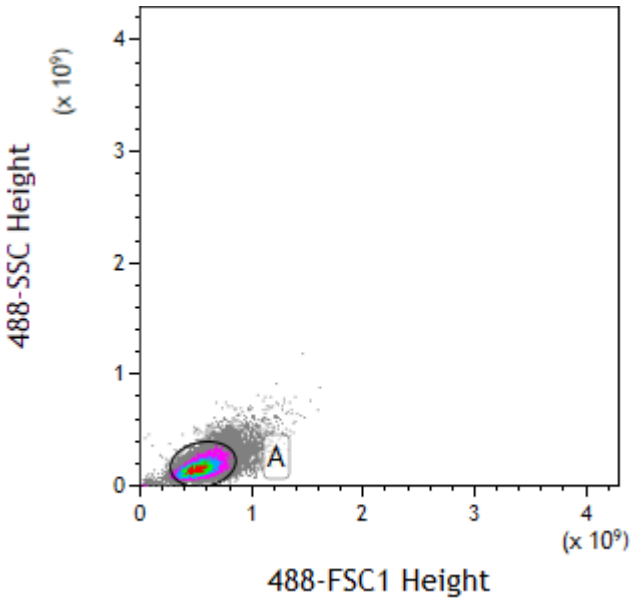


Figure 2.3. Gating of single cells (Gate A) based on forward scatter against side scatter.

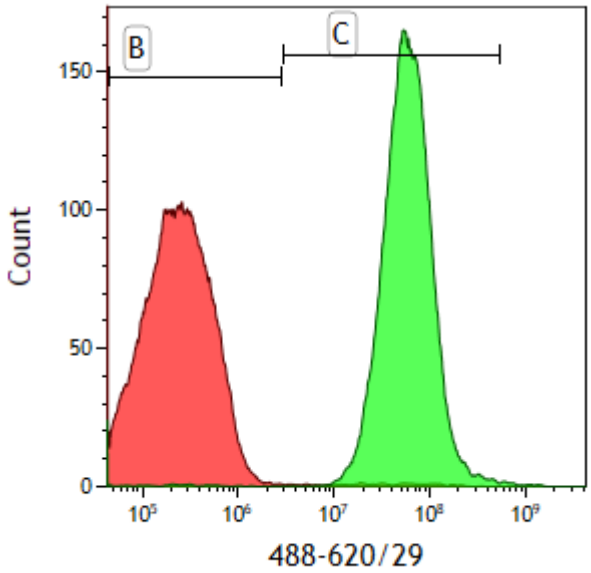


Figure 2.4 Gating of live (Gate B) and dead (or depolarised in the case of BOX staining) cells (Gate C) based on excitation/emission spectrum at 488/620 nm of cells stained with PI.

2.6 Yeast gene expression analysis

2.6.1 Yeast RNA preparation

Yeast RNA extractions were performed using an RNeasy mini kit (Qiagen, UK). Yeast samples (1 mL) were taken at desired timepoints throughout fermentation and transferred to cryovials, which were then centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and the cryovials containing yeast pellets were immediately frozen by placing into liquid nitrogen. Flash-frozen yeast pellets were then stored at -80 ^{o}C until required.

Immediately prior to analysis, frozen yeast pellets were thawed on ice and approximately 1 x 10^{7} cells were lysed mechanically using 0.5 g of 0.45 µM acid-washed glass beads with Buffer RLT (Qiagen, UK). Cell lysate was then fixed to the RNeasy spin column by adding the solution to the spin column and centrifuging at 10,000 rpm for 15 seconds. On-column wash steps were performed by adding 700 µL buffer RW1 and buffer 500 µL RPE; the addition of each was followed by centrifugation of the column at 10,000 rpm for 15 seconds. RNA was eluted from the column by adding 50 µL RNase-free water and centrifuging for 1 minute at 10,000 rpm. The RNA solution was then stored at -20 ^{o}C until required.

2.6.2 RNA sequencing and differential gene expression analysis

RNA concentrations were determined using the Qubit Fluorometer and the Qubit RNA BR Assay Kit (ThermoFisher Scientific; UK), and RNA integrity was assessed using the Agilent TapeStation 4200 and the Agilent RNA ScreenTape Assay Kit (Agilent, UK). For each sample cDNA was generated from 60ng of total

RNA using the QuantSeq 3' mRNA-Seq library prep kit for Illumina (Lexogen, Aurstria). Indexed sequencing libraries were prepared using the Lexogen i7 6nt Index Set (Lexogen; Austria).

Libraries were quantified using the Qubit Fluorometer and the Qubit dsDNA HS Kit (ThermoFisher Scientific; Q32854). Library fragment-length distributions were analyzed using the Agilent TapeStation 4200 and the Agilent High Sensitivity D1000 ScreenTape Assay (Agilent, UK). Libraries were pooled in equimolar amounts and final library quantification performed using the KAPA Library Quantification Kit for Illumina (Roche; UK). The library pool was sequenced on the Illumina NextSeq 500 on a NextSeq 500 High Output v2.5 75 cycle kit (Illumina; 20024906), to generate approximately 5 million 75bp single-end reads per sample. HTseq values were then used as a determinant of gene expression by generating a sequence depth value based on the equation below.

$$Sequence\ depth = \frac{HTseq\ counts}{Total\ nuumber\ of\ reads\ per\ sample}$$

2.6.3 Condition dynamics analysis

Raw reads were trimmed using Cutadapt v3.0 using the parameters -m 35 -a AGATCGGAAGAGCACACGTC --trim-n -a A(Udeh) -a T(18) --nextseq-trim=10. Trimmed reads were aligned to *S. pastorianus* (ASM1102231v1) reference genome using Star v2.7.6a with the

parameters --outFilterType BySJout --outFilterMultimapNmax 20 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.6 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000 --outSAMattributes NH HI NM MD. Aligned reads were counted using HTSeq v0.12.4 using the settings -m intersection-nonempty -s yes -f bam -r pos. The obtained gene counts were further analysed using the standard analysis protocol with DESeq2 1.30.1.

CHAPTER 3: BREWING YEAST STRAIN CLASSIFICATION AND THE EFFECTS OF VERY HIGH GRAVITY FERMENTATION CONDITIONS

3.1 Introduction

Yeast has been utilised for the fermentation of beer and other beverages for many years, although initially its form was largely unknown to the brewer. However, due to the findings of Louis Pasteur in the 19th century, the integral role of yeast in the creation of fermented beverages began to be recognised (Barnett, 2000). This discovery sparked increasing scientific analysis of the process, leading to the evolution of brewing practices and forming a base for the understanding that we have today. Inevitably this also led to diversification within the industry and the creation of different beer types, many characteristics of which stem from the type of yeast used.

There are two main species of yeast that are predominantly employed within the brewing industry and the choice of species defines the broad product category. Strains belonging to the species *Saccharomyces cerevisiae* are used to produce ale-type beers, although it should be recognised that *S. cerevisiae* yeasts are diverse. This broad group of organisms can be used for other alcoholic beverages, baking and biofuels, many of which are not suitable for beer production (Piskur et al., 2006; Gallone et al., 2018). The other key yeast species from a brewing perspective, *Saccharomyces pastorianus*, is employed in the production of the more popular lager-style beer (Boulton and Quain, 2001; Baker et al., 2015). *S. pastorianus* strains are interspecies hybrids derived from two parental species: *Saccharomyces cerevisiae* and what is widely considered to be *Saccharomyces eubayanus* (Dunn and Sherlock, 2008; Libkind et al., 2011; Baker et al., 2015; Morimoto et al., 2016). Lager yeast can be

further sub-divided into two types, known as Saaz (type I) and Frohberg (type II) (Josepa et al., 2000; Dunn and Sherlock, 2008; Walther et al., 2014).

Differences between yeast species, brewing yeast classification, and individual strains within each category can be determined using a wide variety of observational, molecular and genetic approaches. Ale and lager yeasts can be differentiated by their ability to grow at 37^{o}C (Walsh and Martin, 1977), reflecting the ability of ale yeasts to withstand higher temperatures than lager yeast (Boulton and Quain, 2001). Brewing yeasts can also be differentiated based on α-galactosidase activity, determined rapidly using the reagent X-α-gal. This molecule is a homolog of melibiose and the capacity to assimilate this sugar is a trait possessed by lager yeast but not ale strains (Tubb and Liljestrom, 1986; Box et al., 2012). Although lager strains are phenotypically similar, Saaz yeast strains are generally more cold tolerant that Frohberg strains (Gibson et al., 2013). In addition, one of the major attributes of Saaz yeast that differentiate them from Frohberg strains is their inability to efficiently utilise maltotriose (Salema-Oom et al., 2005; Gibson et al., 2013), which is a prolific fermentable constituent of brewer's wort (Briggs et al., 2004).

Classification of yeast strains by phenotypic means is often considered to be a crude method of distinguishing between different yeast types, therefore genetic approaches are typically applied. Genetic characterisation provides the ability to further differentiate between ale and lager strains and between the two types of lager yeast: Saaz (type I) and Frohberg (type II) (Josepa et al., 2000; Walther et al., 2014). Differentiation between type I and type II strains can be

achieved using ITS (Internal transcribed spacer) PCR, whereby conserved ITS regions of the yeast genome are amplified and subjected to RFLP. When these restriction fragments are analysed using agarose gel-electrophoresis, specific fingerprints are generated according to the origin of the yeast strain (Pham et al., 2011). It should be noted that this method of differentiation does not differentiate between *S. pastorianus* Frohberg strains and ale (*S. cerevisiae*) yeast, prompting the use of a combination of genetic and phenotypic analysis to distinguish between the two species. It is however, possible to generate a genetic fingerprint of individual yeast strains through PCR amplification of inter-delta regions of the yeast genome (Legras and Karst, 2003), although other methods such as RFLP analysis of yeast transposons (Wightman et al., 1996) or the yeast mitochondrial DNA profile (Lee et al., 1985). These identification techniques are often used in routine yeast supply and are essential to ensure strains can be correctly identified, as well as allowing detection of variants that may occur during population growth during propagation or repitching over a large number of generations.

Each yeast strain also exhibits its own individual ability to tolerate fermentation-derived stress factors, such as ethanol toxicity, oxidative stress and osmotic stress (Attfield, 1997; Gibson et al., 2007). The capacity to survive under stress conditions may then have an impact on a particular yeast strain's fermentation efficiency, particularly when considering 'intensive' high-gravity fermentation processes (Huuskonen et al., 2010). In order to harness the potential economic benefits of high and very high gravity (VHG) brewing, the yeast strain employed must be able to perform under elevated ethanol

concentrations and osmolality throughout the fermentation in order for the process to be viable. Furthermore, fermentation characteristics are not limited to the central dogma of the process; factors such as yeast-derived flavour and aroma compound production must also be considered. Yeast strains produce a unique flavour profile as a result of wort constituent catabolism, which is also an important factor in yeast strain selection (Quain, 1986). The production and balance of flavour and aroma compounds is highly sensitive to fermentation conditions, and can therefore differ between standard gravity (SG) and high gravity processes (Anderson and Kirsop, 1974).

In this study a variety of commercial brewing yeast strains were selected and assessed for key fermentation-related characteristics, in particular the ability to function under conditions associated with very high gravity worts. Each strain was initially analysed for metabolic, genetic and phenotypic traits in order to reveal strain-dependent characteristics, and to both differentiate and profile each strain. Key growth and fermentation parameters were also evaluated in response to increasing levels of osmotic stress to provide an insight into the link between stress tolerance and fermentation performance at higher gravities. Finally, the sensorial attributes of beers produced from standard and high gravity worts were examined to further understand the behaviour of each yeast and the effects of increased gravity on flavour and aroma compound production.

3.2 Results

Five yeast strains, M2, NCYC 1332, SMCC 100, CBS 1260 and CBS 1774, were selected for analysis as detailed in Section 2.1. Each yeast strain was subjected to a range of characterisation techniques in order to ensure that they were unique, and to determine their brewing classification as either ale or lager. Lager yeast were further differentiated to categorise them as either type I (Saaz) or type II (Frohberg) strains. Finally, each strain was assessed for their ability to ferment standard and high gravity worts, and their capacity to tolerate ethanol and osmotic stress, selected as key stress factors related to very high gravity brewing.

3.2.1 Phenotypic differentiation of ale and lager yeast

A simple method for differentiating ale and lager type yeasts is to cultivate strains at 37^{o}C (Section 1.3.1). It is known that *S. cerevisiae* strains are able to withstand and proliferate at these temperatures, while *S. pastorianus* strains cannot (Walsh and Martin, 1977). Due to their genetic background, lager strains are better adapted to colder conditions (Section 1.3.1) which is reflected in their preferred growth temperature, as well as the temperature range typically applied to lager fermentations (Boulton and Quain, 2001; Walther et al., 2014). Each strain was examined for growth characteristics on solid YPD media, according to the protocol described in Section 2.3.2. Analysis of growth at 25^{o}C indicated that all strains used in this study were able to grow, as expected (Figure 3.1). However, when strains were exposed to a growth temperature of 37^{o}C, only M2 and NCYC 1332 exhibited observable growth,

indicating that these two strains were ale-type yeasts. Conversely, strains SMCC 100, CBS 1260 and CBS 1174 did not exhibit any growth at 37°C, indicating that they were lager strains.

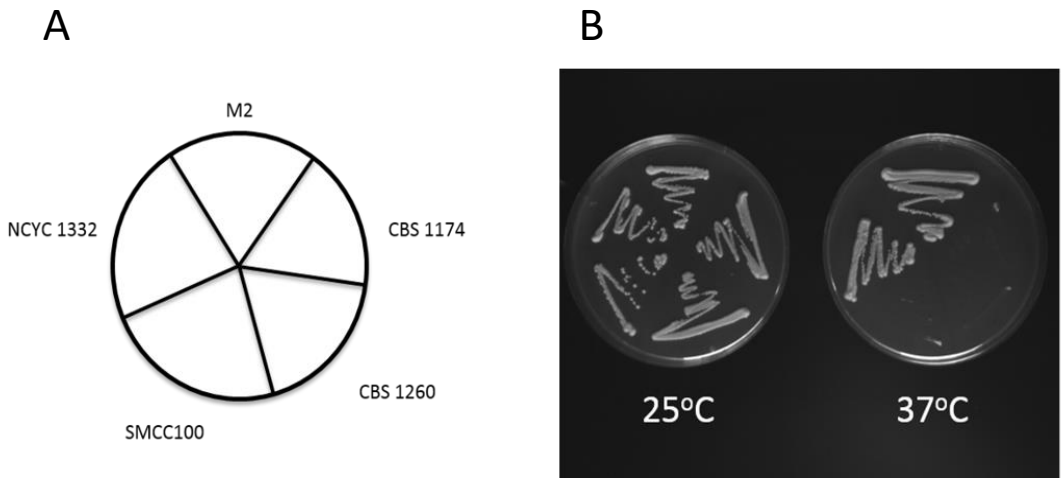


Figure 3.1. (A) Pinwheel depicting layout of agar plate for each strain. (B) Yeast growth on YPD agar at 25°C and 37°C.

As observational growth is a relatively crude method for species identification, further analysis was applied to confirm the taxonomy of each strain being used in this study. For this purpose, the metabolic capability of each yeast to utilise melibiose, through production of the enzyme melibiase, was evaluated using the X-α-gal test (Section 2.3.3). This test utilises a melibiose homologue, 5-bromo-4-chloro-3-indole-α-D-galactopyranoside (X-α-gal), as a colorimetric substrate to determine melibiase activity. As lager strains possess melibiase activity, they are able to cleave the X-α-gal molecule resulting in a blue colour developing from the liberation of the indole constituent of the substrate (Tubb

and Liljestrom, 1986; Box et al., 2012). While a positive result (blue colour) is characteristic of a lager strain, a negative result (no colour change observed) is indicative of an ale yeast. The results shown in Figure 3.2 confirmed that strains M2 and NCYC 1332 were ale yeast, as no colour change was observed, meaning that these strains were not able to utilise melibiose. Since a colour change was visible with strains SMCC 100, CBS 1260 and CBS 1174, it could be concluded that these strains were lager yeast.

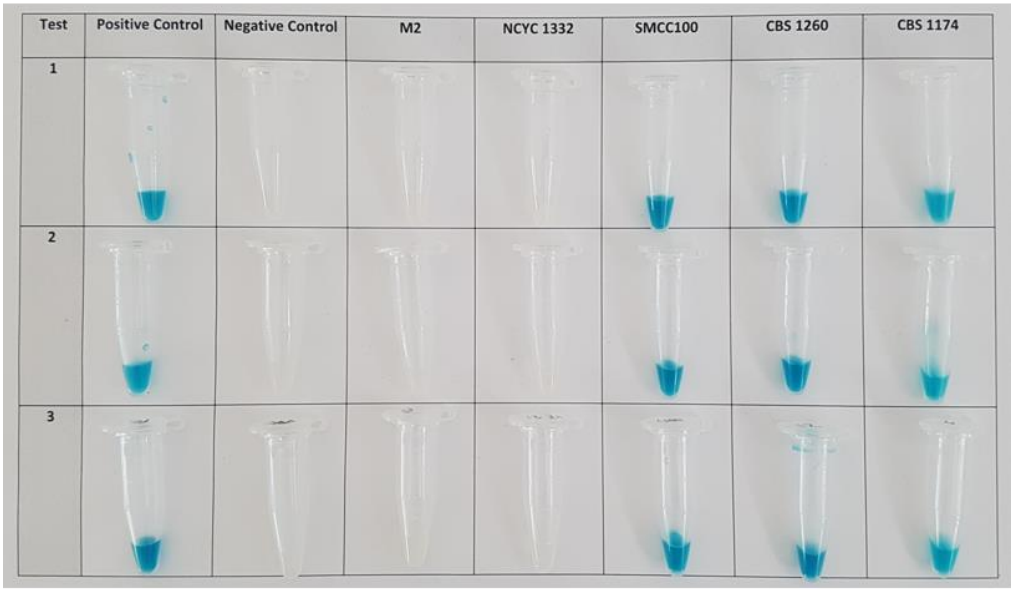


Figure 3.2: Rapid X-α-gal analysis of brewing yeast strains. Blue colour indicates a lager strain and colourless indicates an ale strain. The commercial lager yeast strain W34/70 was used a positive control, with water as a negative control. All tests were performed in triplicate.

3.2.2 ITS-PCR and RFLP for determination of yeast species and differentiation of type I and type II lager strains

Within the classification of lager yeasts, two distinct lineages exist which can be referred to as type I (Saaz) or type II (Frohberg) lager strains. Identification of yeast strains as either type I or type II is important in order to understand the genetic origins of the yeast, and to further understand behavioural characteristics exhibited by each strain type (Gibson et al., 2013). PCR amplification of yeast genomic DNA using ITS-specific primers alone yields non-distinguishable results for domesticated brewing strains, meaning that this method cannot be directly used to differentiate *S. cerevisiae* and *S. pastorianus* (Pham et al., 2011). However, the ITS-PCR product can be subjected to RFLP in order to generate a species-specific fingerprint revealed by separation of fragments using gel electrophoresis, this can be used to determine if a strain is either an *S. cerevisiae* or *S. pastorianus* Frohberg (Type II), or a Saaz type strain (Josepa et al., 2000; Pham et al., 2011). In this study, the restriction enzymes HaeIII, HinfI and CfoI were used in independent reactions to analyse each brewing strain.

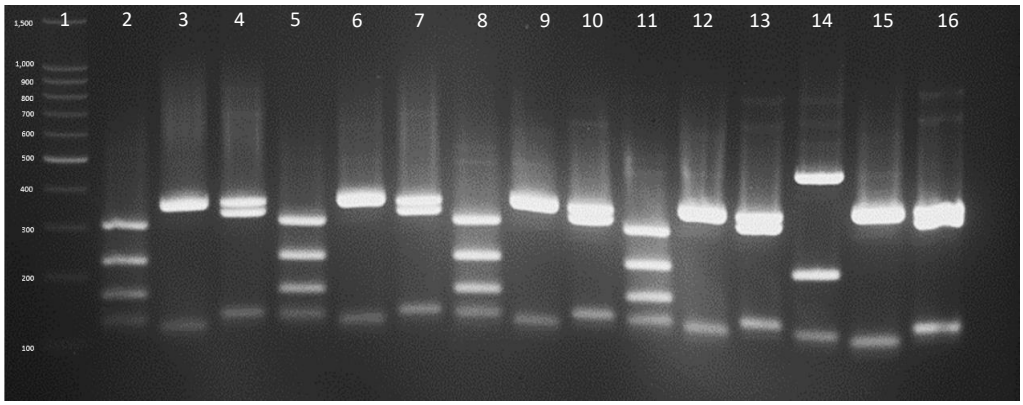


Figure 3.3. Analysis of RFLP profile of brewing yeast strain ITS regions. Lane 1:100 bp DNA ladder. Lanes 2-4: M2 strain ITS region digested with HaeIII, HinfI and CfoI respectively. The order of restriction enzymes is repeated for all strains. Lanes 5-7: NCYC 1332, Lanes 8-10: SMCC 100, Lanes 11-13: CBS 1260 and Lanes 14-16: CBS 1174.

Initially the ITS region of each strain was amplified by PCR using primers ITS1 and ITS4 (Section 2.3.6) to create fragments of approximately 880 bp in size (data not shown). The generated DNA was then cut using the corresponding restriction enzymes (Figure 3.3). It can be seen that restriction fragment analysis of the ITS region using the enzyme HinfI revealed that all strains yielded two fragments of 380 and 120 bp in size. Similarly, the enzyme CfoI generated restriction fragments of 360, 340 and 120 bp for all strains. However, the enzyme HaeIII generated four distinct amplicons of 320, 220, 180 and 140 bp for all strains except CBS 1174. Instead, this strain yielded three amplicons of 450, 220 and 140 bp. From these results, CBS 1174 could be effectively differentiated, with all other strains exhibiting identical restriction profiles. Based on previous studies (Guillamon et al., 1998; Josepa et al., 2000; Pham et

al., 2011), the restriction profile based on the ITS region observed for strain CBS 1174 is indicative of a Saaz lager strain.

Strains M2, NCYC 1332, SMCC 100 and CBS 1260 yielded identical results, matching the profile expected for both *S. cerevisiae* strains and *S. pastorianus* Frohberg type lager yeasts. Using a combination of the results of the X-α-gal test (Section 3.2.1) and the ITS-RFLP analyses shown here, strains M2 and NCYC 1332 strains could be confirmed as ale yeasts, while SMCC 100 and CBS 1260 could be classified as Frohberg type lager strains.

3.2.3 Differentiation of yeast strains by Interdelta PCR

In order to ensure that the individual brewing yeast strains used in this study were unique, PCR amplification of the interdelta region of the yeast genome was used to generate strain-specific fingerprints according to Section 2.3.7. Interdelta PCR is a routine method for identifying yeast strains, whereby multiple discriminatory transposon-flanking regions are amplified and revealed using gel electrophoresis (Ness et al., 1993; Legras and Karst, 2003). This method has been used for identification of a range of industrial strains (Lavalliée et al., 1994) and is the current recommended methods for yeast strain identification according to the ASBC methods of analysis (Zandycke et al., 2008).

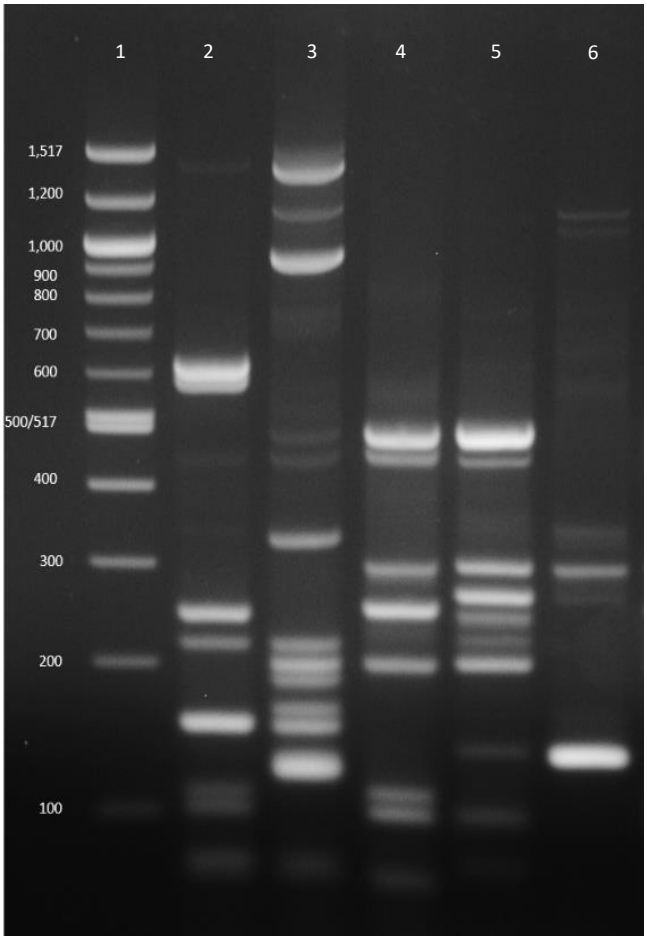


Figure 3.4. Interdelta-PCR analysis of five brewing yeast strains. Lane 1: 100 bp DNA ladder, Lane 2: M2, Lane 3: NCYC 1332, Lane 4: SMCC 100, Lane 5: CBS 1260 and Lane 6: CBS 1174.

As shown in Figure 3.4, the interdelta sequences generated from ale strains M2 and NCYC 1332 differed significantly, with clear discrepancies observed between the two fingerprints. For example, NCYC 1332 shows distinct bands at ~1450, 950 and 330 bp that were not present in the fingerprint of the M2 strain. Similarly, the Saaz lager yeast CBS 1174 produced a distinct fingerprint that was different from all the other strains, with comparatively few distinct bands visible generated by the analysis. In contrast, a high degree of fingerprint

similarity was apparent between strains SMCC 100 and CBS 1260, most likely reflecting their close evolutionary heritage, as both yeast belong to the Frohberg lager group. For these strains, both strains yielded amplicons at 470, 450, 300 and 200 bp. However, despite similarity, the sequence of bands between 200 and 300 bp was sufficiently different to allow for differentiation of the two yeasts.

3.2.4 Osmotic stress tolerance of brewing strains

It is widely accepted that different yeast strains behave differently under specific and defined conditions (Section 1.3.1). A major behavioural trait relevant to brewing and fermentation efficiency is the ability of individual strains to withstand environmental stress (Attfield, 1997; White et al., 2008; Saini et al., 2018). One of the key environmental challenges encountered by fermenting yeast is osmotic stress, caused by elevated wort sugars and resulting in a pressure on the yeast population (Pratt et al., 2003; Gibson et al., 2007). Furthermore, while osmotic pressure might be expected to be alleviated during fermentation as a result of sugar consumption, osmolarity is actually known to increase throughout fermentation. This occurs due to the increasing levels of ethanol concentration within the fermenting wort which exerts a strong osmotic pressure exerted on the yeast population (Hallsworth, 1998; Zhuang et al., 2017).

In order to assess the osmotic stress tolerance of the five brewing yeast strains used throughout this study, growth media was supplemented with a non-assimilable sugar to create an artificial environment of high osmolality. Sorbitol

was selected to illicit an osmotic stress response since this sugar cannot be taken up by brewing yeast and therefore does not alter the nutritional composition of the growth medium. In addition, sorbitol does not dissociate into ions (Hirasawa et al., 2006), making it a more relevant stressor than alternatives such as sodium chloride that are commonly used to elicit an osmotic stress response and may have toxic effects on yeast cells beyond that of their role in altering osmolality (Serrano, 1996). The effect of osmotic stress on yeast strains was elucidated by analysing growth rate, as a direct indication of the ability of cells to tolerate and proliferate under stressful conditions.

The growth of each strain under increasing concentrations (10, 20, 30 and 40% w/v) sorbitol was compared to a non-supplemented base YPD media and data was used to compare tolerance between strains. Yeast growth at 25 ^{o}C was analysed by measuring optical density at 600 nm according to the method outlined in Section 2.3.4. For each of the strains used in this study, the supplementation of the growth medium with sorbitol led to a decrease in the growth rate of the yeast. This decrease in growth was increasingly apparent for strains as higher concentrations of sorbitol were employed (Figure 3.5).

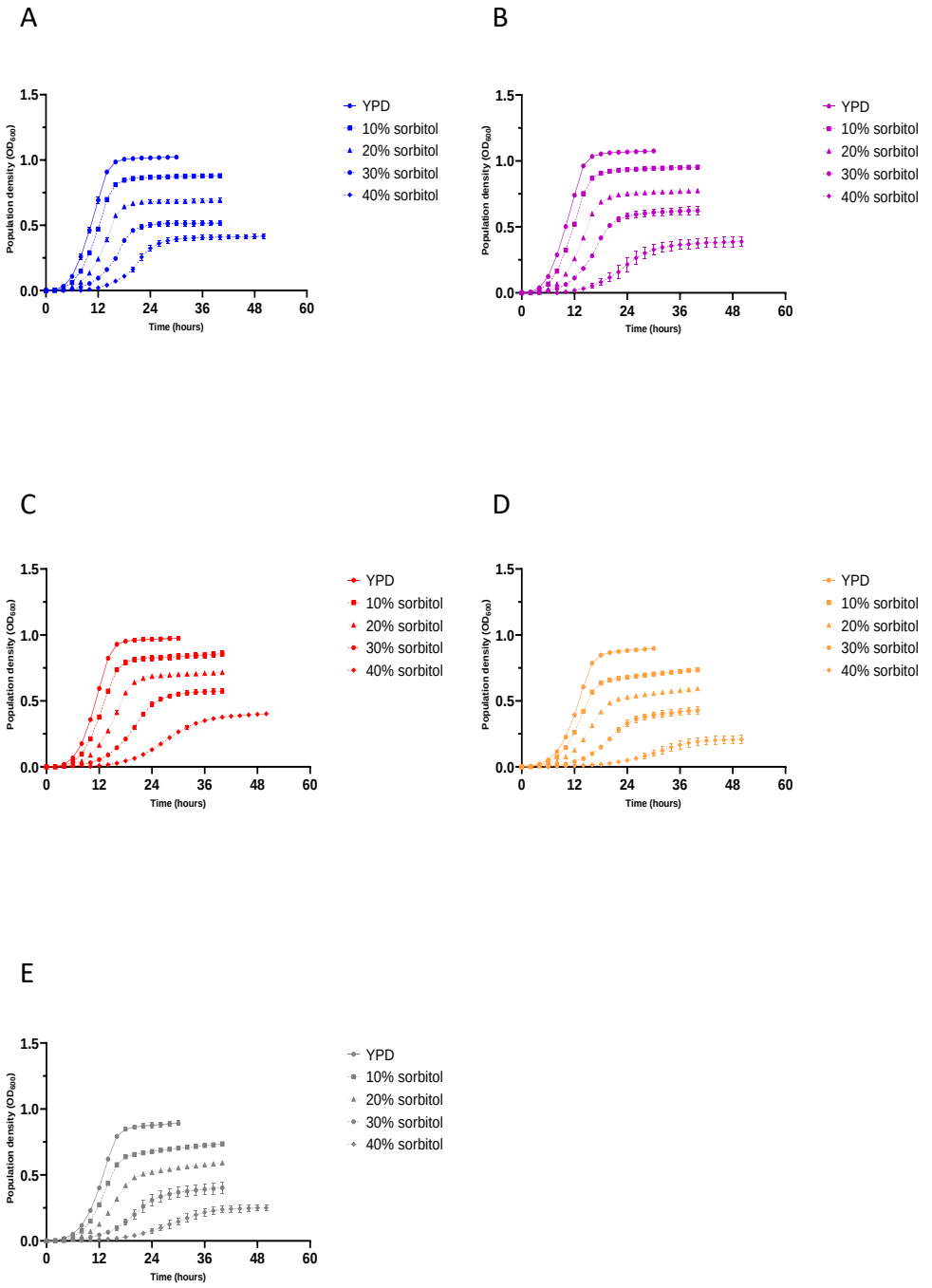


Figure 3.5. Growth analysis of yeast strains (A – M2, B – NCYC 1332, C – SMCC 100, D – CBS 1260, E – CBS 1174) under increasing concentrations of sorbitol. Error bars show the standard deviation between triplicate samples.

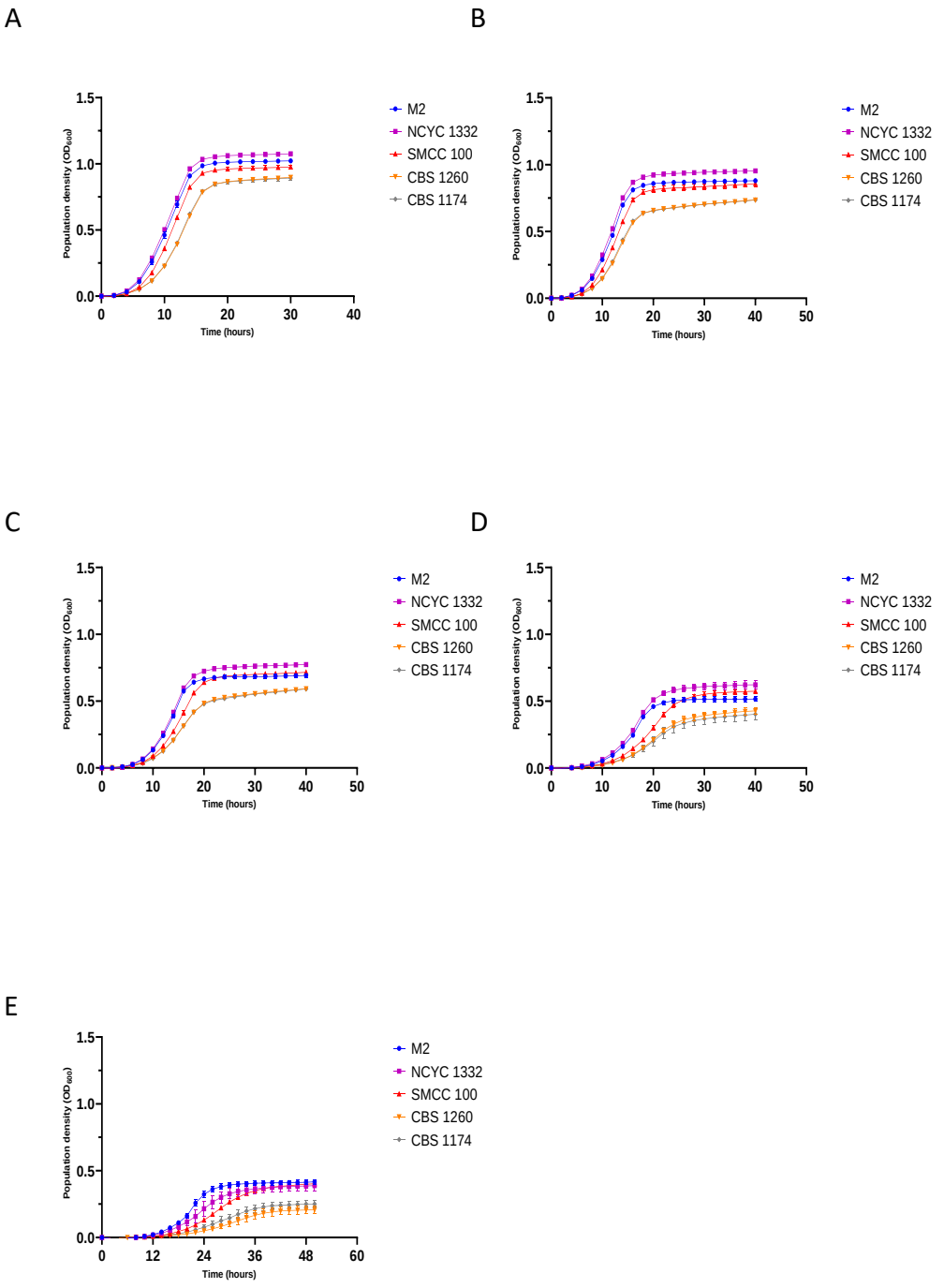


Figure 3.6. Growth analysis of yeast strains M2, NCYC 1332, SMCC 100, CBS 1260, CBS 1174 in YPD media under increasing concentrations of sorbitol (A- 0%, B- 10%, C- 20%, D- 30% and E- 40%). Error bars show the standard deviation between triplicate samples.

In order to compare the stress tolerance of each strain, growth was compared at each concentration of sorbitol. Analysis of biomass production in standard YPD media (Figure 3.6A) showed that ale strains M2 and NCYC 1332 exhibited enhanced growth kinetics when compared to the lager strains. Of the lager strains, SMCC 100 conveyed an enhanced growth performance when compared to CBS 1260 and CBS 1174, with the latter behaving similarly, despite CBS 1260 sharing a closer lineage with the SMCC 100 strain (Section 3.2.2). Generally, it can be seen that each strain reached stationary phase between 14 and 18 hours of growth, with varying levels of maximum growth (optical density) for each strain.

As yeast were exposed to low levels of osmotic stress, induced by supplementation with 10% w/v sorbitol, a similar trend in terms of strain performance was observed. Both of the ale strains outperformed the lager yeasts, while within the lager category SMCC 100 showed faster growth than CBS 1260 and CBS 1174. At this level of stress, the maximum optical density reached by each strain was only slightly reduced when compared to un-supplemented YPD media, indicating that the level of osmotic stress exerted by this concentration of sorbitol did not have a substantial effect on the health of the yeast population. A further reduction in growth rate for all strains was observed when the growth medium was supplemented with 20% w/v sorbitol. Interestingly, at this concentration, although the lager strain SMCC 100 initially appeared to exhibit a sub-optimal growth rate when compared to the ale strains, in the latter stages of the growth cycle the maximum growth capacity was similar to the seemingly more osmotolerant M2 ale strain. Both of the

other lager strains continued to display a slower rate of growth and lower maximum growth overall (Figure 3.6C).

As yeast were exposed to 30% w/v sorbitol, the growth capabilities of all strains became significantly reduced. Using strain M2 as an example, the maximum optical density reached was approximately 50% of that in un-supplemented YPD media (Figure 3.6D). As discussed previously, despite a slower growth rate, strain SMCC 100 showed an enhanced capacity to withstand sorbitol when compared to the other lager strains. Furthermore, at this concentration the maximum growth observed was more than for the ale strain M2. When 40% w/v sorbitol was employed, there was little difference between the maximum growth capacity of strains M2, NCYC 1332 and SMCC 100 (Figure 3.6E). However, differences in growth rate persisted with M2 now being the fastest (compared to NCYC 1332 previously). As for all of the conditions used in this study, strains CBS 1260 AND CBS 1174 were the poorest-performing strains. The capacity of strain SMCC 100 to grow efficiently under osmotic stress indicates that this yeast may have an enhanced ability to adapt to high environmental osmolality, at least when compared to the other two lager strains used in this study.

3.2.5 Fermentation performance at standard and very high gravity

The ability to ferment wort effectively is arguably the most important characteristic of brewer's yeast, and certainly the most direct indication of a yeast strains suitability for brewing. However, individual fermentation characteristics in relation to elevated concentrations of wort, and the impact

that these have on yeast metabolism and flavour generation are also valuable in determining the suitability of a strain for VHG fermentations. In order to assess performance at both standard and very high gravities, each strain was pitched into worts of 16 and 24 ^{o}P gravity in 100 mL 'mini' FVs and monitored for fermentation progression by monitoring weight loss over time according to Section 2.4.2. In the interest of uniformity, all fermentations were carried out isothermally at 15^{o}C, a temperature regime closely associated with the fermentation conditions applied for the industrial strain SMCC 100, and lager yeasts in general. It is acknowledged that this does not reflect the typical temperature associated with fermentation using ale yeasts, however in this instance it was applied to ensure that conditions were the same for all of the strains under investigation, eliminating this as a potential factor influencing the data obtained. Standard gravity conditions included the use of a 16 ^{o}P all-malt, hopped wort in accordance with standard brewing practices, whereas high gravity conditions included the use of a glucose syrup added to the standard wort, increasing the starting gravity to 24 ^{o}P (section 2.2.3).

In each instance, fermentations conducted at 24^{o}P (VHG) took significantly longer to attenuate than at 16^{o}P (SG) (Figure 3.7). This was not unexpected, due to the higher concentration of fermentable sugars present in the starting wort under VHG conditions. Theoretically, more time is needed by the fermenting yeast to convert the sugars present to ethanol and this premise was true for all five yeast strains. However, the time taken for the completion of fermentation under VHG conditions was disproportionately long when compared to that of a SG fermentation for each yeast strain. Using the

industrial strain SMCC 100 as an example (Figure 3.7C), fermentation of a 16 ^{o}P wort was completed after 160 hours, whereas the 24 ^{o}P fermentation was completed after 472 hours. This equates to 2.4 ^{o}P/24 hours for a SG fermentation and 1.22 ^{o}P/24 hours for a VHG fermentation. Although this provides a broad insight into performance, it should be noted that this does not consider the degree of attenuation, or the ABV achieved during fermentation. Based on this, the performance of each yeast under both SG and VHG conditions was compared in order to identify strain-specific properties and to select an appropriate strain for further analysis.

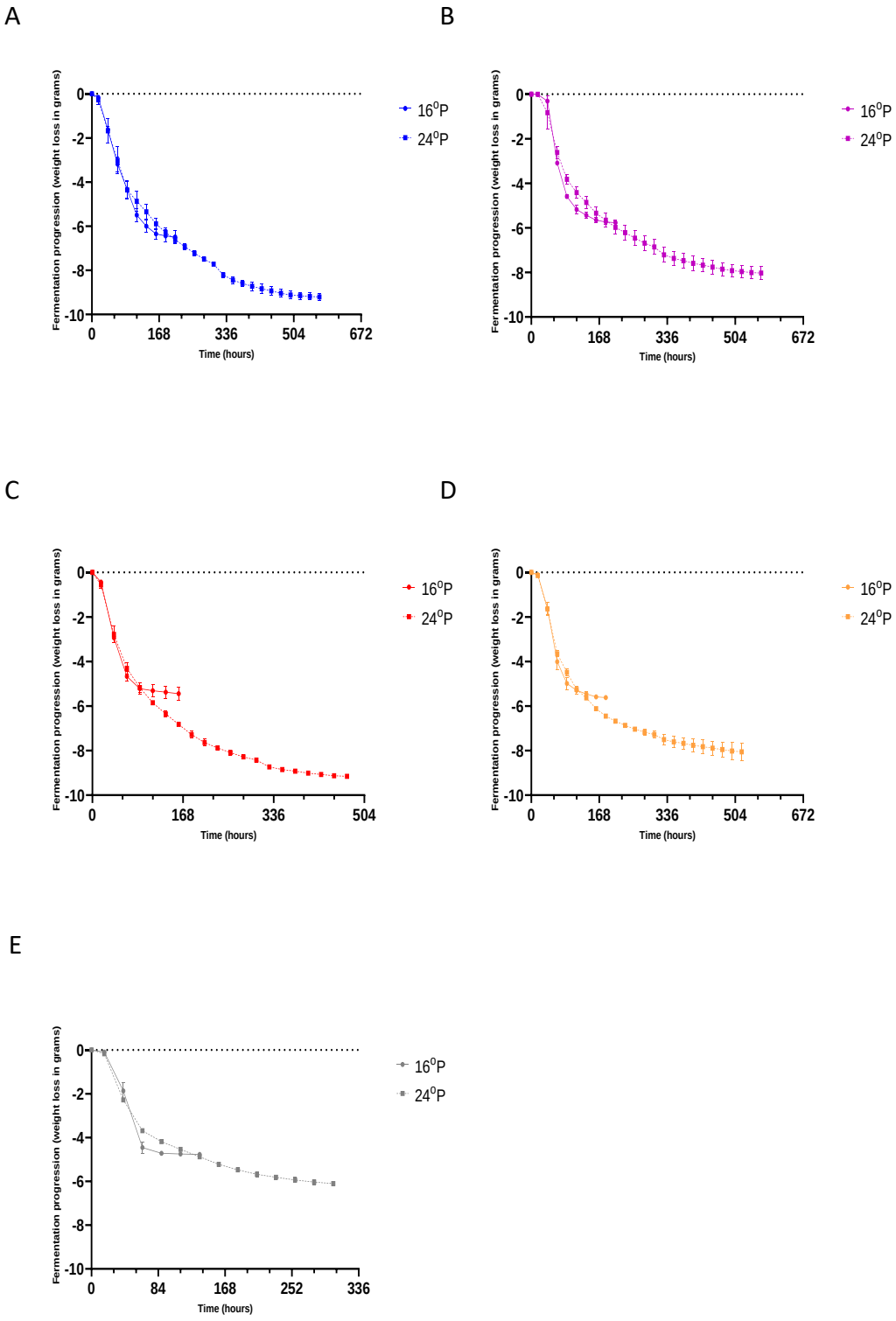


Figure 3.7. Fermentation progression of yeast strains (A – M2, B – NCYC 1332, C – SMCC 100, D – CBS 1260, E – CBS 1174) in standard gravity (16°P) and very high gravity (24°P) wort in 100 mL fermenters. Fermentation progression was monitored

by measuring weight loss over time. Error bars show the standard deviation between triplicate samples.

As seen in Figure 3.8, each yeast strain yielded a distinct fermentation curve at 16^{o}P. Although the two ale yeasts used in this study, M2 and NCYC 1332, share similar properties in terms of their osmotolerance and stress resistance, they exhibited differences in fermentation behaviour. Compared to the other strains investigated in this study, NCYC 1332 showed a relatively sluggish initiation of fermentation with only 0.31 grams ± 0.05 weight loss observed within the first 40 hours after pitching. This was particularly evident when compared to the best-performing strain at this stage of fermentation, strain SMCC 100, which exhibited a weight loss of 2.93 grams ± 0.16 after 40 hours. However, both ale strains attenuated with the highest ABV and relative degree of fermentation (RDF) as shown in Table 3.1, also reflected by total weight loss analysis (Figure 3.8).

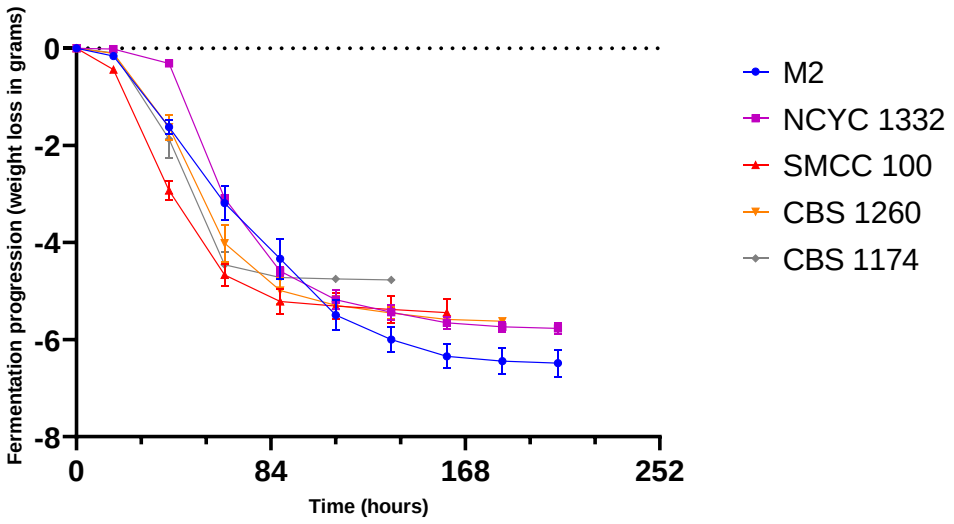


Figure 3.8. Fermentation of standard gravity (16 °P) wort by brewing yeast strains. Wort at 16°P was fermented in 100 mL fermenters and progression was monitored by measuring weight loss over time. Data points represent the mean of triplicate samples, with error bars indicating the standard deviation.

The lager strains used in this study also produced different fermentation characteristics at standard gravity. Frohberg strains SMCC 100 and CBS 1260 yielded similar results in terms of end product specification, with no statistical difference found between the ABVs of the green beer produced at standard gravity by each strain (Table 3.1) ($p \leq 0.05$). However, SMCC 100 showed an enhanced performance when compared to CBS 1260 in terms of the fermentation rate, as indicated by the speed at which attenuation was reached (Figure 3.8), finishing 24 hours sooner. When CBS 1260 and CBS 1174 were compared, a similar trend in fermentation progression was observed within the first 64 hours after pitching, although CBS 1260 completed with a lower final

gravity and higher ABV. Under these conditions, Saaz strain CBS 1174 had the lowest relative degree of fermentation (RDF), undesirable from a yield perspective, albeit with the lowest time to attenuation of all the strains used in this study.

Table 3.1. End of fermentation specifications for green beer produced using each strain. Fermentations were conducted in 16 °P (standard gravity) wort at 15 °C. All values represent the mean of triplicate samples ± the standard deviation.

Strain	ABV (% v/v)	Final Gravity (°P)	RDF (%)
M2	7.52 ± 0.09	2.06 ± 0.08	72.13 ± 0.27
NCYC 1332	7.41 ± 0.14	2.10 ± 0.11	71.85 ± 0.48
SMCC 100	7.00 ± 0.22	2.20 ± 0.08	70.80 ± 0.27
CBS 1260	7.03 ± 0.18	2.20 ± 0.12	70.82 ± 0.78
CBS 1774	6.32 ± 0.00	4.50 ± 0.01	60.15 ± 0.02

As shown in Figure 3.7, increasing wort gravity from SG to VHG also led to an increase in fermentation time. One of the potential contributors to the disproportionately elongated fermentation times observed is the presence of elevated environmental stress associated with VHG conditions (Puligundla et al., 2011). However, as discussed previously (Section 3.2.4.), the response to osmotic stress is strain specific, with some strains exhibiting an enhanced ability to proliferate under such conditions compared to others. As such, it was

anticipated that yeast strain performance under VHG conditions would also be variable. In order to assess the impact of high gravity on performance, each strain was pitched into 24 °P and fermentation characteristics were determined.

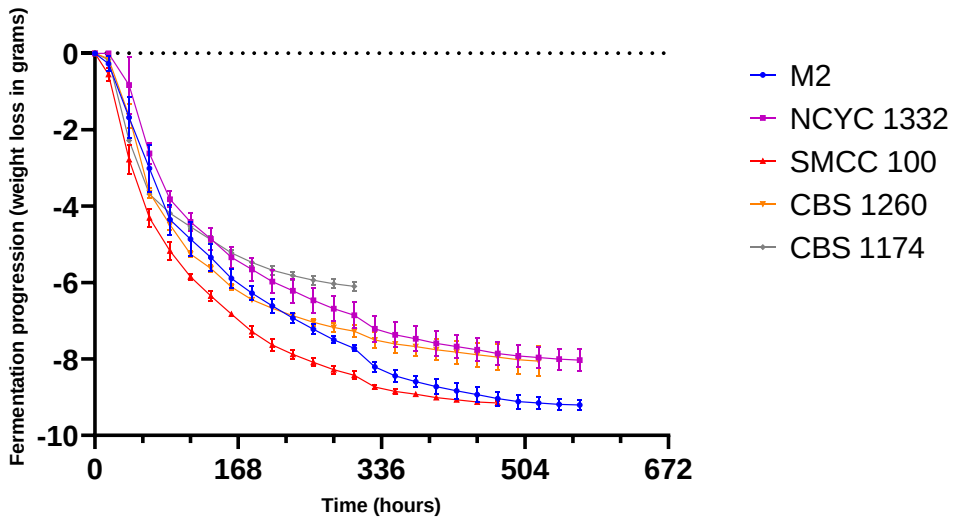


Figure 3.9. Fermentation of 'very high gravity' (24 °P) wort by brewing yeast strains. Wort at 24°P was fermented in 100mL fermenters and progression was monitored by measuring weight loss over time. Data points represent the mean of triplicate samples, with error bars indicating the standard deviation.

Interestingly, although the ale strain M2 achieved the greatest weight loss overall, this was not reflected in the green beer analysis (Table 3.2), with final characteristics (including ABV) being statistically similar to M2, NCYC 1332 and CBS 1260. However, the lager yeast SMCC 100 was the best-performing strain at VHG (Figure 3.9; Table 3.2). Under VHG conditions, this strain produced the highest concentration of ethanol while also reaching attenuation faster than all

of the other strains. The Saaz lager yeast CBS 1774 exhibited the weakest fermentation performance when compared to other strains, attenuating at a final gravity of 10.23 °P ± 0.29 after 304 hours, with a final ABV of 7.91 % (v/v) ± 0.16.

Table 3.2. End-product specifications for yeast strains following fermentation using 24 °P (very high gravity) wort. All values expressed is the mean of triplicate samples.

Strain	ABV (% v/v)	Final Gravity (°P)	RDF (%)
M2	10.42 ± 0.10	5.59 ± 0.37	64.81 ± 1.08
NCYC 1332	10.51 ± 0.16	5.55 ± 0.33	65.05 ± 1.09
SMCC 100	11.58 ±0.20	3.26 ± 0.07	72.46 ± 0.05
CBS 1260	10.37 ± 0.45	5.67 ± 0.66	64.54 ± 2.35
CBS 1774	7.91 ± 0.16	10.23 ± 0.29	49.16 ± 1.00

The performance of the ale strain M2 and the lager yeast SMCC 100 can at least partly be explained by the capacity to tolerate osmotic stress. Both of these strains exhibited the greatest resistance to these stress factors (Section 3.2.4) in each brewing category. Similarly, the poor performance of strain CBS 1774 can likely be explained by the relatively limited capacity of this strain to withstand osmotic stress as well as the inability of this strain to utilise maltotriose (as a Saaz-type lager strain) resulting in a lower degree of extract

uptake resulting in lower ABVs. It is likely that a combination of poor growth and sensitivity to stress resulted in the fermentation arresting at a relatively high gravity. Although SMCC100 performed the best at VHG, with a reduced attenuation time coupled with increased extract uptake and favourable ABV, this was not reflected at standard gravity suggesting that this strain is better suited to fermenting at higher gravities.

3.2.6 Volatile flavour profile of beers produced at standard and very high gravity

Although it is accepted that the production of flavour compounds is strongly influenced by fermentation conditions and raw materials, the yeast strain employed can also result in differences in end-product flavour profile. Related to this, in order for a fermentation regime at VHG to be viably implemented, the flavour profile of the resulting beer must be acceptably similar to that of the beer produced at standard gravity. In order to investigate the differences in flavour compound production at both standard and high gravity for the five yeast strains used in this study, the volatile flavour compounds (esters and higher alcohols; Section 1.3.4) present in the green beer were analysed by GC-MS according to the method outlined in Section 2.4.5. The data obtained was then mathematically normalised to reflect the flavour profile of a 5% ABV beer. Flavour volatile profiles for each strain were compared firstly between SG and VHG conditions, and secondly against each of the other strains used in this study. Statistical differences were determined according to the analysis outlined in Section 2.4.6.

The flavour profiles of beer produced by each of the five strains at standard and very high gravity can be seen in Figure 3.10. In each case, the flavour profile of the beer produced at VHG differed significantly in at least one of the flavour volatiles analysed, when compared to that of a standard gravity-produced beer. For example, isoamyl alcohol levels were lower in all VHG beers, although this difference was not significant for strain CBS 1260. Conversely, the concentration of ethyl acetate was greater for all strains at VHG, although this difference was not significant for CBS 1174. There were also differences in the production of other higher alcohols; levels of isobutanol were found to be elevated in the VHG-produced beers for M2 and CBS 1174, with 1-propanol concentrations reduced in the VHG beer produced with strain NCYC 1332. Although this may suggest a disproportional upregulation of alcohol acetyltransferase genes (Verstrepen et al., 2003b) under stressed conditions, there are many other factors which can contribute to higher alcohol production, including basic fermentation attributes such as yeast growth. Although further analysis would be required to fully elucidate the causes behind the observations here, it should be noted that the goal was not to product match, but to identify key differences between the properties of the 5 yeast strains analysed at SG and VHG.

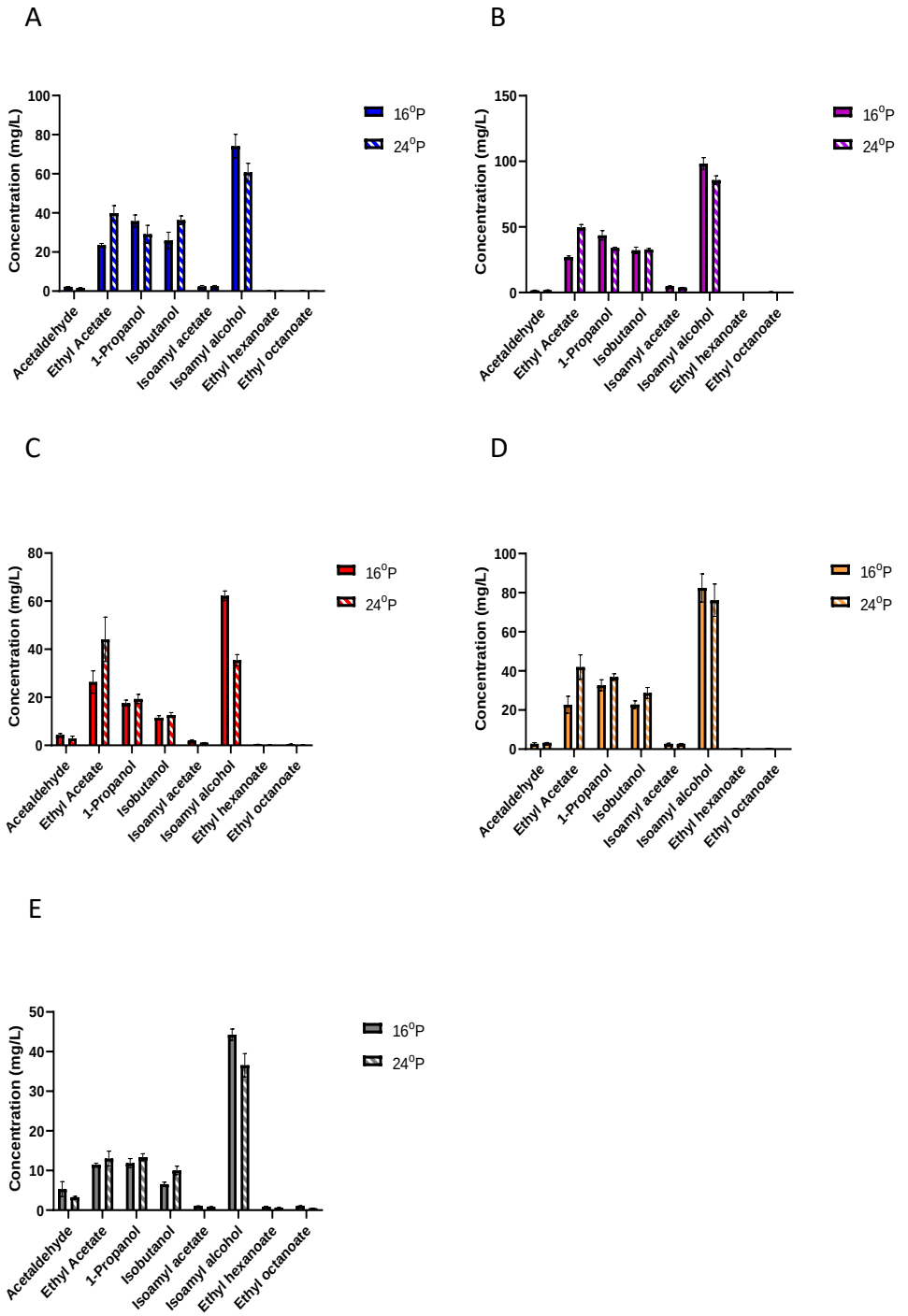


Figure 3.10. The influence of very high gravity fermentation conditions on yeast volatile flavour compound production. Green beer flavour compounds were analysed following fermentations at 16 °P and 22 °P with five brewing yeast strains, M2 (A), NCYC 1332 (B), SMCC 100 (C), CBS 1260 (D) and CBS 1174 (E). The

concentration of each flavour compound was normalised based to a 5% ABV beer. The y axis in each instance was altered to reflect the data present. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

The trends in flavour production between SG and VHG-produced beers were broadly conserved between all strains; compounds that were statistically different in a VHG product were uniformly higher or lower than their SG counterpart. In order to further investigate the differences in flavour volatile production by each yeast strain at standard and very high gravity, the flavour compound profiles of each strain were compared. Although each individual strain produced a unique volatile flavour compound profile overall, analysis of individual flavour compound production revealed some similarities between yeasts (Figure 3.11). In terms of the higher alcohols analysed in 5% ABV beers derived from SG fermentations, no significant differences were found between the concentrations of 1-propanol or isobutanol between M2 and CBS 1260, or between strains SMCC 100 and CBS 1174. However, the concentrations of isoamyl alcohol were found to be unique for all strains used (Figure 3.11A), as all concentrations were found to be statistically different ($p \leq 0.05$). The yields of higher alcohols for strains SMCC 100 and CBS 1174 were constantly lower than for the other strains, while NCYC 1332 yielded the highest concentration in each instance (Figure 3.11A). Contrary to the low levels of higher alcohols produced by CBS 1174, this strain produced higher concentrations of the ethyl esters (ethyl hexanoate and ethyl octanoate) than all of the other strains

(Figure 3.11B.). Despite SMCC 100 sharing a similar trend in terms of higher alcohol production to strain CBS 1174, this resemblance was not reflected in the yields of ethyl esters between the two strains, with lower concentrations produced by SMCC 100 in each case.

The lager strain CBS 1174 remained an outlier in terms of its acetate ester profile, with the strain producing the lowest concentrations of both ethyl acetate and isoamyl acetate (Figure 3.11C). Aside from CBS 1174, there was no significant difference between the concentrations of ethyl acetate produced by the other four strains. Although the mean concentration of isoamyl acetate produced by CBS 1174 was found to be the lowest analysed, this was not a statistically significant event; the concentration of isoamyl acetate produced by all strains was similar overall.

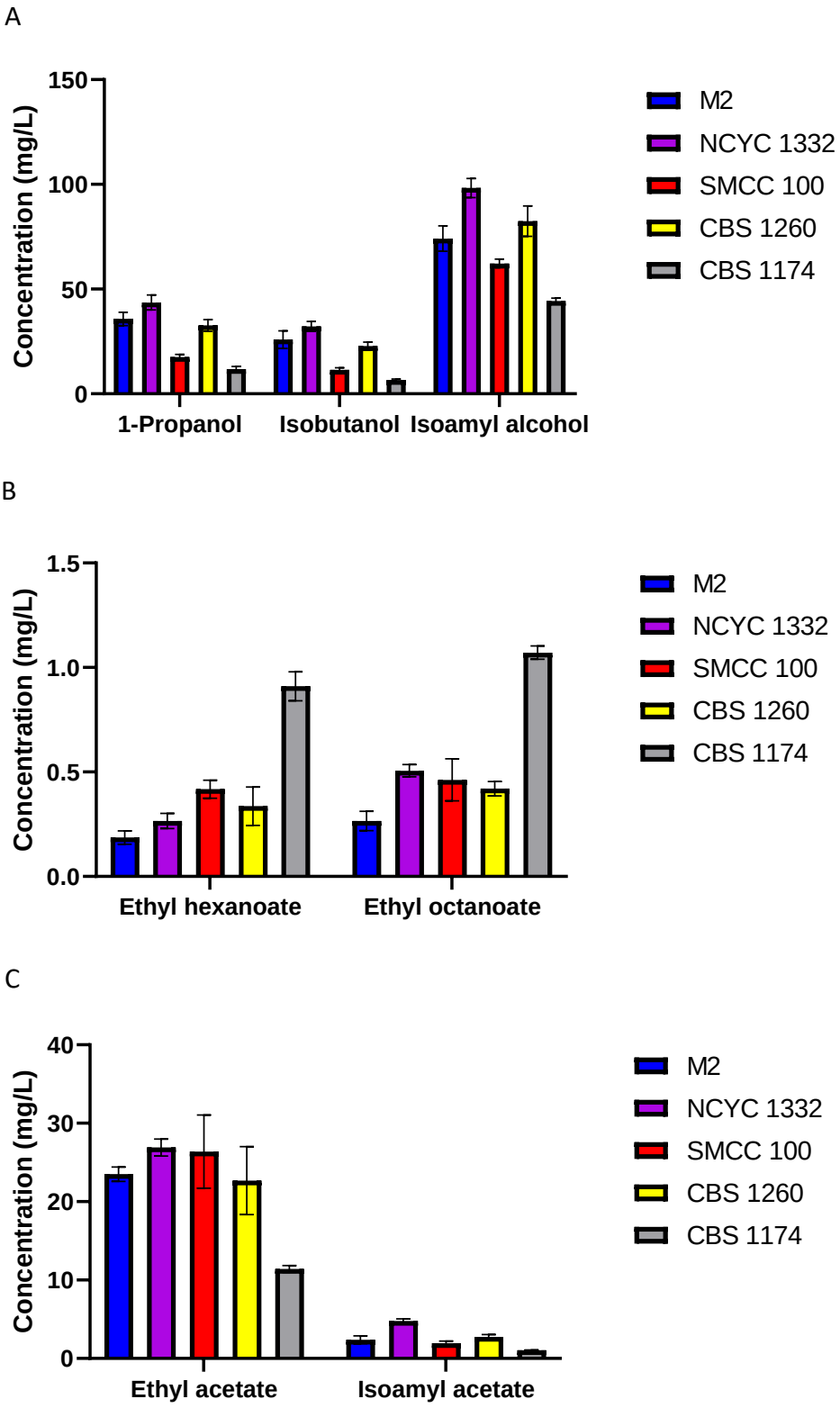


Figure 3.11. Concentration of (A) higher alcohols, (B) ethyl esters and (C) acetate esters as would be present in 5% ABV beers produced by five brewing strains

following fermentation of 16°P wort. Error bars indicate the standard deviation of triplicate samples.

In order to investigate the impact of wort gravity on flavour development, the volatile flavour profile was analysed following fermentation of 24 °P wort. Given that the total ABV in the final beer was elevated in each case, the final concentration of volatile compounds was again normalised to reflect the flavour compound composition of a 5% ABV beer as described previously (Section 2.4.6). The general trend in higher alcohol flavour profile remained consistent between all strains at both high and standard gravity, however some key differences were observed in terms of the relationship between strain flavour compound profiles. Whereas a statistically significant difference was observed in 1-propanol and isobutanol between strains NCYC 1332 and CBS 1260 in beer produced at standard gravity, the concentrations of these compounds were not statistically different ($p \geq 0.05$) at very high gravity (Figure 3.12A). Conversely, at standard gravity, the volatile compound profile of isoamyl alcohol was found to be unique across all strains, however, at very high gravity there was no significant difference between strains SMCC 100 and CBS 1174 with both yielding a comparatively low concentration of this flavour compound (Figure 3.12A).

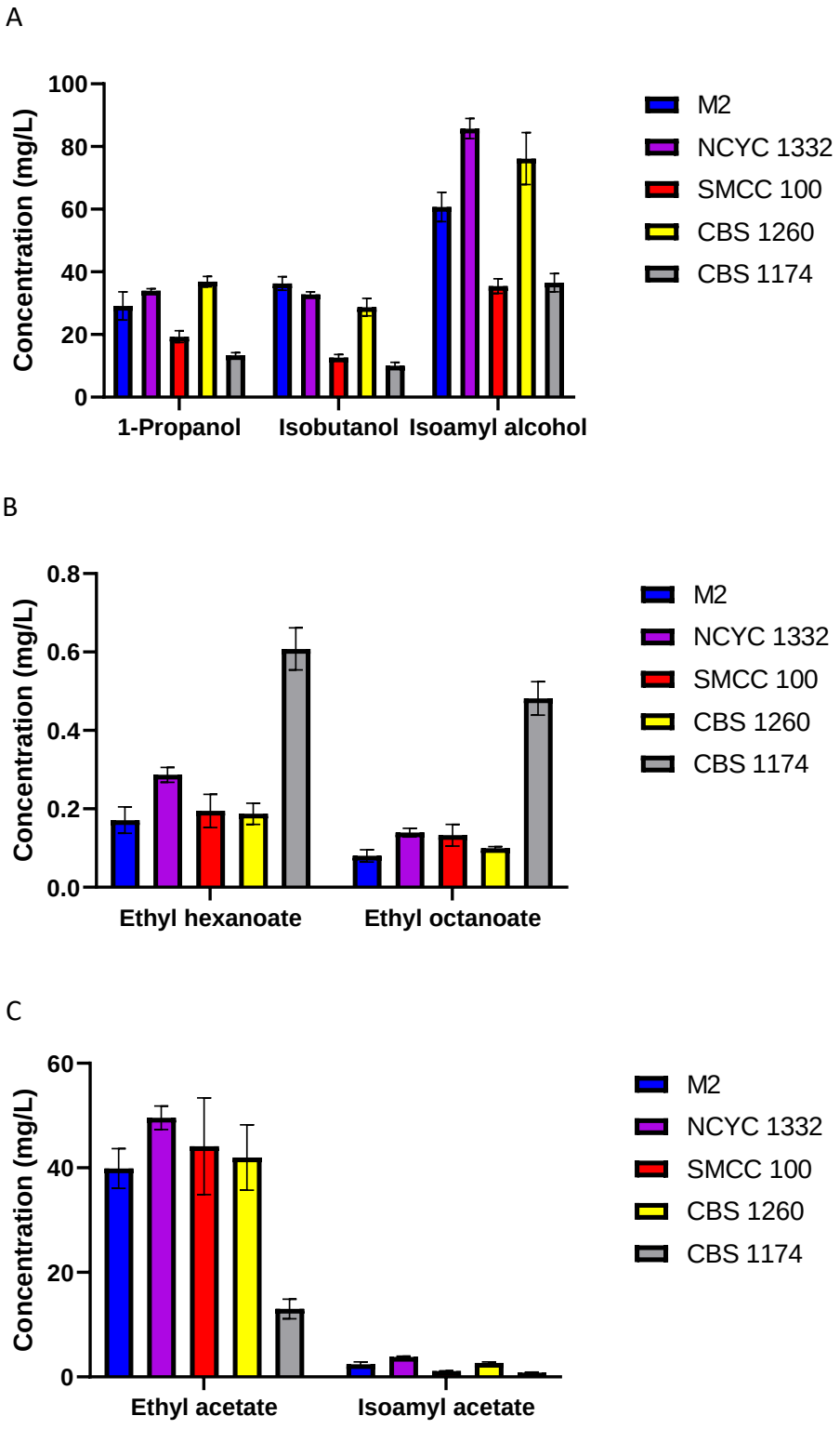


Figure 3.12. Concentration of (A) higher alcohols, (B) ethyl esters and (C) acetate esters as would be present in 5% ABV beers produced by five brewing strains

following fermentation of 24°P wort. Error bars indicate the standard deviation of triplicate samples.

The concentration of both ethyl esters analysed remained statistically higher for strain CBS 1174, similar to the results obtained from the standard gravity beer. However, at high gravity, the concentration of ethyl hexanoate was found to be similar between yeast strains M2, SMCC 100 and CBS 1260, which was not the case for the standard gravity beer. In this instance concentrations were also greater in beer produced using NCYC 1332, but still remained lower than for CBS 1174 (Figure 3.12B). No difference was observed in ethyl octanoate production between strains, aside from CBS 1174, as mentioned above. The trend in acetate ester profile between standard and high gravity was largely similar between yeasts. All strains yielded a similar profile, except for CBS 1174 which continued to be significantly lower in ethyl acetate, and for strain M2 which was statistically lower than NCYC 1332 only (Figure 3.12C). Similar to the results obtained from standard gravity beer, the concentration of isoamyl acetate was not found to be significantly different between any of the yeast strains analysed.

When directly comparing the data obtained from standard and high gravity fermentations, the general trend was that the concentration of higher alcohols and ethyl esters was lower than at standard gravity. Of the acetate esters, isoamyl acetate remained similar, while ethyl acetate was highly elevated (Figure 3.12C). The reasons for this are currently unknown, however it is

suggested that fermentation parameters could be altered to address both the impaired fermentation performance but also flavour compound imbalance. For example, yeast growth is a determinant of dissolved wort oxygen levels, in which wort aeration was not altered between the two conditions. The effect of insufficient wort oxygen levels could therefore have a negative impact on yeast growth which would therefore also impact higher alcohol production. Furthermore, the inhibitory effect of oxygen on yeast esterase gene expression would therefore also be a potential source of flavour imbalance when comparing SG and VHG-produced beers if wort oxygen is not altered accordingly.

Other contributing factors to altered fermentation-derived beer flavour compound production are yeast pitching rate and fermentation temperature. These parameters were kept uniform amongst all strains despite this not reflecting best practice for both VHG fermentations and fermenting using an ale yeast (O'Connor-Cox and Ingledew, 1990; Erten et al., 2007). Therefore. The fermentation regime may not have been appropriate for VHG conditions, which could have a higher requirement for increased wort oxygen levels or pitching rate. This could not only have impacted the flavour development, but also the fermentation performance (Section 3.2.5). Further analysis is needed to confirm the impact of fermentation conditions and alignment of fermentation parameters on yeast flavour production, with the inclusion of other yeast-derived beer flavour compounds such as diacetyl which was not included in the scope of this study.

3.3 Discussion

The aim of this body of work was to characterise and identify the brewing yeast strains M2, NCYC 1332, SMCC 100, CBS 1260 and CBS 1174 and subsequently to investigate the fermentation characteristics of each strain under standard and very high gravity conditions. This was achieved using a combination of phenotypic, metabolic and genetic analysis. Using the ability of ale yeasts to tolerate higher growth temperatures than lager yeast, it was concluded that M2 and NCYC 1332 were ale-type yeasts and that the other strains were lager yeasts. This was further confirmed using X-α-gal analysis, which demonstrated melibiase activity, associated with lager strains, in SMCC 100, CBS 1260 and CBS 1174. Subsequently, PCR amplification and RFLP of the ITS region of the yeast genome identified strain CBS 1174 as a Saaz/Type I lager yeast, with SMCC 100 and CBS 1260 belonging to the Frohberg/Type II group. Finally, fingerprinting using interdelta PCR was able to confirm that each strain was unique.

Once the genetic make-up of strains had been confirmed, stress tolerance was determined. As osmotic stress is one of the most prolific stresses associated with very high gravity brewing (Pratt et al., 2003; Gibson et al., 2007), the ability of each strain to tolerate and proliferate under high extracellular osmolality was assessed. As concentrations of sorbitol increased (and therefore osmotic stress increased), the growth rate of each strain decreased. However, the ale strains M2 and NCYC 1332 generally exhibited enhanced good growth capabilities in the presence of high extracellular osmolality. Of the lager yeasts,

strain SMCC 100 was the most osmotolerant, with CBS 1260 and CBS 1174 performing relatively poorly when exposed to high environmental osmolality.

In order to explore whether osmotic stress tolerance translates to fermentation performance at very high gravity, the fermentation characteristics of each strain were compared using an all-malt standard gravity wort of 16^{o}P and a very high gravity (24^{o}P) wort created using a sugar adjunct. As expected, the key artefact of increasing gravity was to increase fermentation time and the amount of alcohol present in the final product, irrespective of yeast strain. However, at high gravity, strain CBS 1174 performed poorly when compared to the other strains in terms of attenuation limit and alcohol production. Ale strain M2 produced the greatest weight loss, but this was not reflected in final gravity analysis. Furthermore, this strain required an extended period of time to reach attenuation when compared to the lager strains CBS 1260 and SMCC 100. Despite the two ale strains exhibiting higher growth characteristics under conditions of osmotic stress, it was the lager strain SMCC 100 that achieved the highest ABV and the greatest reduction in wort gravity during VHG fermentation. It should be noted that although SMCC 100 did not appear be as osmotolerant as the two ale yeasts overall, the rate and extent of growth in Strain SMCC 100 was observed to be less impacted under elevated concentrations of sorbitol (i.e. the strain performed worse than the ale strains under conditions of weak stress, but more similarly when high levels of stress were applied), so this result was not entirely unexpected. When compared to the other strains, SMCC 100 attenuated approximately 48 hours faster than CBS 1260 and around 96 hours faster than the two ale yeasts.

In addition to fermentation performance, the production of flavour-active compounds produced at different gravities was also considered. Due to the differing final beer characteristics, the volatile flavour compound data was normalised to the amount of alcohol generated and standardised to a 5% ABV beer. The production of some flavour compounds, including isoamyl alcohol, yielded highly variable results for all strains. Conversely, compounds such as isoamyl acetate were found to be very similar, with no significant difference between the yeast strains analysed. Only some of the trends observed at standard gravity were present in the beer analysed following very high gravity fermentations, with key differences in the absolute concentrations present in some flavour compounds. As the resultant beers were not tasted, the sensorial impacts of the concentration of flavour compounds is speculative.

Based on the data here it can be seen that strain-specific tolerances to osmotic stress did not directly correlate to improved fermentation performance at VHG. As VHG fermentations invoke a variety of increased stress factors, including ethanol toxicity and oxidative stress, other strain-specific tolerances may play an important role. For example, the capacity of a yeast strain to effectively regulate stress-response mechanisms such as the high osmolarity glucose (HOG) pathway (Section 1.5.2), to produce protective compounds such as glycerol and trehalose (Albertyn et al., 1994; Hounsa et al., 1998; Saito and Tatebayashi, 2004; Prick et al., 2006; Bandara et al., 2009), or to mitigate stress via intra-cellular activity such as autophagy and repair (Cebollero and Gonzalez, 2006; Prick et al., 2006; Knorre et al., 2013b). Furthermore, the ability of a yeast cell to adapt its internal physiology in response to stress, for example to

preserve or reinforce key structures such as the plasma membrane, the vacuole and the mitochondria, as well as other sites that are affected by fermentation-derived stresses will likely equip a yeast strain to effectively tolerate stressful conditions. These fundamental aspects of cell physiology were selected for further analysis in response to stress. Based on the data obtained, strain SMCC 100 was identified as displaying favourable fermentation properties under VHG conditions, as well as being a valuable commercial yeast. Consequently, this yeast was selected for detailed physiological analysis in subsequent Chapters.

CHAPTER 4: THE IMPACT OF OSMOTIC STRESS ON YEAST HEALTH, FERMENTATION PERFORMANCE AND ORGANELLE MORPHOLOGY

4.1 Introduction

In order for a yeast population to survive the challenging environment encountered during fermentation, it must rely on the ability of individual cells to adapt their functionality, physiology and morphology in response to stress factors (Zakrzewska et al., 2011). One of the principle stresses encountered during fermentation is osmotic stress (Hounsa et al., 1998; Pratt et al., 2003), arising from high levels of sugar in wort upon pitching, but also associated with the increase in ethanol concentration that occurs as fermentation progresses (Pratt et al., 2003; Zhuang et al., 2017). Thus, there is significant overlap between the osmotic impact of sugar in wort and the increased osmolarity encountered as a result of elevated ethanol concentrations. Yeast are able to sense changes in osmotic pressure and react rapidly, triggering a cascade of gene pathways involved in the adaptation of physiology to tolerate the new conditions (Causton et al., 2001; Zakrzewska et al., 2011). When the gravity of a wort is increased (i.e. characterised by a higher concentrations of sugar than is typically applied), yeast are exposed to increased extracellular stress leading to impaired yeast health (Gibson et al., 2007). Despite the capacity to respond to stress, the increase in osmotic pressure can manifest in a number of ways, including reduced yeast viability, vitality, and impaired fermentation performance (D'Amore et al., 1988; Pratt et al., 2003; White et al., 2008).

Within the brewing industry it is common practice to assess the health of a yeast population by analysis of viability using methylene blue (Pierce, 1970).

However, while useful from a production perspective, this technique gives little insight into the physiological state of the yeast. In order to greater understand the effects of stress on the yeast cell, other methods can be employed. Many of these involve the use of fluorescent staining procedures to visualise key components of the cell. In this way, recent applications of live cell fluorescent imaging techniques and flow cytometry have enhanced the understanding of the effects of various stress factors on aspects of yeast health, by analysing plasma membrane depolarisation (Simonin et al., 2007; Capusoni et al., 2019), mitochondrial membrane potential (Ludovico et al., 2001) and cell vitality (Hernlem and Hua, 2010). This has led to insights into the role of mitochondrial membrane potential in maintaining yeast health and viability (as well as mitochondrial health) and the impact of cellular damage on the plasma membrane. Although organelle morphological changes in response to environmental stress have been well documented in laboratory strains, little is known about the effects of fermentation-related stress on organelle morphology in polyploid industrial yeast strains, and their potential link to fermentation performance. Although all of the components of the yeast cell are essential for functionality, two particularly important subcellular constituents involved in the yeast stress response are the vacuole (Izawa et al., 2010) and the mitochondria (Knorre et al., 2013a). Furthermore, the yeast plasma membrane is an essential component of cellular functionality, and one that is negatively impacted by osmotic stress (Simonin et al., 2007).

Vacuoles are dynamic organelles that act not only as storage organelles, but also play a major role in autophagy through the adoption of lysosomal

functionality (Journo et al., 2008; Li and Kane, 2009). Mitochondrial involvement in fermentation remains somewhat of a mystery, due to the process being mainly anaerobic (O'Connor-Cox et al., 1996; Boulton and Quain, 2001). However, studies have linked mitochondrial dynamics and functionality to adaptation to environmental stress (Shutt and McBride, 2013; Kitagaki and Takagi, 2014), suggesting that mitochondria may also play an integral role in determining survival of cells when exposed to fermentation-related stress factors. The plasma membrane is also essential for yeast survival, however permeabilisation and depolarisation of the plasma membrane as a result of extracellular stress can result in cell death (Mizoguchi and Hara, 1998).

Both the vacuole and mitochondria can be visualised using fluorescent staining coupled with confocal microscopy. The yeast tonoplast can be stained using FM 4-64™, which initially binds to the plasma membrane and then is endocytosed during a pulse-chase staining procedure where it binds to vacuole membranes within the cell (Vida and Emr, 1995). Mitochondrial morphology can also be studied with confocal microscopy using fluorescent probes, which can vary in their mode of action. Mitochondrial stains such as rhodamine 123 function based on mitochondrial membrane potential (MMP), while others including Mitotracker Green FM bind to mitochondrial proteins within the matrix (Dinsdale et al., 1995; Chazotte, 2011). The aim of this study was to examine the physiological effects of heightened extracellular stress and the impact of stress on yeast vacuole morphology, mitochondrial dynamics and plasma membrane health. Furthermore, the consequences of heightened stress on fermentation performance and cell physiology will be assessed.

4.2 Results

In order to determine the effects of osmotic stress on organelle morphology, YPD media and brewing wort were independently supplemented with 30% w/v sorbitol. Sorbitol is a non-assimilable carbohydrate that induces osmotic pressure on a yeast population without altering the nutritional composition of the growth or fermentation medium or inducing toxic effects on yeast (Hirasawa et al., 2006). Consequently, it can be used to effectively study the isolated effect of osmotic stress on yeast quality and physiology. The concentration of sorbitol used was selected due to preliminary data indicating that the level of osmotic stress exerted on the yeast population was sufficient to impair yeast growth, while not completely inhibiting it. Furthermore, previous work has shown that the osmotic effect of 30% w/v sorbitol is approximately the same as the maximum osmolarity experienced during a VHG fermentation (Zhuang et al. (2017). In this study, the yeast strain SMCC 100 was selected for analysis due to its industrial relevance, but also due to the enhanced performance of this strain under VHG conditions as reported in Chapter 3.

4.2.1 Effect of sorbitol-induced osmotic stress on yeast growth

The effects of heightened osmotic stress on yeast growth and fermentative performance were examined through the use of growth media supplemented with 30% w/v sorbitol as described in Section 2.3.4. Yeast was inoculated at a concentration of 1 x 10^6 cells/mL and incubated at 25 ^oC, shaking at 100 rpm, as described in Section 2.2.1. Once the yeast had been inoculated into the

appropriate medium, growth was determined by measuring the optical density of the inoculum until stationary phase had been reached (Section 2.3.4). The effect of sorbitol supplementation was then studied to assess the effect of extracellular osmotic stress on cell proliferation, against a control sample prepared in standard un-supplemented YPD media.

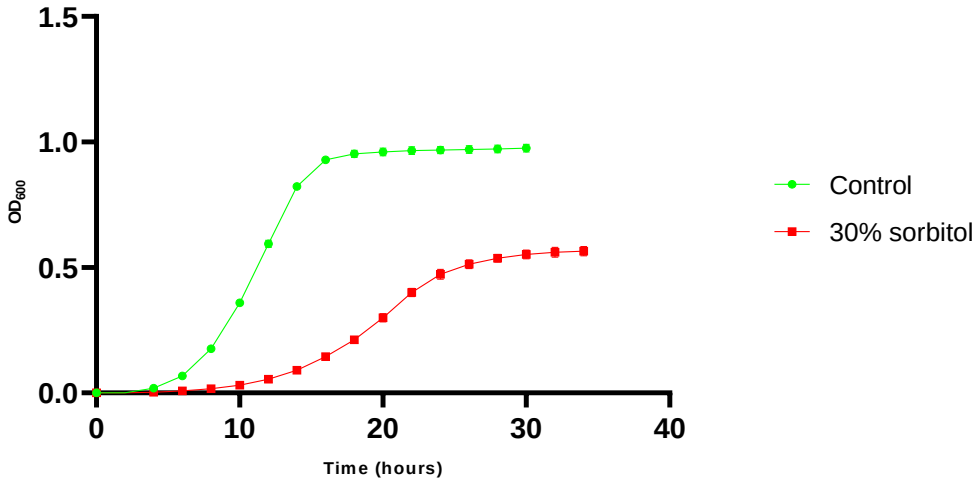


Figure 4.1. The effect of osmotic stress on yeast cell growth. Oxidative stress was created through supplementation of YPD growth medium with 30% (w/v) sorbitol. Yeast cell growth was measured by monitoring growth in 96 well plates and determined through analysis of optical density at 600 nm. Data points represent the mean of triplicate samples, with error bars indicating the standard deviation at each time point. In cases where the error bars are too narrow, these will not be visible in the figure.

When yeast was exposed to standard growth medium without any stress, growth was observed to occur rapidly as shown in Figure 4.1. Under these

conditions, yeast entered the log phase of growth approximately 8 hours after inoculation. This phase lasted for approximately 8 hours when cell growth began to decelerate. Following a short lag phase, yeast cell growth arrested at around 20 hours, by which time the population had reached a maximum cell density, corresponding to an optical density of approximately 1. It can be seen that when yeast cells were stressed through the addition of 30% sorbitol, yeast growth rate was dramatically reduced. The initial lag phase was extended until approximately 18 hours post-inoculation. This was followed by a relatively short log phase during which a minimal increase in optical density was observed compared to the control conditions. At 32 hours, yeast growth began to plateau at an optical density amounting to ~50% of the control conditions, indicating that the influence of osmotic stress induced by this concentration of sorbitol was sufficient to reduce yeast growth by half.

4.2.2 Effect of osmotic stress on yeast viability and cell membrane health

In order to investigate the effects of increased osmotic stress on yeast viability and plasma membrane health, yeast cells were exposed to YPD media supplemented with 30% w/v sorbitol for 24 hours as described in Section 2.2.1. In parallel, control samples were cultivated in standard un-supplemented YPD for the same period of time. Cells from both conditions were then analysed for viability and membrane depolarisation as a result of cellular damage using the fluorescent stains propidium iodide (PI) and $DiSBAC_2(3)$ (BOX) respectively (Section 2.5.1), with high levels of membrane depolarization and a high

percentage of dead cells within the population indicating negative yeast health . PI enters dead cells where it binds to DNA (Deere et al., 1998) and is used in this instance as a measure of cell viability. The fluorescent stain BOX also enters cells via compromised plasma membranes where it binds to intracellular membranes, however it can enter living cells with damaged membranes (Epps et al., 1994; Hewitt and Nebe-Von-Caron, 2001). Stained cells were visualised and quantified using flow cytometry according to Section 2.5.5. Using these two stains, the proportion of the yeast population that was still viable, but had sustained damage to the plasma membrane could be estimated. This was achieved through the use of PI to indicate which cells were live or dead, and BOX to determine membrane health through analysis of depolarization where increased BOX-straining indicates membrane damage based on the ability of the stain to enter cells with compromised membranes (Hewitt and Nebe-Von-Caron, 2001; Simonin et al., 2007; Capusoni et al., 2019), which also accounts for living cells that have sustained membrane damage.

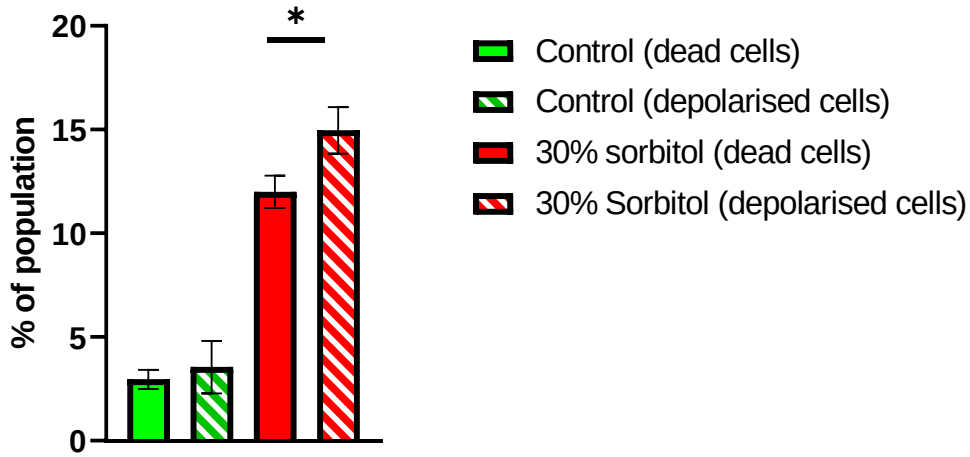


Figure 4.2. The effect of osmotic stress on yeast viability and cell membrane health. Sorbitol (30%) was used to create osmotic stress (red) against an un-supplemented YPD control (green). PI and BOX were used to determine cell viability and membrane depolarisation respectively. Cells stained with PI were considered dead (block colour) and cells stained by BOX were considered to exhibit depolarised membranes (striped). Error bars indicate the standard deviation of triplicate samples. (*) denotes a significant difference ($p \geq 0.05$) between dead cells and depolarised cells.

It can be seen from Figure 4.2 that the presence of 30% sorbitol caused an increase in the percentage of dead cells (12.00 % ± 0.78) within the population after 24 hours, when compared to control cells grown in standard YPD media (2.96 % ± 0.47). Exposure to 30% sorbitol for 24 hours also increased the number of depolarised cells within the yeast population (14.96 % ± 1.12), indicating a greater proportion of cells with poor membrane health when compared to the control cells (3.58 % ± 1.27). Plasma membrane damage as a result of osmotic stress is caused by dehydration of the cell exerting physical pressure on the membrane (Rapoport et al., 1982; Simonin et al., 2007; Capusoni et al., 2019). In stressed yeast samples, cells displayed damaged membranes as indicated by increased depolarisation against control samples. For the experimental sample, as the total percentage of depolarised cells was significantly greater than that of dead cells, it can be concluded that this data was not purely reflective of cell death, but that a number of living cells also

exhibited compromised or damaged membranes. There was no significant difference between the number of dead cells and the number of depolarised cells in the control group, suggesting that live cells had not sustained any membrane damage (Figure 4.2). This set of data was perhaps unsurprising since it is known that osmotic stress acts upon the plasma membrane by causing physical strain to the structure as a result of cell dehydration (Adya et al., 2006; Simonin et al., 2007). However, this does indicate that the presence of an osmotic challenge due to growth environment can result in a significant amount of damage to the plasma membrane of brewing yeast, resulting in a reduction in both the viability and vitality of cells under these conditions.

4.2.3 Vacuole morphology in response to osmotic stress

The cell vacuole has been closely studied in relation to both hyper- and hypo-osmolarity. Although vacuoles are often primarily considered to be storage organelles, their role in cell homeostasis and degradation of cellular components (both of which are required for cellular survival under stressful conditions) is well established (Klionsky et al., 1990; Thumm, 2000). It is known that exposure of laboratory yeast strains to hypertonic environments can cause the vacuole to 'fragment', in which larger vacuoles divide into a greater number of smaller vacuoles within the cell (Zieger and Mayer, 2012). To investigate this phenomenon in industrially-relevant polyploid brewing yeast strains, cells were exposed to YPD supplemented with 30% w/v sorbitol and stained with FM 4-64™ to visualise vacuole morphology by confocal microscopy according to the

methodology outlined in Section 2.5.4. Yeast cells grown in un-supplemented YPD media were used as an unstressed control.

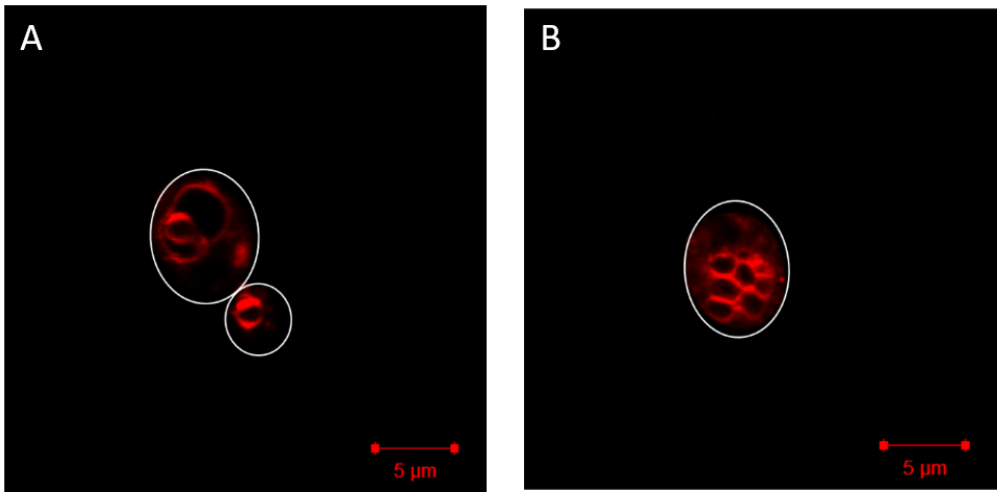


Figure 4.3: The impact of osmotic stress on yeast vacuole morphology. Yeast cells were analysed for vacuolar structure under standard (A) and stressful (B) conditions by staining with FM 4-64™ and subsequently analysed using confocal microscopy. Experimental samples were exposed to media supplemented with 30% w/v. The vacuole membrane is indicated by the red colouration. White circles represent artificially generated outlines of yeast cells.

It can be seen from Figure 4.3A that under standard (unstressed) conditions, vacuoles could be visualised as large structures of relatively low abundancy within the cell, suggesting that this morphology is typical of a healthy cell. When high levels of sorbitol were present in the growth medium, brewing yeast vacuoles developed a fragmented state (Figure 4.3B). The fragmentation or fission of vacuoles was similar to that observed in laboratory strains and is

believed to occur in order to maintain the osmotic balance within the cell by adapting the surface area to volume ratio of the organelle (Zieger and Mayer, 2012). It has been suggested that vacuole fragmentation is an immediate response to osmotic shock and can occur as rapidly as 10 minutes after exposure to hyperosmolarity (Weisman, 2003). Interestingly, it has been suggested that yeast are able to regain a characteristically healthy vacuole morphology as they adapt to stress (Li and Kane, 2009). However, this phenomenon was not observed here. This may indicate that the levels of osmotic stress caused by the addition of 30% sorbitol were sufficiently high so as to not allow the yeast population to fully adapt. Alternatively, it is possible that an extended period of time may have been required for this to occur under the sustained conditions applied. Further work would be needed to investigate the influence of different levels of osmotic stress and incubation time on vacuolar morphology in order to track vacuolar changes more precisely and pinpoint the 'tipping point' in which cells are able/unable to revert to a 'standard' morphology. Despite this, it was clear from the experiment performed that the conditions provided were sufficient to elicit a physiological response which could be analysed further in subsequent Chapters.

4.2.4 Mitochondrial dynamics in response to osmotic stress

Mitochondria are dynamic organelles that serve a range of cellular functions. While they are primarily associated with the production of ATP via aerobic respiration, studies have also shown that there is a link between mitochondrial physiology and functionality, and the yeast stress response due to the role of

mitochondria in reactive oxygen species (ROS) generation (Kanki et al., 2015), lipid processing (Mitra and Lippincott-Schwartz, 2010) and programmed cell death (Fannjiang et al., 2004). Given that mitochondrial fission and fusion events are related to cell activity (Jensen et al., 2000; Berman et al., 2008), and that mitochondrial membrane potential is important in determining the efficient functioning of the organelle (Vayssier-Taussat et al., 2002), it is possible that mitochondrial membrane potential and morphology may be influenced by stress. In order to assess the occurrence and extent of this in brewing yeast strains, mitochondrial morphology and membrane potential were investigated using a combination of confocal microscopy and flow cytometry. The mitochondrial membrane potential dependant dye, rhodamine 123 (R123) was applied to visualise yeast mitochondrial morphology using confocal microscopy and to determine mitochondrial activity using flow cytometry. For the latter, R123 functions to identify active mitochondria since these emit a higher fluorescence than less-active mitochondria, due to increased membrane potential (Ludovico et al., 2001). It should be noted that the increase in fluorescence emitted could also be due to an increase of mitochondrial biomass. To take this into consideration, MitoTracker™ Green FM was applied in order to monitor total mitochondrial biomass, since binding of this molecule occurs independently of membrane potential.

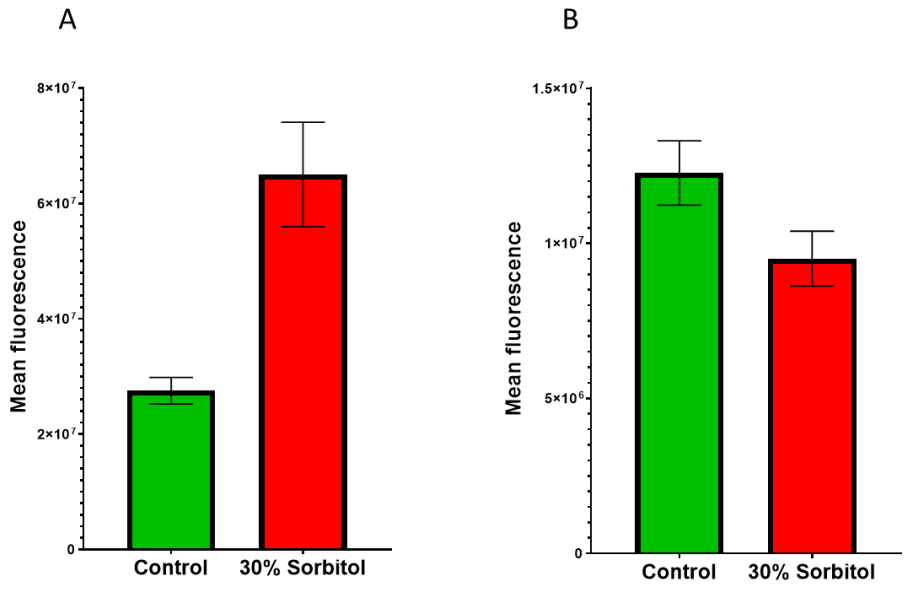


Figure 4.4. The effect of osmotic stress on mitochondrial membrane potential (A) and mitochondria mass (B). Yeast cells were exposed to YPD media supplemented with 30% sorbitol for 24 hours and assessed for mitochondrial dynamics. Stressed cells were compared against a control sample comprising cells cultivated in standard YPD media. The mitochondrial membrane potential (A) was measured using rhodamine 123 and mitochondrial mass (B) was determined by means of MitoTracker™ Green FM. Data represents the mean of triplicate samples, with error bars indicating the standard deviation.

.

When exposed to an environment containing heightened levels of osmotic stress caused by supplementation with 30% sorbitol, a significant increase in yeast mitochondrial membrane potential was observed ($p \leq 0.005$), indicating that mitochondrial activity increased in response to stress. This increase in activity was attributed to increased functionality and was not due to variations in biomass. This could be clearly demonstrated, as staining with MitoTracker™

Green FM (figure 4.4B) indicated that mitochondrial mass decreased upon exposure to osmotic stress ($p \leq 0.005$). This result was surprising and it the underpinning causes are currently unknown. However, it is suggested that the reduction in mitochondrial mass could be due to the removal of spent or damaged mitochondria, or impaired mitochondrial biogenesis, while the increased activity could be reflective of the role of mitochondria in generating components required to protect the cell, such as production of membrane-bound lipid structures (Zinser et al., 1993; Schweizer and Hofmann, 2004; Tehlivets et al., 2007) or protein assembly (Herrmann et al., 2013).

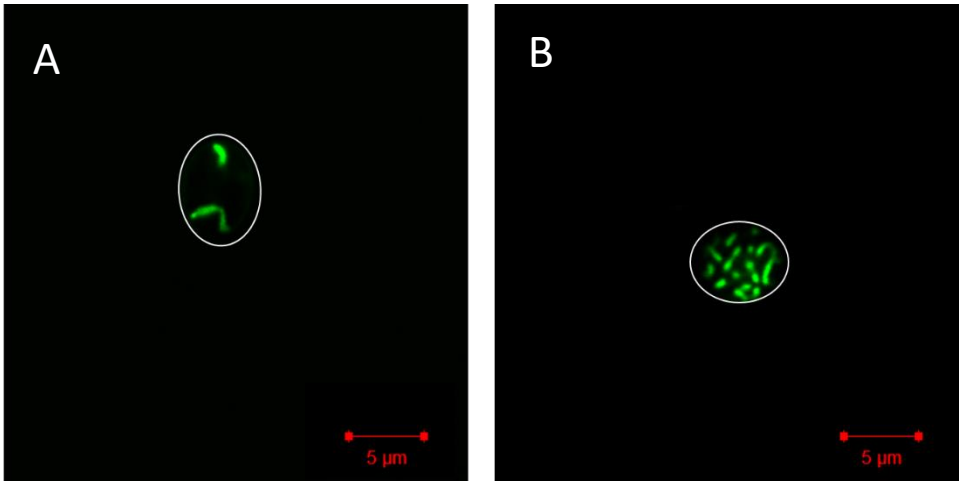


Figure 4.5. The effect of osmotic stress on mitochondrial morphology. Analysis of mitochondrial (green) morphology under normal conditions (A) and stressful conditions (B). Cells were exposed to either YPD media or YPD supplemented with 30% sorbitol for normal and stressful conditions respectively before staining with rhodamine 123 and visualised by confocal microscopy. White circles represent artificially generated outlines of yeast cells.

The increase in activity suggested by fluorescence intensity and membrane potential was supported by visual analysis of yeast cell mitochondria using confocal microscopy. Yeast cells which had been cultivated under standard non-stressed conditions contained mitochondria that were present in a fused (elongated) state (Figure 4.5A). The mitochondrial equilibrium between fused and discrete form has been suggested to be indicative of environmental stress (Youle and van der Bliek, 2012; Knorre et al., 2013a), although there have been conflicting reports on the relationship between morphology and different environmental conditions (Berman et al., 2008; Westermann, 2012; Gomes and Scorrano, 2013; Viana et al., 2020). However, there is strong evidence to suggest that the equilibrium of fusion and fission events leans towards fission when environmental stress is applied to yeast cells, which is believe to be partly due to the energetic inefficiency of mitochondria in the fused state (Knorre et al., 2013a). Indeed, this phenomenon was observed when growth media was supplemented with 30% sorbitol. Mitochondria appeared as more frequent, discrete structures within the cell (Figure 4.5B), when compared to cells under normal conditions. This suggests a link between mitochondrial form and an increase in overall mitochondrial membrane potential within the cell, likely due to increased mitochondrial function.

The potential reasoning behind the physiological changes observed in Figure 4.5. could be to increase mitochondrial efficiency in order to support mitochondrial activity in response to stress as discussed above. Increased mitochondrial functionality or efficiency could potentially be required to fulfil the stress-related aspects to mitochondrial metabolism outlined previously

(lipid metabolism, ROS generation, protein processing). However, the nature of this activity remains unclear and further work is required to fully elucidate the reasoning behind the increased mitochondrial activity and altered physiology in response to stress. As the occurrence of unfused mitochondria in the presence of stress coincides with an increase in mitochondrial membrane potential, with no decrease in mitochondrial mass, these results suggest that mitochondrial fission is a determinant of increased mitochondrial activity/productivity.

4.2.5 Osmotic stress, fermentation performance and organelle morphology

Previously it was demonstrated that osmotic stress caused a series of detrimental effects on yeast, including a reduction in growth rate, poor viability and plasma membrane damage (Section 4.2.2). Subsequently it was demonstrated that vacuolar and mitochondrial morphology were impacted by stress (Sections 4.2.3-4.2.4), potentially leading to both physiological and metabolic changes that could impact on fermentation performance. Consequently, in order to further examine the effects of heightened osmotic stress on yeast fermentation characteristics and cell physiology, yeast populations were inoculated into a 16 ^{o}P all-malt wort supplemented with 30% sorbitol, and compared to a non-supplemented 16 ^{o}P control wort. The rationale for this approach was, as before, to allow the effects of osmotic stress to be investigated without altering the nutritional composition of the wort; a novel approach to studying the relationship between stress and organelle

morphology during fermentation. It should be noted that data was normalised based on the concentration of fermentable sugars, hence the starting gravity of the sorbitol-enhanced wort appears identical to the standard 16 °P control in the data shown here.

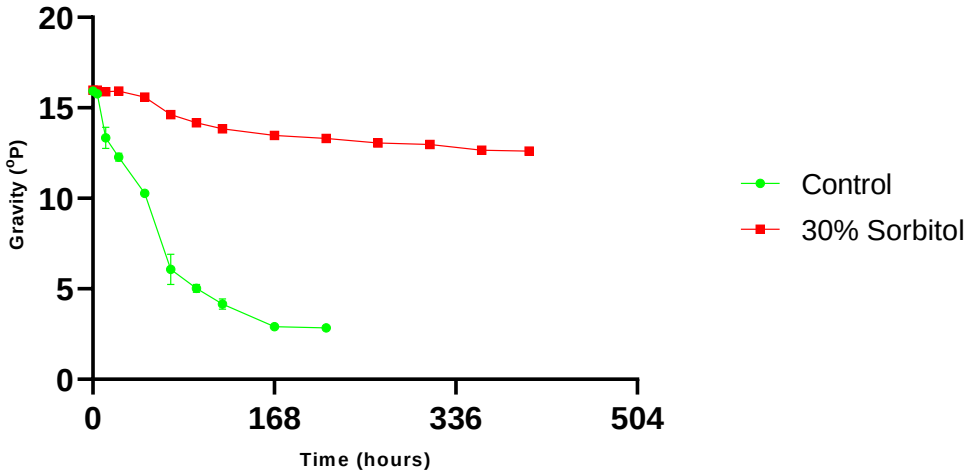


Figure 4.6. The effect of heightened osmotic stress on fermentation performance. Osmotic stress was induced by supplementation of 16 °P wort with 30% (w/v) and compared with a control using a 16 °P wort without the addition of sorbitol. All gravities are shown as normalised values to the control sample, negating the increase in gravity as a result of sorbitol supplementation. All data represent the mean of triplicate samples, with error bars indicating the standard deviation at each time point. In cases where the error bars are too narrow, these will not be visible in the figure.

Under control conditions, the gravity of the un-supplemented wort reduced rapidly during the initial stages of fermentation. This rate of fermentation progression continued until 72 hours, by which point approximately 75% of the

total gravity reduction has occurred. Extract uptake rate slowed after this point, with an end gravity of 2.84 ^{o}P achieved after a total of 216 hours (Figure 4.6). When analysing the performance of yeast under conditions of heightened stress through supplemented with 30% sorbitol, initiation of fermentation is delayed and no reduction in gravity was observed within the first 24 hours. Following this point, wort gravity began to decrease, albeit at a slower rate than that observed in the control fermentation (Figure 4.6). It can be seen therefore that the effects of sorbitol supplementation were manifested in reduced fermentation activity, which also resulted in sluggish fermentation and a reduced overall extract conversion. After 404 hours of fermentation the final gravity remained at 12.61 ^{o}P, after which no further reduction was observed and the fermentation was deemed to have 'stuck' at this point indicating that yeast cells were unable to function due to the stress present.

In order to identify physiological changes in organelles occurring over the course of fermentation, yeast cell samples were taken from both control and experimental fermentations at specific time points as indicated in Figure 4.7A. These time points were selected based on what were considered to be key stages in fermentation. At each stage, cells were analysed for organelle content and morphology to determine the effect of heightened stress on both the vacuole and the mitochondria over time.

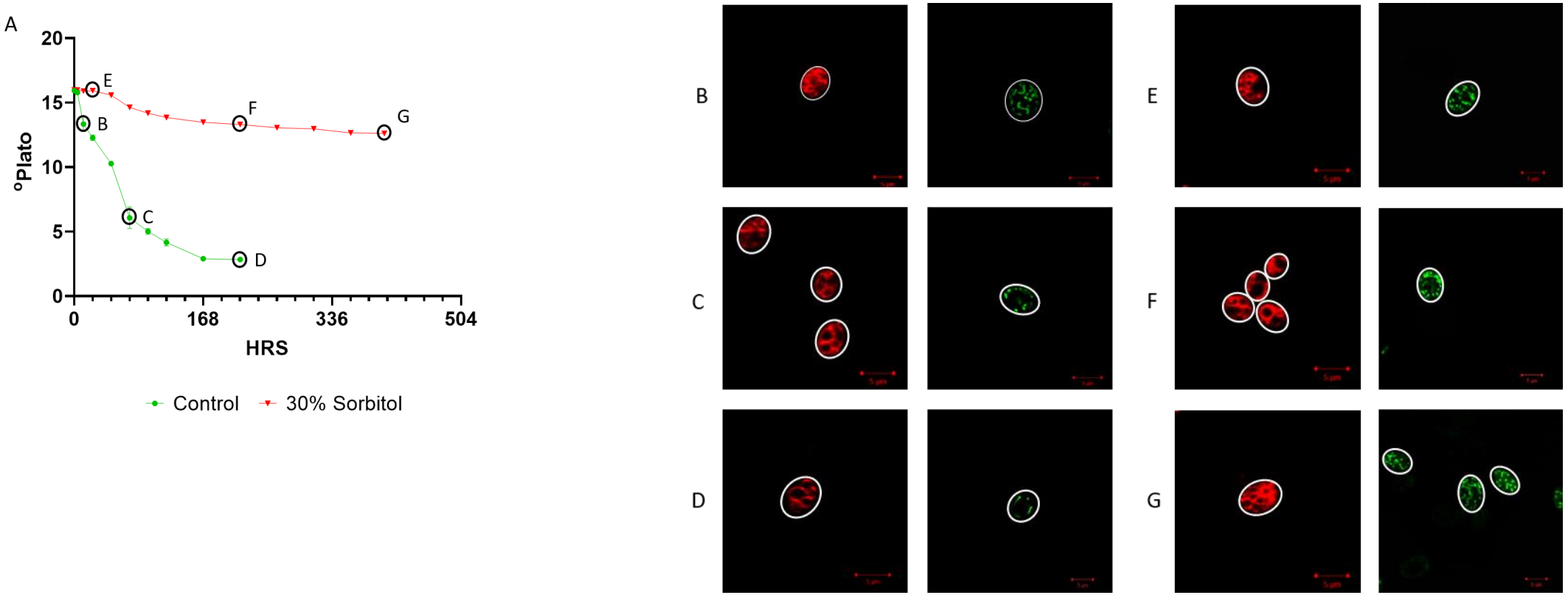


Figure 4.7. The effect of osmotic stress on organelle morphology during fermentation. The vacuole (red) and mitochondria (green) morphology at each annotated timepoint throughout fermentation (A) in 16°P standard wort (sample points B-D) and 16°P wort supplemented with 30% sorbitol (sample

points E-G). White circles represent artificially generated outlines of yeast cells. Fermentations were carried out in 2 L stirred bioreactors at 15 °C . Samples taken at denoted timepoints were them subjected to analysis by fluorescent staining with either FM 4-64™ (vacuole staining) or rhodamine 123 (mitochondria staining) confocal microscopy. Approximately 50-100 cells, or groups of cells, were assessed for morphology of each organelle and images were selected based on representative physiologies.

In the control fermentations it can be seen that after 24 hours the vacuolar structures began to become fragmented. Smaller pro-vacuoles were visible throughout the cell with a relatively high frequency (Figure 4.7B). At the same time, mitochondria developed into a characteristic unfused or discrete morphology (Figure 4.7B). These were also present at a relatively high frequency, with smaller mitochondria visible as well as slightly more elongated structures indicating that the process of mitochondrial fission was occurring. At the mid-point of fermentation yeast vacuoles still appeared to be fragmented (Figure 4.7C), although less so than at the beginning of fermentation. At this point some vacuoles appeared as the more dominant structure within the cell, with other smaller vacuolar structures distributed throughout the cell. Mitochondria at this point appeared to be present in the unfused state as before. These small structures were typically located towards the periphery of the cell, which may implicate them in the cell surface response, for example through their role in creating compounds such as fatty acids and sterols important for membrane health (Alexandre et al., 1994; Learmonth, 2012; Flis and Daum, 2013; Ishmayana et al., 2017). At the fermentation endpoint, vacuole physiology no longer appeared to be fragmented, with a more 'typical' morphology observed, similar to that associated with the inactive or non-stressed state. Similarly, mitochondria also adopted an elongated fused physiology, more closely related to that of unstressed cells (Figure 4.7D).

When increased osmotic pressure was exerted on the yeast population by the addition of 30% sorbitol, both vacuolar and mitochondrial morphology were similar to the control sample after 24 hours. Vacuoles appeared fragmented

and mitochondria also showed a discrete morphology (Figure 4.7E). Similarly, at the fermentation midpoint, small vacuolar structures continued to be apparent throughout the cell. Mitochondria continue to adopt an unfused morphology at this stage in the fermentation as in the control sample (Figure 4.7E). At the designated fermentation endpoint, after which no further decrease in gravity was observed, both mitochondrial and vacuolar morphology reflected a stressed physiology (Figure 4.7G), indicating that the yeast population had not been able to recover a characteristically healthy morphology, despite activity being arrested.

4.3 Discussion

Throughout fermentation, the environment and variety of stresses that yeast cells are exposed to is constantly changing. Although each of these different stress factors can contribute to yeast health and performance, osmotic stress is arguably the most relevant since it reflects the combined presence of both sugars and alcohol (Zhuang et al., 2017). Due to this, osmolarity is a constant stress during fermentation, unlike the effects of starvation or ethanol toxicity which only occur towards the latter stages of a batch fermentation (Gibson et al., 2007; Zhuang et al., 2017). In this study the impact of osmotic stress on cell growth and fermentation performance were investigated. Furthermore, potential sub-cellular targets of stress were studied in order to investigate the relationship between osmotic pressure and organelle physiology.

Initially, it was observed that cell growth was impaired in the presence of osmotic stress. During growth phases under extracellular stress, stress

response pathways are required to maintain survival of the cells. This in turn requires the production of stress protectants and compatible solutes (Section 1.5.2) which diverts the assimilated carbon to these pathways rather than the generation of biomass, thus reducing the growth rate of the yeast population (Hounsa et al., 1998; Shen et al., 1999; Hohmann, 2002). Furthermore, negative impacts on population viability and damage of the plasma membrane affecting cell health/vitality will reduce the ability of a cell to effectively proliferate (Hewitt and Nebe-Von-Caron, 2001; Dupont et al., 2011; Kono et al., 2016). The relationship between osmotic stress and yeast growth and yeast health is important since cell proliferation is a key performance indicator in brewing and other fermented beverages. The ability of a yeast population to successfully grow during the initial stages of fermentation is essential for a rapid and efficient fermentation (David and Kirsop, 1973; Boulton and Quain, 2001). As shown here, the presence of excessive osmotic stress can significantly reduce the growth rate of a yeast population and the total biomass achieved. It is likely that this is the primary cause of the poor performance of cells during fermentation of wort supplemented with sorbitol. While it is acknowledged that the amount of stress applied in this instance was relatively severe, it was interesting to note that the predominant effects were to slow down the rate of sugar uptake and to cause the fermentation to arrest before completion. This latter suggests that the health of the population was severely compromised as reported previously (D'Amore et al., 1988; Pratt et al., 2003), or that cells ceased activity and remained dormant in response to the stress.

When individual cellular components were analysed in response to osmotic stress, it was observed that plasma membrane health deteriorated, as indicted by an increase in the percentage of depolarized cells. Osmotic stress is known to exert physical stress on the yeast cell due to the efflux of water from the cell and loss of cell turgor (Mager and Siderius, 2002). This is a passive process and involves rapid shrinkage of cell upon exposure to high osmolality (Hohmann, 2002) which causes depolarisation of the plasma membrane (Hewitt and Nebe-Von-Caron, 2001; Capusoni et al., 2019). It is likely that the effect of this in brewing yeast is multifaceted. Membrane health is important to prevent the free diffusion of solutes, which can lead to further damage (Mizoguchi and Hara, 1998; Simonin et al., 2007). In addition, it is known that the membrane has a key role in the transfer of desirable nutrients such as sugars (D'Amore et al., 1989; Meneses et al., 2002), nitrogen (Garrett, 2008) and metal ions (Walker et al., 2006). Disruption to the membrane is likely to impair these mechanisms causing the individual cell to display reduced vitality. However, it is possible that cells can make adjustments to the membrane to withstand or mitigate the impacts of stress (Alexandre et al., 1994; Abe and Hiraki, 2009; Dupont et al., 2011). For example, it is known that cells can incorporate sterols (Thomas et al., 1978) and sugars such as trehalose (Wiemken, 1990; Hounsa et al., 1998; Avonce et al., 2006)to protect membrane structures. The capacity of cells to perform this efficiently may be key to withstanding osmotic stress.

Analysis of vacuole morphology in response to osmotic stress and during fermentation indicated that there was a tendency to undergo fragmentation. Cells typically exhibited a small number of large vacuoles in the absence of

stress, but rapidly displayed a much larger number of smaller pro-vacuoles under conditions of stress. This phenomenon has been widely reported in laboratory strains and it is known that mechanical stress related to water efflux from the cell can trigger fragmentation of the yeast vacuole in order to maintain proper osmotic balance within the cytosol (Li and Kane, 2009). This is believed to be due to a release of water from a large vacuole in order to maintain the osmotic pressure within the cytoplasm, which causes smaller vacuoles to be formed. Hyperosmotic shock is sensed by the vacuole cation channel protein Yvc1p (Zieger and Mayer, 2012), which mediates the efflux of calcium stored in the vacuole to the cytosol in order to mitigate the effects of osmotic stress (Denis and Cyert, 2002). By increasing the vacuole surface/volume ration in response to osmotic stress, an increase in the ability to sequester Ca^{2+} ions into the cytosol via vacuole H^{+} -ATPase pumps (Li et al., 2012) in conjunction with the HOG pathway, triggering detoxification mechanisms (Hohmann, 2002), calcineurin-signalling pathways (Moser et al., 1996; Cyert, 2003) and compatible solute production (Saito and Posas, 2012; Gonzalez et al., 2016) required for cell survival under osmotic stress. Vacuole morphology is therefore a direct indicator of osmotic stress and the organelle is actively involved in the response and survival of yeast cells.

Interestingly, at the end point of standard (control) fermentations, the yeast vacuole no longer appeared to be in a hyper-fragmented state, despite conditions remaining osmotically charged. This indicates that yeast cells were able to recover their normal cell physiology and adapt to the extracellular stress exerted on the cell. This suggests that the morphological changes occurring to

the vacuole during fermentation were positive and allowed the cell to thrive before reverting to its basic form. In contrast, under highly stressful conditions, cells were not able to recover and the fragmented vacuolar structure remained. This inability to adapt to the stress and maintain normal physiology suggests a potential link between the poor fermentation performance exhibited and vacuole dynamics.

Analysis of the yeast mitochondria in response to stress yielded a similar but distinct response. Under osmotic stress, yeast mitochondria rapidly switched from a fused network to a discrete morphology. However, similar to that observed with vacuole, the reoccurrence of the characteristically 'healthy' morphology was observed at the end of fermentation. At this stage mitochondria appeared as elongated,fused structures. This change in form suggests that yeast were able to efficiently adapt to stresses presented during fermentation and regain their normal cell physiology. However, when wort was supplemented with sorbitol to create additional stress, mitochondrial fission was observed to persist through to the end of fermentation. It is likely that this is a reflection of damage to the cell in this instance. This hypothesis is supported by the increase in membrane potential observed under osmotic stress. It is known that there is a link between mitochondrial form and mitochondrial membrane potential (Fehrmann et al., 2013; Vevea et al., 2014) and that segregation and inheritance of mitochondria from a mother to a daughter cell can also be restricted when membrane potential is low (Boldogh et al., 2001). It has also been suggested that the equilibrium between the fused and unfused state is related to osmotic stress (Pastor et al., 2009; Knorre et al.,

2013a) and survival under ethanol toxicity (Kitagaki et al., 2007).. Under stressful conditions, the equilibrium shifts towards mitochondrial fission as a pro-survival mechanism (Youle and van der Bliek, 2012), implicating mitochondrial fission in the yeast stress response. This could potentially occur as an effort to increase mitochondrial efficiency, both due to the involvement of mitochondria in the adaptation to the extracellular stress (potentially through increased lipid metabolism required to fortify the plasma membrane), but also to compensate for any loss of mitochondrial mass as a result of the stress. Mitochondria fusion requires constant energy input from the cell to maintain this physiology (Jensen et al., 2000; Berman et al., 2008), and therefore as stress is applied to the cell, this energy may be redirected to support other cellular functions necessary for survival. A loss of mitochondrial mass has been suggested to be related to the removal of damaged mitochondria by mitophagy, in which case mitochondrial fission would also occur to prevent depolarisation of the whole mitochondrion as a result of mitochondrial damage (Mendl et al., 2011). Alternatively, it is possible that impaired mitochondrial biogenesis or inheritance during division as a result of stress could result in a reduction in mitochondrial mass per cell over time, however further experimental procedures would need to be applied to fully elucidate these mechanisms.

CHAPTER 5: THE IMPACT OF WORT SUPPLEMENTATION ON YEAST PERFORMANCE FOR VERY HIGH GRAVITY FERMENTATION APPLICATIONS

5.1 Introduction

In modern brewing, the utilisation of high gravity worts (16-18^{o}P) for lager production has become common practice. While the implementation of high gravity substrates has attributed to a global increase in brewery productivity and reduction of costs and water usage, efforts to further increase output by implementing very high gravity procedures have been somewhat unproductive (Puligundla et al., 2011). This is partly due to the increased levels of stress exerted on the yeast population during a VHG (18-22^{o}P) fermentation, which results in poor cellular health and overall performance (Pratt et al., 2003; Gibson et al., 2007). Although fermentation-related stress factors contribute heavily to the shortfalls associated with VHG brewing, an additional issue is nutritional limitation (Casey et al., 1983; Gibson, 2011). Brewing yeast strains have distinct nutritional requirements which, at low gravity, are largely fulfilled by the malt component of brewers wort (Boulton and Quain, 2001). These include metal ions, lipids, vitamins, and nitrogenous compounds, as well as dissolved oxygen added immediately prior to fermentation (MacWilliam, 1968; Dekoninck et al., 2013; He et al., 2014b). At higher gravities, the concentrations of these nutrients can often be insufficient to support optimum yeast functionality. This is largely a result of supplementation with sugar syrups which lack the range of nutrients associated with malted barley (Casey et al., 1984; Younis and Stewart, 1999). Thus, VHG fermentations often result in sluggish or 'stuck' fermentations with poor yeast activity.

Nutritional supplementation of wort is a common mechanism for achieving positive results from fermentation, with key additions including a range of metal ions, as well as lipids and nitrogen supplements (Bafrncová et al., 1999; Walker, 2004; Gibson, 2011). Addition of metal ions is not a novel process; worts are often lacking in sufficient quantities of zinc and this is a particularly common nutritional supplement (De Nicola and Walker, 2011; Gibson, 2011). Zinc plays a key role in the formation of ethanol, acting as a cofactor for alcohol dehydrogenase, as well as other enzymes supporting yeast metabolism (Ganzhorn and Plapp, 1988; Walker, 2004). As one of the most abundant metal ions involved in biological processes, magnesium also plays an integral role in yeast functionality and cellular integrity and stress tolerance (Rees and Stewart, 1997; Walker, 1998). When magnesium ions are present at sub-optimal concentrations in wort, the supplementation of wort with magnesium poses a potential opportunity to improve performance (Rees and Stewart, 1997; Walker, 1998). Nitrogen limitation has also been reported to be a prominent deficiency, and is particularly associated with higher-gravity fermentations prepared using adjunct sugars (Casey et al., 1983; Bafrncová et al., 1999). Free amino nitrogen (FAN) derived from malted barley is necessary for yeast growth during the initial stages of fermentation and factors which impair growth can yield negative effects on performance (Hill and Stewart, 2019).

Although present in relatively small quantities when compared to other wort nutrients, the concentration of vitamins in wort can also have an effect on fermentation rate and yeast viability (Alfenore et al., 2002). Specifically,

thiamine (vitamin B_1) has been identified as having a positive effect on yeast activity and glucose utilisation in industrial yeasts (Stambuk et al., 2009). Furthermore, the importance and role of biotin (vitamin B_7) in metabolism is well established, with this vitamin playing an important role in carbohydrate and amino acid metabolism as well as fatty acid synthesis (Lardy et al., 1949; Moat and Lichstein, 1954). However, little is known about the effects of thiamine or biotin supplementation on brewing yeast fermentation. Similarly, although pyridoxine (vitamin B_6) has been well characterised as a yeast growth stimulant, due to its role as an enzyme cofactor in amino acid and carbohydrate metabolism (Ough et al., 1989; Perli et al., 2020), its impact on brewing yeast performance remains unknown.

Efforts to improve fermentation efficiency through supplementation of the fermenting medium with a combination of sterols and unsaturated fatty acids (UFAs) have also proven successful (Casey et al., 1983; Gibson, 2011). As the most abundant sterol found in the yeast plasma membrane, ergosterol has been the subject of a number of previous studies aimed at improving yeast performance (Thomas et al., 1978; Casey et al., 1983; Casey et al., 1984; Dupont et al., 2011). Furthermore, supplementation of fermentations with ergosterol has been used in an attempt to circumvent issues of yeast growth and the decreased solubility of oxygen in higher gravity wort, thus aiding in the prevention of stuck or incomplete fermentations (Casey et al., 1983). Although the precise mechanism of improved performance is not fully know, it is widely accepted to be due to the integral role of sterols in maintaining plasma membrane fluidity, a key determinant in stress tolerance and required to

support cell growth which is important in generating biomass and ensuring cell activity (Alexandre et al., 1994; Soustre et al., 2000; You et al., 2003; Abe and Hiraki, 2009; Ishmayana et al., 2017).

The aim of this study was to assess the impact of nutritional supplements on fermentation performance at VHG. Specifically, the aim was to investigate the impact of metal ions (zinc and magnesium), as well as ergosterol, nitrogen (through diammonium phosphate or DAP) and vitamins (biotin, pyridoxine and thiamine) on fermentation performance and yeast health post attenuation. In addition, an alternative approach to sugar addition was taken based on the hypothesis that reducing or eliminating stress could act as an alternative mechanism for ensuring performance. To achieve this, a 'sugar top-up' regime was applied in an effort to reduce the amount of osmotic stress experienced by yeast when pitched directly into very high gravity wort. This approach is somewhat uncommon in the brewing industry, but has been applied in fed-batch fermentation systems previously, for example those used in other biotechnological fermentation systems including bioethanol and for the production of certain flavoured alcoholic beverages. The effect of the optimisation approaches summarised here were assessed for their overall impact on fermentation performance, yeast health and their applicability to the brewing industry in terms of ease of use and their potential to allow VHG brewing to become a viable and practical option.

5.2 Results

In order to assess the effect of supplementation on fermentation performance at high and very high gravity, the impact of individual nutrient additions were assessed in the context of an industrially-relevant fermentation regime. Based on the current upper limits of wort gravity associated with the product streams and yeast strains investigated here, a 17.5 ^{o}P wort was used as a control wort, produced from a 15 ^{o}P all-malt hopped wort base, supplemented with 20% of corn adjunct as described in Section 2.2.3. Very high gravity conditions were created by supplementing the control wort with high maltose syrup (HMS) to yield a final VHG wort of 22 ^{o}P. Fermentations were carried out in either 100 mL mini FVs, or in 2 L vessels according to the methods outlined in Section 2.4.2 and Section 2.4.3 respectively. Briefly, all fermentations were conducted at a constant temperature of 15 ^{o}C with pre-aerated wort according to the method outlined in Section 2.4.3. Yeast strain SMCC100 was selected based on previous data suggesting stress tolerance and promising performance at VHG (Chapters 3-4) and its industrial relevance. All cultures were pitched at a rate of 1.2 x 10^{6} cells/mL/^{o}P to reflect the standard practice associated with this yeast strain at a commercial level.

5.2.1 Analysis of yeast fermentation performance at high and very high gravity

The mechanism applied to create 22 ^{o}P wort essentially acts to dilute the malt portion of the pitching wort leading to a reduction in macronutrients available to the yeast, while also increasing the amount of osmotic stress provided

through sugar addition. In order to determine the effect of elevating gravity from HG to VHG, the performance of strain SMCC100 was assessed by analysis of fermentation progression using 17.5 ^{o}P and 22 ^{o}P worts respectively.

Under both conditions a characteristic and expected lag phase was observed for around 24 hours. After this period, a steady reduction in gravity was observed as fermentation progressed. It can be seen that the predominant effect of increasing wort gravity on fermentation was an elongation of fermentation time (Figure 5.1). The 17.5 ^{o}P control (HG) fermentation attenuated 280 hours after pitching, reaching a final gravity of 3.91 ^{o}P. The VHG 22 ^{o}P fermentation attenuated after 376 hours with a final gravity of 5.30 ^{o}P. The overall rates of extract uptake for the 17.5 and 22^{o}P fermentations were 1.165 ^{o}P/day and 1.066 ^{o}P/day respectively, with VHG conditions yielding a slower rate of extract utilisation throughout the fermentation. To some extent, this simply reflects the increased time needed to convert the higher quantity of sugars to ethanol. However, the time to attenuation became disproportionately longer over time, likely to be as a result of the increased stress factors related to elevated osmotic stress and ethanol toxicity exerted on the fermenting yeast population (as discussed in Chapter3 and Chapter 4) (Puligundla et al., 2011; Zhuang et al., 2017). These results therefore support previous observations of impeded yeast performance as a result of adjunct supplementation and accompanied increase in gravity. This was therefore somewhat expected; however, it should be noted that very high gravity procedures have typically been compared to standard gravity (below 15^{o}P), essentially creating a large difference in starting wort concentration. In this

study, the fermentation performance between high and very high gravity fermentation was assessed in order to evaluate if increasing wort gravity beyond current practice was a viable strategy. While the data obtained indicated that this approach was promising, improvements to fermentation speed were desirable to ensure that this would represent a viable commercial strategy.

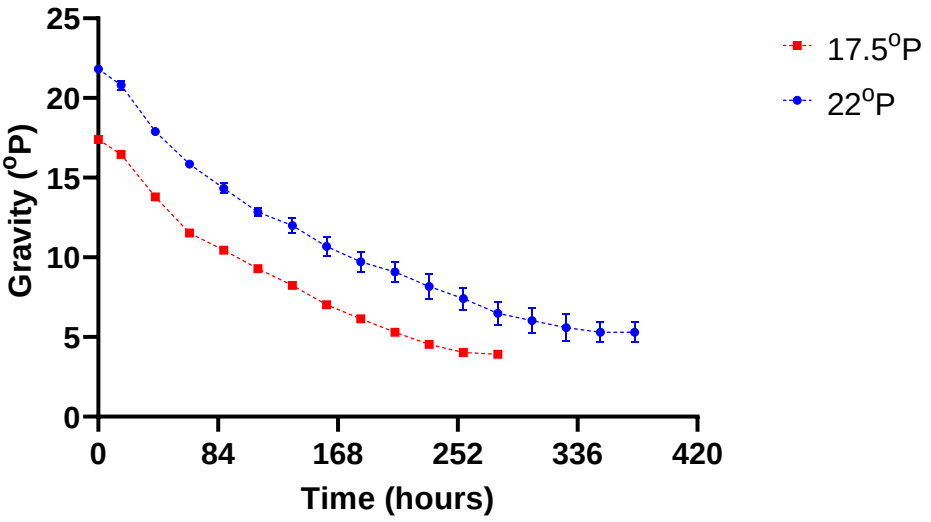


Figure 5.1. Fermentation performance of yeast strain SMCC100 in 17.5°P and 22°P worts. Data points represent the mean of triplicate samples with error bars denoting the standard deviation. Fermentations were carried out in 2L FVs and progression was monitored daily by monitoring wort gravity over time. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

5.2.2 The effect of nutrient additions on fermentation performance

Based on the initial data obtained for strain SMCC1000 under VHG conditions, nutrient additions were screened for their effect on time to attenuation and fermentation rate. Each nutrient was assessed individually and was supplemented at three concentrations: 1X, 2X and 5X. The base dose rates were conceptualised based on previous literature that has discussed the optimal concentrations of each nutritional supplement for applications to brewery fermentations (Casey et al., 1983; Rees and Stewart, 1997; Walker, 1998; Walker, 2004; De Nicola and Walker, 2011; Hucker et al., 2016). Where previous literature was scarce in regards to specific nutrient additions, dosing concentrations were assessed based on the concentrations of each nutrient typically found in brewer's wort (MacWilliam, 1968; Casey et al., 1984; Bamforth, 2003; Briggs et al., 2004). In order to account for variation during early generations of serial repitching, yeast cultures were consecutively repitched into fermentations with identical conditions for three generations and data from the third set of fermentations was used to assess the effects of each nutrient on fermentation rate. As such, data from the third generation of fermentations are shown below (Figure 5.2- 5.6), however the data from the first and second fermentation generations are also discussed (Figure 5.7). Initial concentrations of wort zinc, magnesium and free amino nitrogen (FAN) are outlined below (Table 5.1), these were measured by inductively coupled plasma mass spectrometry (ICP-MS) for zinc and magnesium (Section 2.2.4) and using the O-phthaldialdehyde method for FAN (Section 2.2.5).

Table 5.1. Nutritional composition of a 17.5 °P adjunct wort and a 22 °P wort (17.5 °P base wort plus high-maltose syrup).

Wort gravity	Zinc concentration (ppb)	Magnesium concentration (ppm)	Free amino nitrogen (mg/L)
17.5 °P	202.64	115.59	312.62
22 °P	162.12	92.47	250.09

Initially, zinc sulphate (heptahydrate) was added to the wort as a source of zinc at 1.32 mg/L, 2.64 mg/L and 6.60 mg/L based on the ratios 1X, 2X and 5X respectively. This corresponded to Zn^{2+} additions of 300.12 ppb (1X), 600.23 ppb (2X) and 1500.58 ppb (5X). Supplementation of very high gravity wort with zinc sulphate had a beneficial effect on fermentation performance when implemented at a concentration of 1.32mg/L, which successfully reduced attenuation time by 24 hours when compared to the unsupplemented fermentation (Figure 5.2). Interestingly, increasing the concentration of zinc supplement to 2.64 mg/L did not have any further beneficial effects on fermentation performance. In fact, this resulted in there being no difference in attenuation time when compared to the unsupplemented fermentation. When the concentration of zinc sulphate heptahydrate was increased further, to 6.60 mg/L, fermentation was negatively affected whereby attenuation time was increased when compared to the unsupplemented fermentation (Figure 5.2). This observation is likely due to the concentration threshold of zinc toxicity being surpassed under these conditions; concentrations of zinc greater than

500 ppm have been suggested to have a negative influence on yeast health and performance, albeit depending on yeast strain and fermentation conditions (Rees and Stewart, 1998).

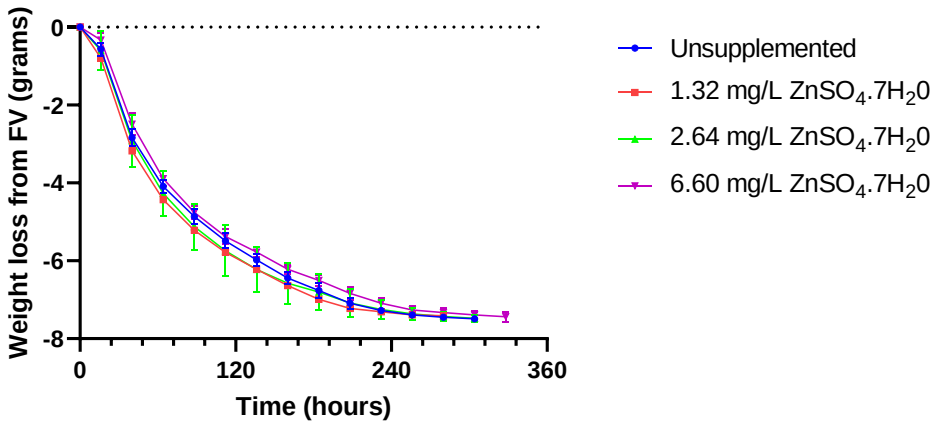


Figure 5.2. Effect of zinc sulphate supplementation on fermentation performance of yeast strain SMCC100 in 22 °P wort. Fermentations were carried out in 100 mL FVs and progression was monitored daily by measuring weight loss over time. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

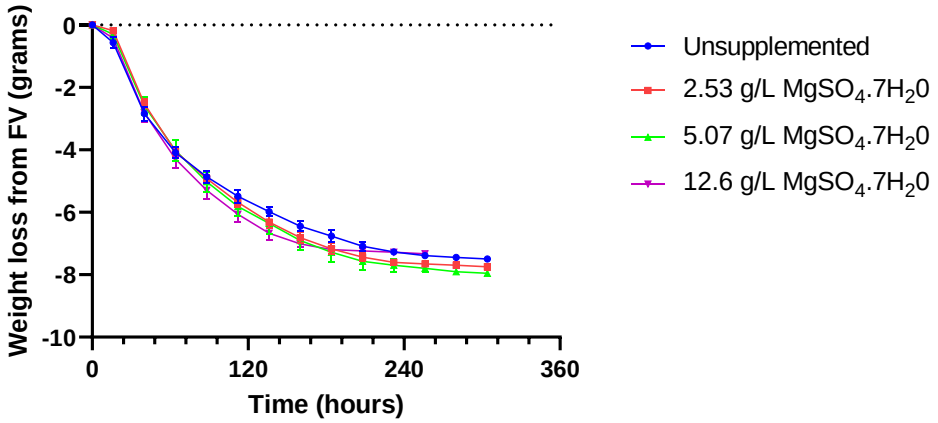


Figure 5.3. Effect of magnesium sulphate supplementation on fermentation performance of yeast strain SMCC100 in 22 ^{o}P wort. Fermentations were carried out in 100 mL FVs and progression was monitored daily by measuring weight loss over time. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

As with zinc supplementation, wort magnesium levels were increased through the addition of magnesium sulphate (heptahydrate) at rations of 1X, 2X and 5X corresponding to 2.53 g/L, 5.07 g/L and 12.6 g/L respectively. This corresponded to Mg^{2+} additions of 249.97 ppm (1X), 499.94 ppm (2X) and 1249.86 ppm (5X). Supplementation of the high gravity wort with all of the concentrations of magnesium sulphate used in this study appeared to enhance fermentation performance, with greater weight lost from the FV at the latter stages of fermentation when compared to the un-supplemented control fermentation (Figure 5.3). The increase in fermentation rate was particularly apparent when a concentration of 12.6 g/L of magnesium supplement was employed, however

although fermentations were completed more quickly at this concentration, the greatest uptake of extract was observed when a concentration of 5.07 g/L magnesium supplementation was applied (Figure 5.3).

To assess the impact of nitrogen concentration on fermentation performance, worts were supplemented with diammonium phosphate (DAP), a low-cost nitrogenous supplement typically employed in oenology and other fermentation systems (Blateyron and Sablayrolles, 2001; Adams and Vuuren, 2010). As before for the other nutrient additions, three concentrations of DAP were applied: 0.5 g/L (1X), 1 g/L (2X) and 2.5 g/L (5X). This corresponded to wort FAN additions of 19.2 mg/L (1X), 38.4 mg/L (2X) and 96 mg/L (5X). All concentrations of DAP used in this study yielded positive results in terms of fermentation performance, showing that increasing wort FAN successfully reduced the time to attenuation under VHG conditions (Figure 5.4). Supplementation of wort with the maximum concentration applied (2.5 g/L) yielded the greatest improvement, increasing the fermentation rate and reducing the final attenuation time by 96 hours compared to the unsupplemented control (Figure 5.4).

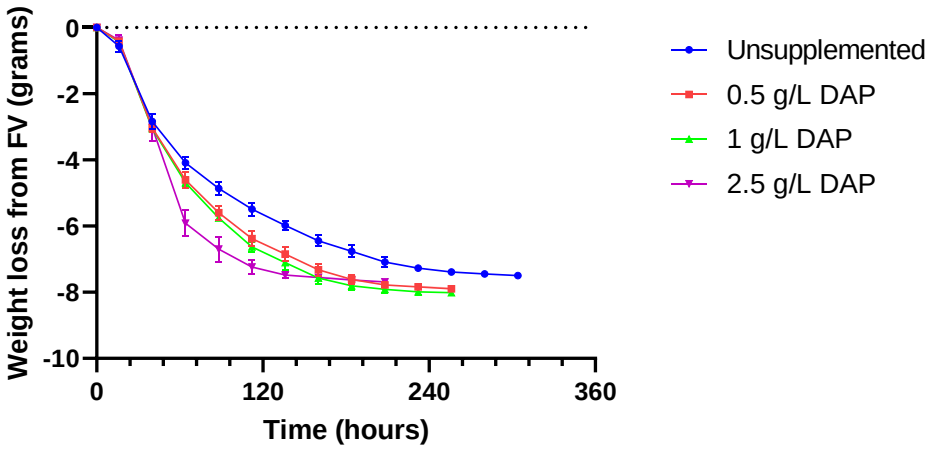


Figure 5.4. Effect of diammonium phosphate (DAP) supplementation on fermentation performance of yeast strain SMCC100 in 22 °P wort. Fermentations were carried out in 100 mL FVs and progression was monitored daily by measuring weight loss over time. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

Once the impact of metal ions and nitrogen had been evaluated, the impact of B vitamin supplementation was investigated. Although there have been previous reports on the benefits of biotin supplementation in wine fermentations (Bohlscheid et al., 2006), the impact of general vitamin B supplementation in brewing worts has not previously been investigated, and certainly not at VHG. Typical wort concentrations of thiamine (B_1), biotin (B_7) and pyridoxine (B_6) range from 0.8 to 155 µg/100mL (Briggs et al., 2004). Consequently, the concentrations applied were calculated from this base level and were therefore largely arbitrary in nature, being calculated from the expected wort composition rather than from the known requirements of yeast

cells. However, since B vitamins are both present and required in relatively small concentrations, and the role of biotin in particular is important in many key metabolic pathways in brewing yeast, their impact was assessed related to VHG fermentations.

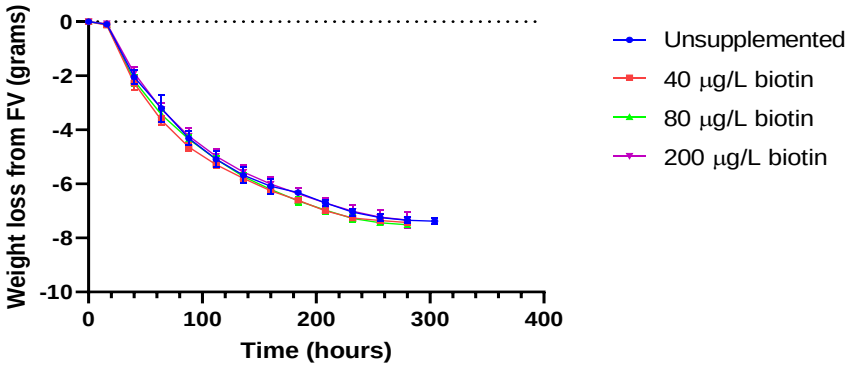
It can be seen that a decrease in the time required to reach attenuation was observed when biotin (B_7) was supplemented to worts at concentrations of 40 and 80 µg/L (Figure 5.5A). Supplementation at 200 µg/L did not provide any further benefit, being similar to the control sample. It should be noted that there was also no difference in fermentation rate observed between 40 and 80 µg/L biotin, which suggests that the optimum impact of biotin addition can be achieved with 40 µL of biotin. In both instances fermentations were completed 24h faster than in the control sample (Figure 5.5A).

Generally, supplementation of worts with pyridoxine hydrochloride (B_6) yielded a beneficial effect on fermentation rate and attenuation time when compared to the unsupplemented control (Figure 5.5B). Supplementation with 40 µg/L and 200 µg/L of pyridoxine hydrochloride reduced fermentation time by 24 hours under these conditions. However, the greatest improvement in fermentation time was observed when supplementing the fermenting wort with 80 µg/L, which reduced fermentation time by a total of 48 hours when compared to the unsupplemented control. This improvement was manifested in terms of an enhanced rate of fermentation, which became apparent during the initial stages (between 64 and 88 hours after pitching). Although the

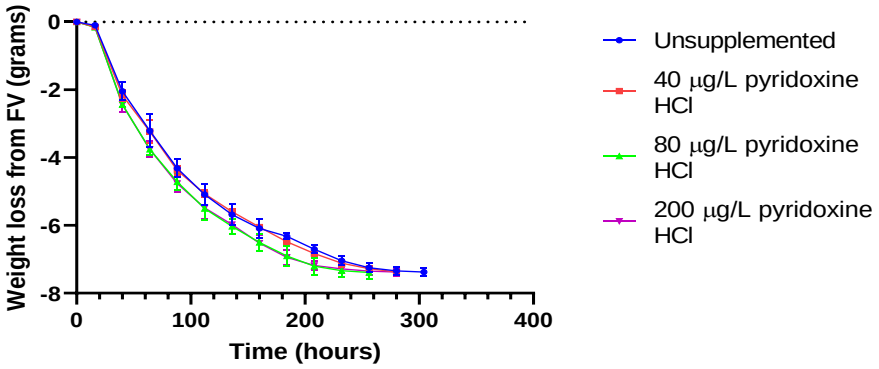
fermentation rate with 200 µg/L of pyridoxine hydrochloride was similar to this, total time to attenuation was not impacted to the same extent (Figure 5.5B).

Similar to the other B vitamins, when VHG worts were supplemented with thiamine (B_1), fermentations exhibited a reduction in the time required to reach attenuation and an increased fermentation rate when compared to the unsupplemented control (Figure 5.5C). In all cases the fermentations attenuated 48 hours earlier than the control group, however, there was no significant benefit in utilising a concentration of thiamine hydrochloride higher than 100 µg/L, with no further reduction observed at the higher concentrations used in this study.

A



B



C

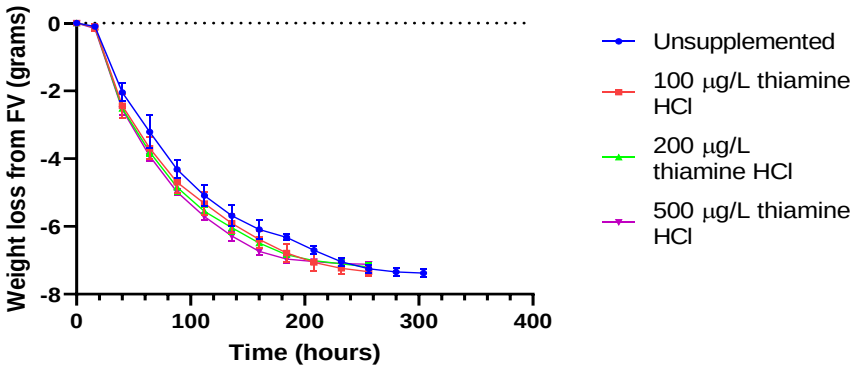


Figure 5.5. Effect of vitamin B_7 biotin (A), B_6 pyridoxine (B) and B_1 Thiamine (C) supplementation on fermentation performance of yeast strain SMCC100 in 22 °P wort. Fermentations were carried out in 100 mL FVs and progression was monitored daily by measuring weight loss over time. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

In order to determine the impact of wort sterol concentration on yeast fermentation performance at VHG, worts were supplemented with ergosterol at 100 mg/L, 200 mg/L and 500 mg/L. It can be seen from Figure 5.6 that all experimental conditions caused a reduction in attenuation time by 72 hours when compared to the unsupplemented control. Interestingly, within the first 88 hours of fermentation, all of the ergosterol-supplemented fermentations exhibited a sluggish start to fermentation, with an extended lag phase and little weight loss (CO_2 evolution) until after 16 hours of fermentation. The extent of this elongated lag in fermentation initiation was such that yeast in the unsupplemented fermentation initially outperformed those under experimental conditions. However, in the latter stages of fermentation, the ergosterol-supplemented fermentations can be seen to greatly enhanced, with faster fermentation rates, reduced time to attenuation, and a greater total weight loss from the FV, indicating greater fermentable extract assimilation overall (Figure 5.6). The reason for the effects early in fermentation are unknown. However, given the role of ergosterol in assuring yeast division, it might have been expected that yeast growth would be enhanced leading to a shorter lag phase. However, given that the opposite was true it is suggested that this may be a consequence of the inhibition of ergosterol uptake through SUT1 repression caused by the presence of oxygen (Kwast et al., 1998).

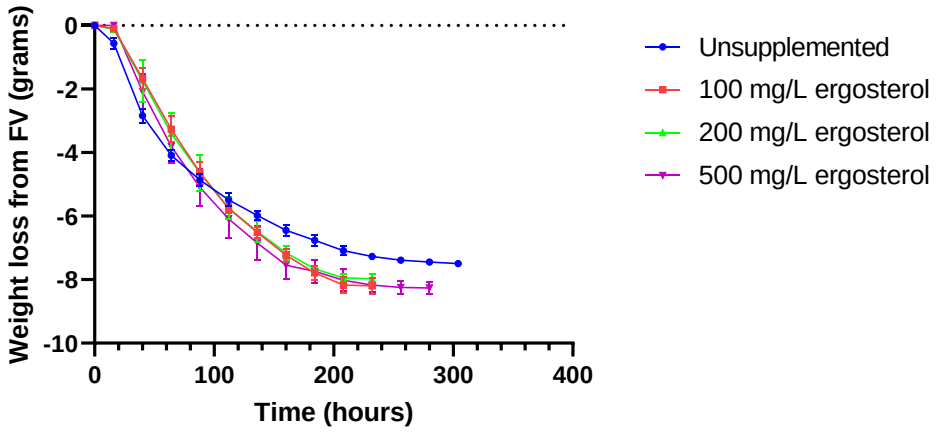


Figure 5.6. Effect of ergosterol supplementation on VHG fermentation progression. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

It should be noted that the data above reflects analysis of the 3rd fermentation in a series. This practice was conducted to remove any issues associated with the use of generation 0 yeast. However, each of the first two fermentations were also evaluated, focusing purely on the time taken to reach attenuation. Based on this, some data could be obtained regarding the benefits of supplementation over the course of serial repitching, and to determine if the effects were consistent over time. It can be seen that, generally speaking, the application of nutrients had an enhanced effect as the number of repitchings increased (F1-F3; Table 5.7). For example, the beneficial effects of zinc supplementation at both 1.32 mg/L and 2.64 mg/L were minimal in the first two fermentations, before being more pronounced during F3. Similarly, the detrimental effects of applying 6.60 mg/L zinc sulphate were not observed until

the third fermentation, potentially indicating a build-up of internal reserves over time. Magnesium sulphate (at a concentration of 12.6 g/L) was the only metal ion observed to consistently improve attenuation time over the course of three consecutive fermentations.

Similar to metal ions, analysis of the impact of B vitamins over time indicated that a positive effect was most obvious in the 3rd fermentation (F3). This was true for biotin supplementation, which only yielded an improvement in fermentation rate on the third generation (F3) of yeast, irrespective of concentration (Figure 5.7). Despite this, some improvement was observed when pyridoxine (80 µg/L) and thiamine (100 µg/L) were used in both the second and third fermentations (F2-F3), with F1 yeast appearing similar to the control.

The most significant and sustained improvements in attenuation time were observed with DAP and ergosterol. DAP supplementation had a positive effect in reducing fermentation time at all concentrations over the course of all of the three repitchings, except at a concentration of 0.5 g/L for generation 2 (F2) yeast. In each case, the highest concentration of DAP had the greatest positive effect on fermentation time (reduced fermentation time) for each generation (Figure 5.7). Similarly, ergosterol at 100 mg/L had a positive effect over the course of serial repitching. A similar, albeit less pronounced, effect was seen with 200 mg/L ergosterol. Interestingly, the addition of 500 mg/L of ergosterol proved extremely detrimental to yeast performance in the first fermentation

(F1). This reduction in fermentation time was however alleviated in the subsequent fermentations (Figure 5.7).

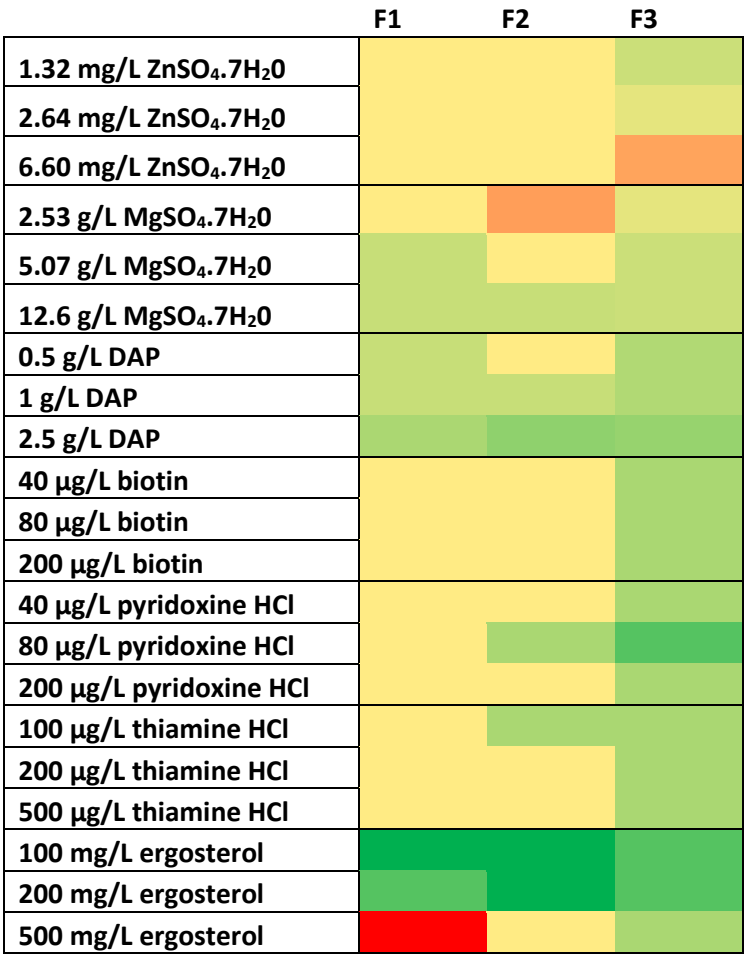


Figure 5.7. Heatmap displaying the effect of each nutrient addition on attenuation time against an unsupplemented control over three fermentation generations (F1, F2 and F3) whereby shades of red indicate longer attenuation times, yellow indicates no change and shades of green indicate a reduction in attenuation time. Colour indicators were assigned relatively based on the range of attenuation times from the dataset, whereby the greatest decrease in attenuation time was assigned the

darkest shade of green and the greatest increase was assigned the darkest shade of red.

5.2.3 The impact of nutrient additions on brewing yeast viability

The aim of this series of experiments was to not only investigate strategies for the improvement of fermentation performance under VHG conditions, but to improve yeast quality post-fermentation. This is largely because of the negative impact of VHG conditions on yeast health and the fact that cultures are typically only reused if the percentage of living cells within the population is considered sufficient (typically >90%). In order to assess the impact of nutritional supplements on yeast health, cell samples were taken from the third generation fermentation in each instance and analysed using methylene blue staining as described in Section 2.3.1. The third fermentation generation was taken as a representative of the effects of each nutrient supplement condition of yeast viability, however this important quality parameter was also monitored throughout the previous fermentations as yeast was cropped and repitched. This was to ensure the longevity of any potential benefits.

It has previously been reported that zinc supplementation is beneficial to yeast, but that high levels of zinc can have toxic effects, with some lager yeast strains unable to tolerate zinc concentrations in excess of 1310 ppb of Zn^{2+} resulting in reduced viability post-fermentation (Rees and Stewart, 1998). In the current study, supplementation of wort was conducted at a much lower rate, but with more positive results. When 1.32 mg/L of zinc sulphate was applied (total wort zinc concentration of 462 ppb) this resulted in significant ($p=0.032$) increase in

post-fermentation viability when compared to an unsupplemented fermentation (93.59% compared to 89.91% respectively) (Figure 5.8). Conversely, the addition of 6.60 mg/L of zinc supplement significantly reduced yeast viability ($p=0.003$). The results from analysis of yeast viability closely aligns with the effects of zinc supplementation on fermentation performance, with the most beneficial supplement concentrations also having the most positive effect on viability.

Magnesium supplementation has been shown to reduce fermentation time at similar concentrations to those used in this study, likely due to the integral role of magnesium in yeast metabolism and as a stress protectant (Walker, 1998). However, in this instance, there was no statistically significant change in yeast viability between experimental or control groups (Figure 5.9), although the mean viability was observed to increase slightly as a result of supplementation in each case.

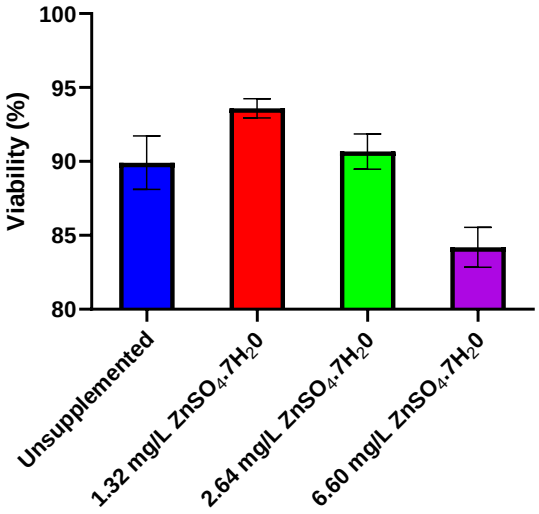


Figure 5.8. Effect of zinc sulphate heptahydrate supplementation on yeast viability post-fermentation. Values represent the mean of triplicate samples with error bars indicating the standard deviation.

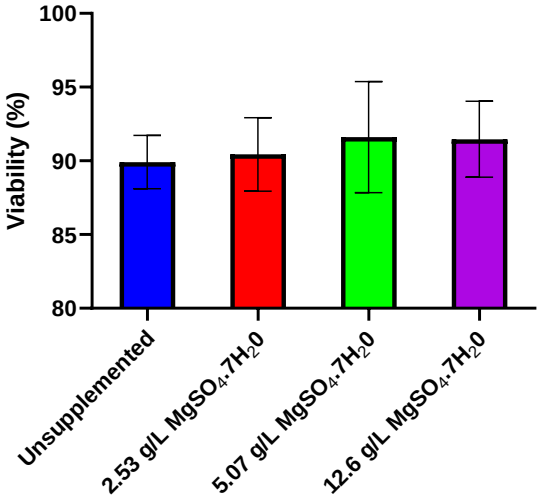


Figure 5.9. Effect of magnesium sulphate heptahydrate supplementation on yeast viability post-fermentation. Values represent the mean of triplicate samples with error bars indicating the standard deviation.

Increasing wort FAN levels is commonly associated with an improved performance of yeast under VHG conditions, due to the requirement of nitrogen for sustaining yeast growth and metabolism (Lekkas et al., 2007; Gibson, 2011; Hill and Stewart, 2019). However, further to improving the rate of extract uptake and attenuation time observed above (Section 5.4), the supplementation of worts with DAP also led to a statistical increase ($p \leq 0.05$) in yeast viability post fermentation (Figure 5.10). However, there was no statistical difference between the DAP supplemented fermentations, suggesting that there was no additional benefit to yeast viability by increasing the concentration of DAP supplementation beyond 0.5 g/L.. As the concentration of FAN in the unsupplemented VHG wort was 250.02 mg/L

(Table 5.1), the increased viability and decreased attenuation times observed in Figure 5.10 and Figure 5.4 respectively indicate that this level of wort FAN is sub-optimal under these conditions.

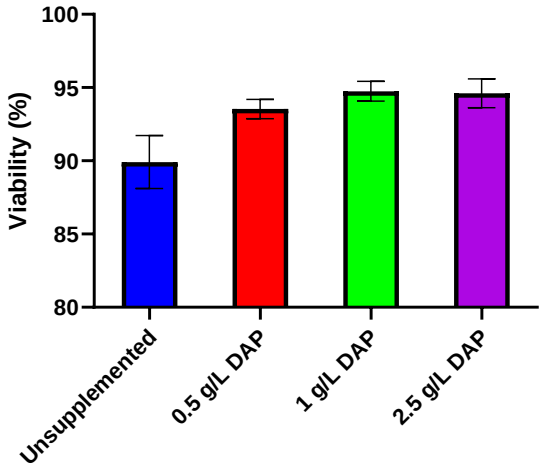
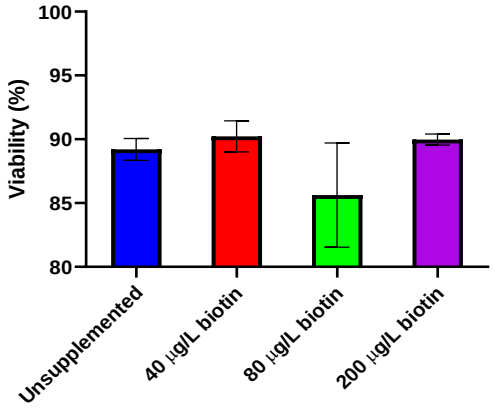


Figure 5.10. Effect of DAP supplementation on yeast viability post-fermentation. Values represent the mean of triplicate samples with error bars indicating the standard deviation.

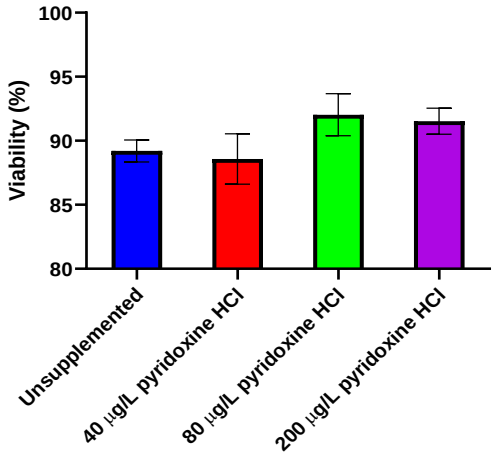
Interestingly, despite the improvement in fermentation performance reported above (Section 5.2.2), supplementation of wort with any of the B vitamins (biotin, thiamine and pyridoxine) did not have an effect on yeast viability following VHG fermentations. In all instances there were no statistical difference between experimental conditions and the unsupplemented fermentation. Although there were some minor changes to the mean viabilities

in some cases, these were not consistent across sample groups and there were no trends related to the concentration of vitamin supplied. This suggests that the primary role of vitamins was to enhance performance and metabolism rather than to affect the capacity of yeast cells to survive fermentation related stress factors.

A



B



C

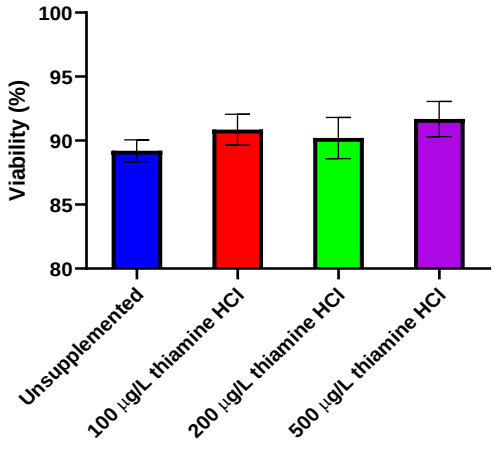


Figure 5.11. Effect of vitamin B_7 biotin (A), B_6 pyridoxine (B) and B_1 Thiamine (C) supplementation on yeast viability post-fermentation. Values are displayed as the mean of triplicate samples with error bars indicating the standard deviation.

Previously it was shown that the addition of ergosterol had the most marked impact on fermentation performance (Section 5.2.2). However, it is interesting to note that this improvement was not a result of improved yeast viability. In all instances, yeast samples supplemented with ergosterol yielded yeast post-fermentation that was either similar in viability to the control or in fact displayed a reduced viability (Figure 5.12). The exception to this was when 100 mg/L ergosterol was used, although in this instance the difference was not statistically significant. When the concentration of ergosterol supplementation was increased to 500 mg/L, yeast viability post-fermentation decreased significantly. This trend goes some way to explaining the slower attenuation time observed in fermentations observed with this concentration of ergosterol (Figure 5.6), although the underpinning causes behind why ergosterol resulted in a reduced viability are currently unknown. Further analysis would be required to determine if this is related to wort gravity alone, whether it is strain dependent, and to confirm that it is a consistent phenomenon for this particular set of experimental parameters.

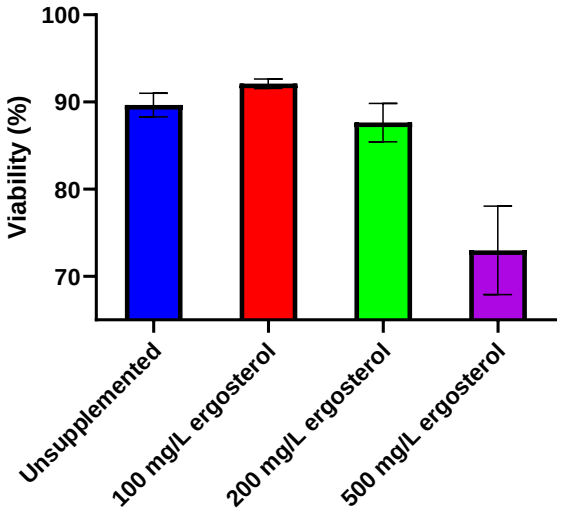


Figure 5.12. Effect of ergosterol supplementation on yeast viability post-fermentation. Values represent the mean of triplicate samples with error bars indicating the standard deviation.

It should be noted that the data above reflects analysis of the 3rd fermentation in a series. This practice was conducted to remove any issues associated with the use of generation 0 yeast. However, each of the first two fermentations were also evaluated briefly such that a summary of the data displayed above, along with data from F1 and F2 fermentations can be found in Figure 5.13 The relative impacts of each supplement on cell viability post-fermentation are indicated by a heatmap where shades of green reflect positive changes and shades of red indicate a negative impact. It can be seen that, generally speaking, the application of nutrients had a positive effect on cell viability post-fermentation and that this was more beneficial as yeast were serially repitched

(F1-F3; Figure 5.13), with only high additions of zinc and ergosterol yielding a reduction in viability.

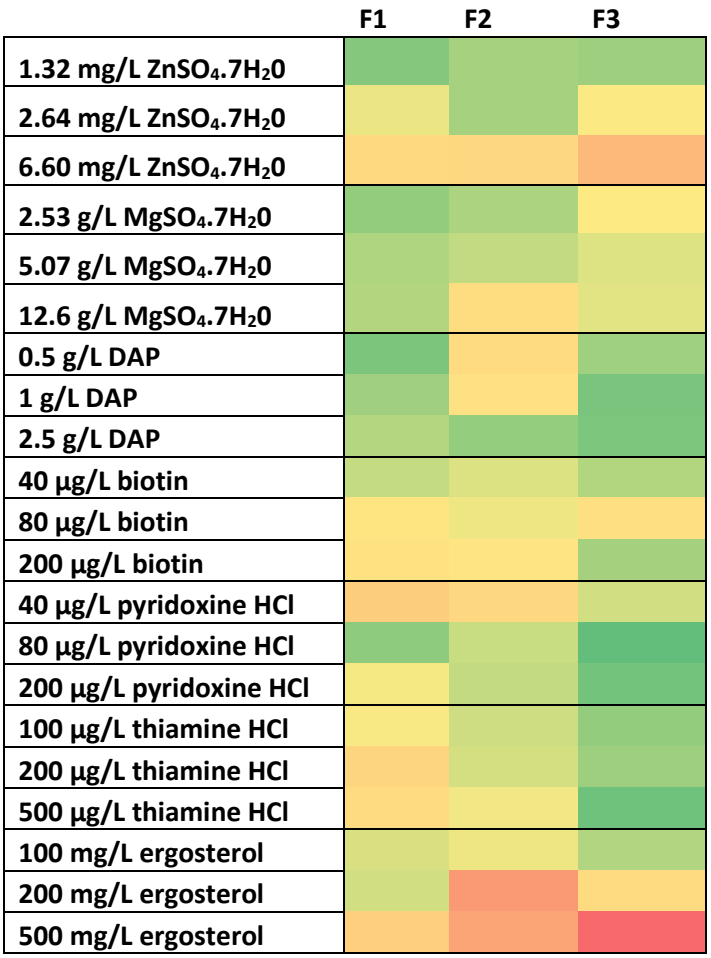


Figure 5.13. Heatmap displaying the effect of each nutrient addition on yeast viability post-fermentation against an unsupplemented control over three fermentation generations (F1, F2 and F3) whereby shades of red indicate decreased viability, yellow indicates no change and shades of green indicate an increase in viability. Colour indicators were assigned relatively based on the range of viabilities from the dataset, whereby the greatest increase in viability was assigned the darkest shade of green and the greatest decrease was assigned the darkest shade of red.

5.2.4 Effect of nutrient supplementation on yeast performance, growth and viability

Based on the data reported above, the concentration of each nutrient supplementation that had the most beneficial effect on fermentation performance and yeast viability was then selected and combined to produce an optimum 'nutrient mix' as indicated in Table 5.2. This nutrient mix was used to supplement a 22^{o}P VHG wort and yeast fermentation performance was analysed in comparison to that of an unsupplemented wort under identical conditions. In this study, the exact same base wort was used as in previous experiments to ensure that the starting nutritional content of the worts were identical. However, in this instance 2 L fermentations were employed to be more reflective of industrial practices, to allow for samples to be taken and for a more precise estimation of gravity reduction to be obtained (Section 2.4.3) as well as assessments of cell concentration and viability (Section 2.3.1). In addition to monitoring fermentation progression, yeast cell number and viability were measured throughout fermentation.

Table 5.2. Concentration of each nutrient selected.

Nutrient supplement	Concentration
Zinc sulphate heptahydrate	1.32 mg/L
Magnesium sulphate heptahydrate	5.07 g/L
Diammonium phosphate	2.5 g/L
Biotin	40 µg/L
Pyridoxine hydrochloride	80 µg/L
Thiamine hydrochloride	100 µg/L
Ergosterol	100 mg/L

It can be seen that the nutrient supplemented fermentations exhibited a marked increase in fermentation rate and a significant reduction in time to attenuation (Figure 5.14). Interestingly, within the first 16 hours after pitching, the nutrient addition did not affect fermentation rate, but that enhanced efficiency was clearly observed after this point. For the supplemented fermentation, the logarithmic phase ceased after 112 hours and fermentation began to plateau until 184 hours, when the fermentation attenuates. For the unsupplemented media, attenuation was not reached until 376 hours, indicating a total time saving of 192 hours through the use of supplements.

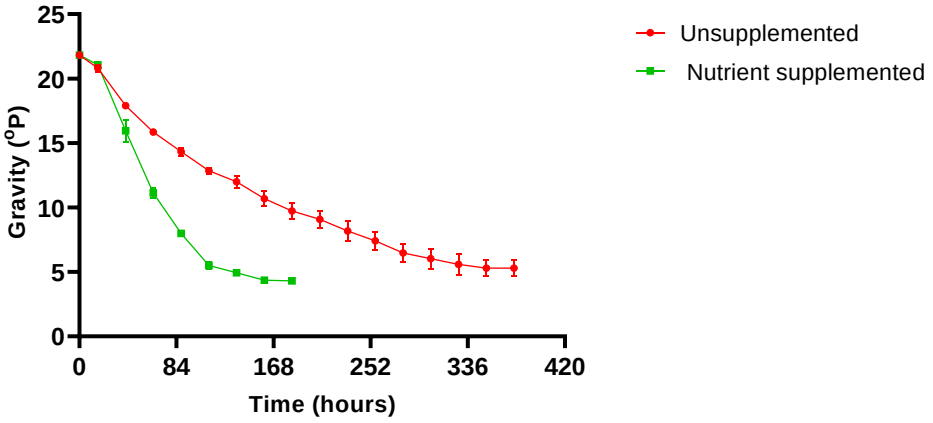


Figure 5.14. The effect of combined nutrient supplementation on fermentation progression at 22°P. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

Interestingly, after pitching yeast into fermentations, an initial decrease in population viability was observed (Figure 5.15). This phenomenon may be related to the very high gravity brewing environment, as high concentrations of sugars present in the starting wort exert osmotic pressure on the pitched yeast, potentially causing the death of weaker cells and a reduction in yeast viability. Following this initial decrease in viability, both experimental conditions showed an increase in yeast viability, likely attributed to biomass production and growth. Viability of cells within the nutrient supplemented fermentation were then maintained at approximately 96-98% until attenuation. However, viability in the unsupplemented fermentations were

lower at around 90-93%, and declined gradually until the fermentation endpoint.

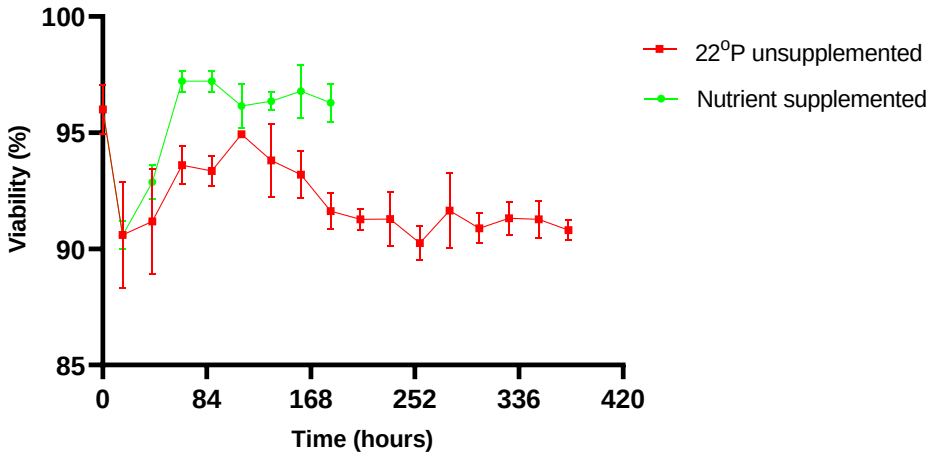


Figure 5.15. Effect of combined nutrient supplementation on yeast viability throughout fermentation. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

It should be noted that both sets of fermentations were inoculated using the same yeast pitching rate. However, analysis of yeast count indicated that the supplemented fermentation exhibited a significantly greater cell concentration than the unsupplemented fermentation after approximately 48 hours when division had ceased (Figure 5.16). Consequently, it is likely that the greater rate of fermentation was promoted by cell number. However, it is clear that this was not the only factor, since fermentation times were significantly longer in unsupplemented media, which would not be expected for yeast retaining >90%

viability. Hence the nutrient mixture was able to boost yeast performance through sugar uptake and metabolic rate rather than solely having an impact on resistance to stress. Despite this hypothesis, it is acknowledged that these two effectors are not mutually exclusive. It is possible that enhanced metabolism can only be achieved once the effects of stress are mitigated in some form, whether that be by the capacity to repair damage, to prepare the cell such that it has greater tolerance to stress. Further analysis would be useful to determine if the nutrient addition was able to reduce the susceptibility of brewing yeast cells to stress challenges related to VHG brewing, such as ethanol and osmotic stress. Note that flavour production is also a key performance indicator and determinant of commercial viability, and this will be discussed in Section 5.2.6 below.

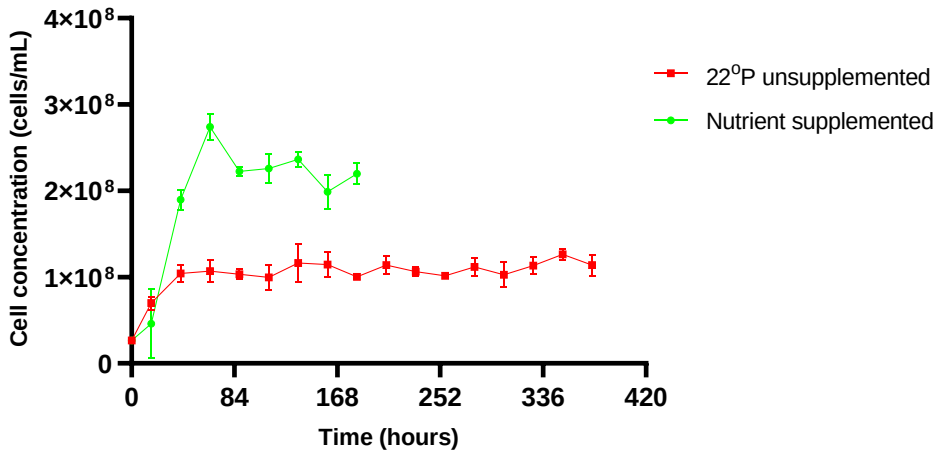


Figure 5.16. Effect of combined nutrient supplementation on yeast growth. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

5.2.5 Effect of sugar supplementation ('sugar top-up') on fermentation performance, yeast health and biomass formation

Although it has been shown that nutritional supplementation has major potential for improving yeast performance at VHG (Section 5.2.4), it should be noted that this approach inevitably incurs an additional expense to the brewer, as well as potential issues with legislation surrounding the addition of non-conventional brewing ingredients. Despite improvement in performance, this avenue for maximising potential therefore needs to be the subject of a cost-performance analysis to determine whether it is commercially viable. As an alternative strategy, it was decided to test an alternative form of supplementation, whereby sugar was added at stages during fermentation to alleviate stress associated with the initial conditions associated with batch VHG fermentations. The premise of this approach was to prevent yeast from being exposed to an initial high sugar concentration and, by avoiding the initial osmotic shock upon pitching, to allow yeast health and fermentation performance to be maximised.

In order to reduce osmotic stress exerted on the pitched yeast, a base wort of 17.5 ^{o}P, prepared as described in Section 2.2.3, was supplemented with a maltose-based sugar adjunct at the mid-point of 2 L fermentations. The base wort and sugar adjunct employed was identical to that used to prepare the 22 ^{o}P wort applied in previous experiments (Sections 5.2.1-5.2.4), and as such this 'unsupplemented' wort was also used as a control for this series of experiments. It should also be noted that the point of addition was determined

based on preliminary trials where a series of precise time points were investigated. Based on this, additions at 24 hours and 48 hours were noted to yield poor results in terms of fermentation rate and attenuation time (data not shown). Consequently, sugar top-up was performed at 88 hours after pitching and the amount of sugar added was such that the total complement was the same as for the control sample; 22 ^{o}P of extract was provided overall in both instances. Similarly, the amount of oxygen provided and the pitching rates were adjusted at the start of fermentation such that they were identical and based on a 22 ^{o}P fermentation regime.

It can be seen that in the top-up regime fermentation, the initial gravity of the pitching wort was 17.5^{o}P. Following an initial lag phase after pitching, wort gravity decreased at an accelerated rate when compared to the 22^{o}P unsupplemented fermentation (Figure 5.17). Gravity then increased at 88 hours as the sugar adjunct was added to the fermenting wort. Although the fermentation trajectory closely matched that of the unsupplemented regime after this point, the fermentation was deemed to be complete 24 hours sooner (Figure 5.17).

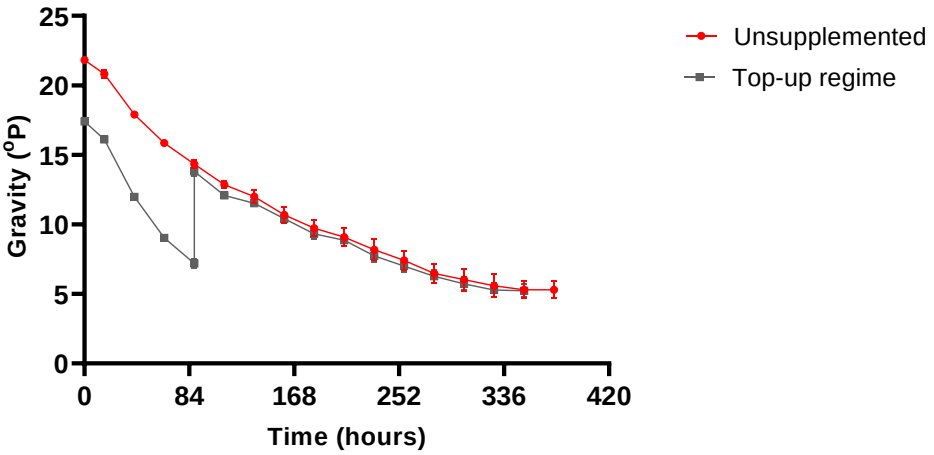


Figure 5.17. The effect of a sugar top-up regime on yeast fermentation performance at VHG. At 88 hours, maltose-based sugar adjunct was added to the top-up regime fermentation (grey line), increasing the fermenting wort gravity. Data points represent the mean of triplicate samples with error bars indicating standard deviation of the samples.

As was noted previously (Section 5.2.4), an initial decrease in viability was observed immediately after pitching (Figure 5.18). However, in the top-up regime that was less that than observed in the unsupplemented fermentation. This could be due to the lower gravity of the pitching wort, reducing the osmotic shock of which yeast are exposed to upon inoculation. Interestingly, at the point where sugar adjunct was added to the fermentation media (88 hours), there was a small spike in yeast viability. Although the reason for this is unclear, it is interesting to note that a steady decline in viability was then observed as fermentation progressed. However, by the fermentation endpoint,

the viability observed in the top-up regime was significantly greater than that of the unsupplemented fermentation ($p \leq 0.05$) (Figure 5.18).

Analysis of cell concentration over the course of fermentation indicated that more cells were present in the top-up regime than that of the unsupplemented fermentation (Figure 5.19). Even though initial pitching rates were identical, this is likely to be a result of the reduced volume of media at this time point. The higher cell concentration was observed during the yeast growth phase until the maximum cell concentration was reached after approximately 40 hours. At the 88 hour timepoint, as the sugar adjunct was added to the vessel, the total volume of the fermenting wort was then increased to the same volume of the unsupplemented fermentation, thus reducing the cell concentration to approximately the same concentration as the unsupplemented fermentation. The cell concentration of the top-up regime then remained constant for the remainder of the fermentation (Figure 5.19).

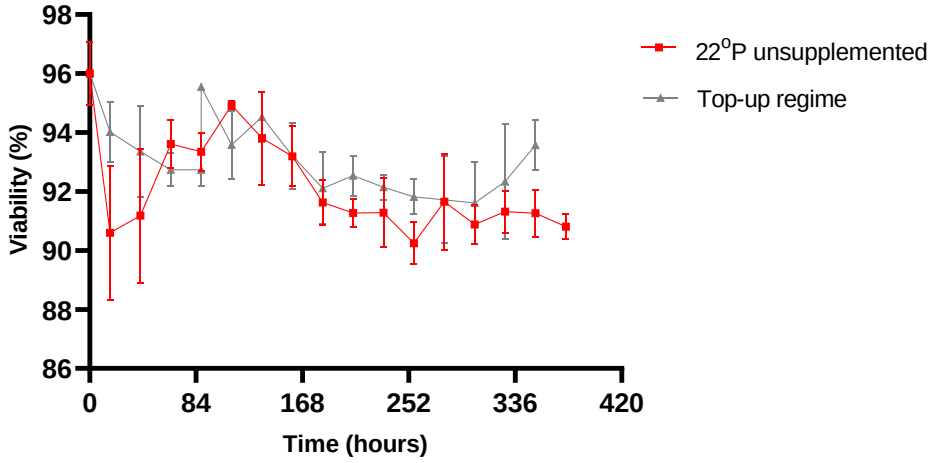


Figure 5.18. The effect of a sugar top-up regime on yeast viability under VHG conditions.

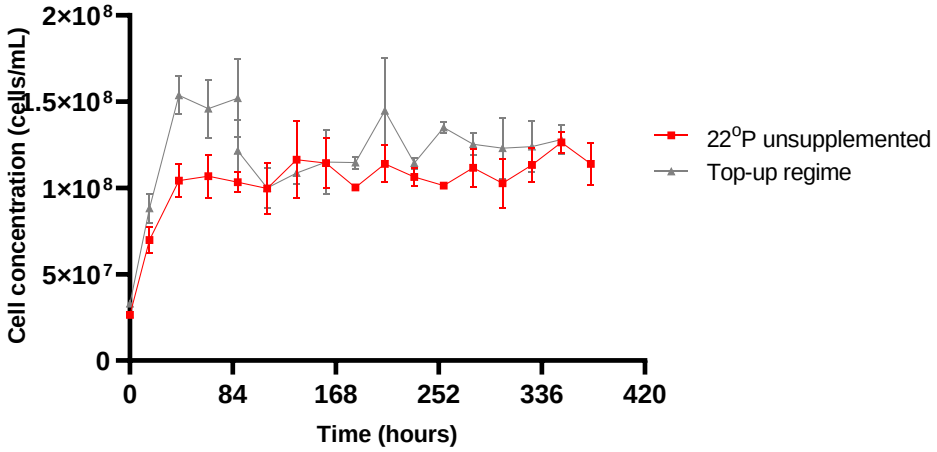


Figure 5.19. Effect of sugar top-up regime on yeast cell concentration throughout a VHG fermentation.

5.2.6 The impact of supplementation and sugar top-up on flavour development and end product characteristics

Although sugar utilisation speed and final attenuation limits are key attributes by which fermentations can be assessed, in terms of brewing and beverage applications the consequences of process optimisation on the flavour profile of products should also be considered. As such, the yeast-derived higher alcohol and ester profile of VHG beers produced via nutrient supplemented and sugar-top up regime fermentations were analysed, along with those obtained from standard unsupplemented worts. The resulting flavour profiles were then adjusted via mathematically to a standard final alcohol by volume (ABV) of 5% to reflect a typical lager ABV and compared with those obtained from a 17.5^{o}P control fermentation in order to assess the potential of each fermentation optimisation procedure.

Flavour profiles of beers produced using each regime were analysed and concentrations of higher alcohols (Figures 5.20), acetate esters (Figure 5.21) and both acetaldehyde and diacetyl (Table 5.3) were determined. Typically, VHG conditions are regarded to induce an environment by which the production of flavour-active volatiles occurs disproportionately in comparison to a standard gravity fermentation (Anderson and Kirsop, 1974; Saerens et al., 2008; Puligundla et al., 2011). However, in this instance, the concentration of higher alcohols and acetate esters (Figure 5.20 and Figure 5.21 respectively) were not observed to differ in the final beer (adjusted to 5% ABV) produced at 17.5 ^{o}P and 22 ^{o}P. Additionally, no significant difference was found between

the higher alcohol or ester profiles of beer produced using control fermentations and the top-up fermentation regime (Figure 5.20 ad Figure 5.21 respectively).

However, when wort was supplemented with a nutrient mix, higher alcohol and acetate esters production was significantly affected. All of the higher alcohols and esters analysed were present at different concentrations when compared to the 17.5 ^{o}P control fermentation. Typically, higher alcohols were present in elevated concentrations, perhaps indicative of the greater amount of yeast growth observed (Erten et al., 2007; Saerens et al., 2008; He et al., 2014a; Pires et al., 2014), while ester production was restricted. The concentrations of both 1-propanol and isoamyl alcohol were significantly higher than those found in the control beer ($p \leq 0.005$). Conversely, ethyl acetate levels in the nutrient supplemented fermentation were significantly lower than in the control beer ($p \leq 0.005$). There was no significant difference in the concentrations of isoamyl acetate amongst the different fermentation conditions.

Analysis of the concentration of diacetyl in the green beer indicated that these were extremely high in the nutrient supplemented fermentation (Table 5.3). For these fermentations, the amount of diacetyl (204.33 ± 51.64) was significantly above the flavour threshold and significantly higher than present in all of the other fermentations conducted ($p \leq 0.005$). However, there was no significant difference in green beer diacetyl levels amongst the other fermentations, although they all were above the flavour threshold (Table 5.3;

Section 1.3.4), indicating that extended maturation of these products would be required. Additionally, there was no significant difference amongst the concentrations of acetaldehyde produced during fermentation under all conditions used in this study. Glycerol levels were significantly higher in nutrient-supplemented beers when compared to the other fermentation conditions in this study ($p \leq 0.005$). The reasons for this are unknown, but are likely to reflect enhanced production and release of glycerol by yeast cells. This could be related to enhanced 'fitness' of the population caused by the presence of key nutrients, or alternatively could reflect an increase in glycerol synthetic enzyme activity, caused by the presence of elevated co-factors. Further experimentation would be required to determine the relationship between aspects of yeast metabolism and carbon flux, nutrient addition and glycerol production.

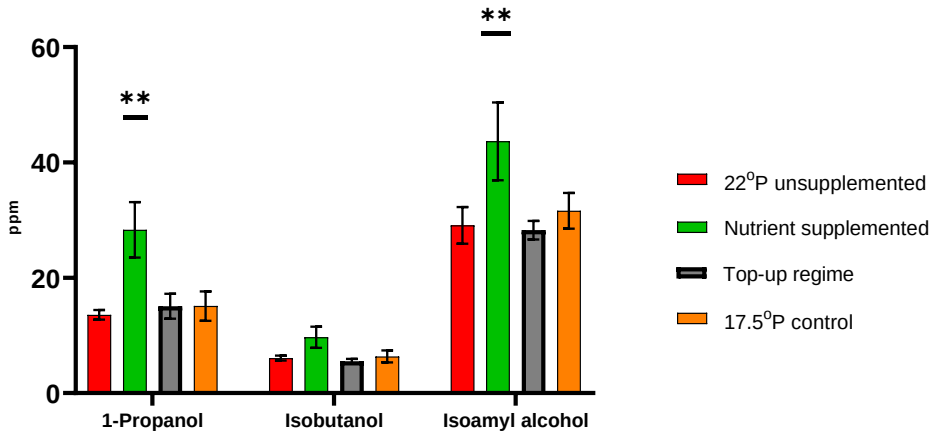


Figure 5.20. Higher alcohol flavour profile of beers produced by an unsupplemented 22°P fermentation, a nutrient supplemented 22°P and a 17°P control fermentation with all data normalised to represent the concentration of each flavour compound within a 5% ABV beer.

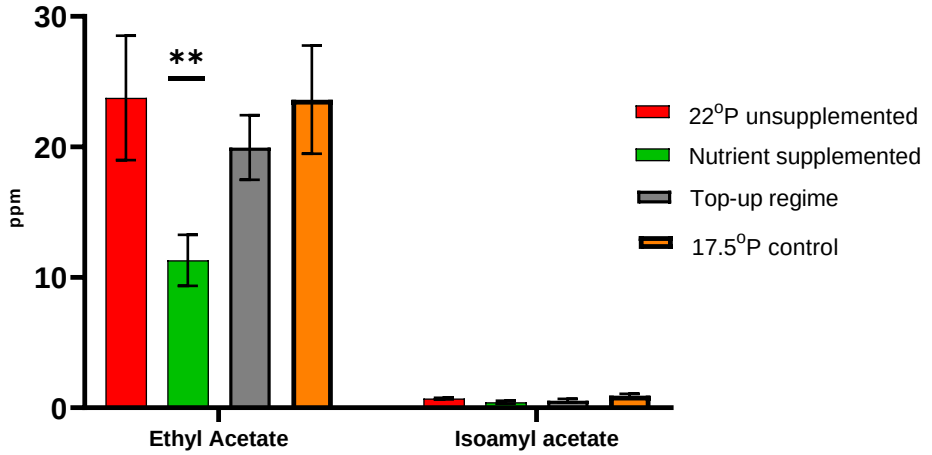


Figure 5.21. Acetate ester flavour profile of beers produced by an unsupplemented 22°P fermentation, a nutrient supplemented 22°P fermentation, a top-up regime

22°P fermentation and a 17.5 °P control fermentation with all data normalised to represent the concentration of each flavour compound within a 5% ABV beer.

Table 5.3. Residual nutritional composition of green beer following a 22 °P unsupplemented fermentation, a nutrient supplemented 22 °P fermentation, a 22 °P top-up regime and a 17.5 °P control fermentation.

Condition	Zinc concentration (ppb)	Magnesium concentration (ppm)	Free amino nitrogen (mg/L)
22 °P unsupplemented	5.61 ± 0.59	75.33 ± 2.99	144.19 ± 2.60
Nutrient supplemented	23.15 ± 8.34	532.89 ± 8.25	144.37 ± 7.41
Top-up regime	7.09 ± 2.02	82.45 ± 0.57	169.33 ± 9.98
17.5 °P control	7.38 ± 1.16	95.99 ± 1.16	254.17 ± 3.66

Analysis of additional final product characteristics indicated that the ABV of the green beer following nutrient supplemented fermentations was significantly higher than that of both the unsupplemented and top-up regimes ($p \leq 0.05$) (Table 5.4). Additionally, there was no significant difference between the amount of ABV attained through unsupplemented or top-up fermentations, supporting previous data obtained in terms of extract utilisation (Section 5.2.5). Interestingly, the top-up regime incurred a lower degree of FAN uptake than the unsupplemented regime ($p \leq 0.05$). Furthermore, the uptake of zinc (zinc levels in wort minus zinc levels in the green beer) was significantly lower in both the 22°P unsupplemented fermentation and the top-up regime than the 17.5

^{o}P control, which is likely due to the reduced zinc levels in the wort due to the inclusion of the nutrient-deficient sugar adjunct. Additionally, the significantly higher uptake of zinc observed in the nutrient supplemented fermentation suggests that under these conditions, zinc levels are severely insufficient in the 22^{o}P wort without any zinc supplementation.

Magnesium levels remained relatively high in the green beer compared to the starting wort (Table 5.3), with relatively low quantities removed from the wort by yeast during fermentation (Table 5.4). The nutrient supplementation resulted in a significantly higher degree of magnesium uptake ($p \leq 0.005$) than all other conditions. Despite this, magnesium levels remained very high in the green beer following the nutrient supplemented fermentation (533.89 mg/L ± 8.25). There was no significant difference in magnesium uptake between the unsupplemented and top-up fermentation regimes.

Table 5.4. End-product characteristics and fermentation performance of green beer produced via different HG and VHG fermentation regimes.

Condition	Attenuation time (hours)	Final gravity (°P)	ABV (% v/v)	FAN uptake (mg/L)	Zinc uptake (µg/L)	Magnesium uptake (mg/L)	Diacetyl (mg/L)	Acetaldehyde (mg/L)	Glycerol (mg/L)
17.5°P control	280	3.91 ± 0.04	6.43 ± 0.44	58.45 ± 18.06	195.27 ± 1.43	19.16 ± 3.74	19.00 ± 3.00	4.28 ± 0.32	1.83 ± 0.27
22°P unsupplemented	376	5.30 ± 0.62	8.73 ± 0.19	124.49 ± 8.27	156.50 ± 0.73	17.14 ± 3.67	8.33 ± 0.58	6.05 ± 1.88	1.96 ± 0.15
Nutrient supplemented	184	4.30 ± 0.03	9.96 ± 0.25	201.17 ± 6.72	439.09 ± 10.21	59.53 ± 10.10	204.33 ± 51.64	6.12 ± 1.30	3.86 ± 0.38
Top-up regime	352	5.22 ± 0.46	8.51 ± 0.28	80.69 ± 6.31	155.02 ± 2.48	10.02 ± 0.70	11.00 ± 2.00	3.15 ± 0.27	1.91 ± 0.38

5.3 Discussion

It is well documented that nutrient additions in the form of zinc and magnesium can have a beneficial effect on fermentation performance and yeast quality post-fermentation (Walker et al., 2006), however the optimum concentration of these minerals can be highly dependent on the process, yeast strain and content of the existing wort (Rees and Stewart, 1998). For example, it has been shown that zinc concentrations exceeding 665 µg/L can result in adverse effects on yeast health a reduction in viability (Rees and Stewart, 1998). In this study, the optimum concentration of zinc sulphate heptahydrate was identified to be 1.32 mg/L (addition of 300.12 ppb of Zn^{2+}, total of 462.23 ppb in wort). Any further increase in wort zinc concentration did not yield any beneficial effects in terms of fermentation time or viability. In the case of the addition of 6.60 mg/L of zinc sulphate (total wort zinc concentration of 1663 ppb), fermentation time was elongated coupled with a decrease in viability. The extent of this decline in viability became accentuated as the number of times the yeast was repitched increased. Although the results from this study suggest that the optimum concentration of magnesium sulphate heptahydrate to be 12.6 g/L (total wort magnesium concentration of 1332 mg/L), practical issues were encountered with the solubility of the magnesium supplement in the nutrient mix. This prompted the consideration of 5.07 g/L magnesium sulphate heptahydrate (total wort magnesium concentration of 592 mg/L), as the appropriate concentration for the combined nutrient approach. It should be noted that this concentration of magnesium aligns with those applied within a

study by Rees et al. (1998), in which fermentation of 20 ^{o}P wort was enhanced by supplementing with 500 mg/L magnesium.

The addition of 0.5 g/L of DAP was sufficient to improve yeast viability post-fermentation and reduce fermentation time, however the highest addition used in this study of 2.5g/L DAP (total wort FAN 346 mg/L) further reduced fermentation time. However, there was no significant difference between the DAP-supplemented fermentations at all concentrations. This suggests that the higher concentrations of DAP are more beneficial to fermentation rate than to yeast viability, however the benefits to fermentation rate outweighed the lack of additional benefit to yeast viability, since this was not negatively affected. As wort FAN is a necessary requirement to facilitate yeast growth (Boulton and Quain, 2001; Hill and Stewart, 2019), it is likely that this supplementation contributed to the increased rate of biomass production in the combined nutrient optimisation approach. This enhanced growth could therefore also contribute to the increased fermentation performance observed both in the DAP-supplemented fermentations and the combined nutrient approach. As elevated wort FAN was one of the likely contributors to the increase in yeast growth observed, this could have potentially caused the imbalance of higher alcohol flavour production, which is highly influenced by yeast growth (Anderson and Kirsop, 1974; Saerens et al., 2008; He et al., 2014a; He et al., 2014b).

Beyond the impact of biotin (Lardy et al., 1949; Moat and Lichstein, 1954; Ough et al., 1989), the influence of B vitamin additions is scarcely addressed in the

literature. Due to their individual benefits on yeast attenuation time, combined with the relatively low concentrations required for a desired effect, addition of these vitamins makes them an attractive option for further study. Pyridoxine reduced fermentation time more effectively than the other two B vitamins investigated, as well as preserving yeast viability to a greater extent. Their combined positive effect on fermentation performance indicators is likely due to their roles in cellular metabolism, in which a general increase in metabolism as a result of these exogenous additions would theoretically increase extract uptake and alcohol production. More work is needed to elucidate the effects of thiamine, biotin and pyridoxine to elucidate their influence on fermentation-derived products and their effects on yeast physiology.

Ergosterol supplementation is a difficult practice for large-scale brewing operations. This is largely due to its insolubility in water, requiring the use of a mixture of ethanol, tween 80 and ergosterol in order to prepare the supplement for use. However, as yeast growth is limited in oxygen-depleted environments (Kirsop, 1974), the addition of ergosterol has the potential to circumvent the impact of growth-related issues caused by environmental stress factors in VHG environments (Ohno and Takahashi, 1986; Boulton and Quain, 2001). In this study it was found that supplementation with ergosterol had a largely positive effect on fermentation speed and yeast performance. The optimum concentration of ergosterol supplementation of 100 mg/L was synonymous with the findings of Casey et al. (1983).

Similar to ergosterol, DAP was also shown to be beneficial to yeast performance, serving to increase the yeast growth capacity. This suggests that yeast growth plays a major role in performance at VHG. However, as previously discussed, altering yeast growth has the potential to influence the flavour profile of the resulting beer due to the relationship between biomass production, carbon flux and the Ehrlich pathway. It is tempting to conclude that this would eliminate the potential for DAP (and ergosterol) in fermentation, however it is possible that further analysis including a detailed study of oxygenation rate, pitching rate and supplement addition would provide more practical data for the brewer and to facilitate flavour matching of a pre-existing beer product. This would also allow alteration of nutrient concentrations or ratios to minimise the effects on flavour production, while also assessing the resulting effect on fermentation performance such that a compromise between the outcomes could be reached.

Primarily due to the flavour discrepancies observed in beer produced using the nutrient-mixture, an alternative fermentation optimisation procedure was assessed, involving sugar addition. The top-up regime utilised in this study was successful in reducing fermentation time and improving yeast quality post-fermentation. Although these were not achieved at the same degree as the nutritional supplement approach, there was no significant difference found in the beer flavour profile when compared to the control beer and this approach was therefore considered to be of more commercial benefit. By preventing yeast from being exposed to the initial stress levels associated with the VHG pitching wort, yeast health was preserved, which manifested in a higher

viability post-fermentation than both control HG and VHG fermentations. This could potentially be attributed to the enhanced fermentation performance observed during the initial stages of fermentation during the top-up regime, due to the disproportionately high cell density at this stage, which also was likely to be a cause of the reduction in total fermentation time.

In summary, the nutritional supplementation regime arguably displays the greatest promise for long term optimisation of VHG brewing. However, this approach requires further refinement for use in routine brewing fermentations, due to the adverse effect on beer flavour profile, including high VDK levels in the green beer. This was observed despite the extremely positive results regarding yeast health and fermentation rate when the strategy is implemented. Therefore, the top-up regime was selected as being the most attractive regime out of the two strategies assessed in this study. While the time-saving benefits were not as pronounced as for the nutrient mixture, the flavour similarity was a positive outcome. It should be noted that further work in this area could include the refining of nutrient additions, perhaps to reduce them in order to trade speed with product character. In addition, the alternative sugar to-up approach could be further refined to consider the potential for multiple adjunct additions, as well as assessing the timing of sugars or supplementary nutrient additions at various points throughout fermentation.

CHAPTER 6: THE INFLUENCE OF VERY HIGH GRAVITY FERMENTATION OPTIMISATION ON YEAST ORGANELLE MORPHOLOGY AND GENE EXPRESSION

6.1 Introduction

From an industrial perspective, yeast populations are typically subjected to rapidly changing environments which pose a series of challenges for the individual cell. In brewing fermentation environments these include ethanol stress (Casey et al., 1983; Devantier et al., 2005a; Saini et al., 2018), osmotic stress (Devantier et al., 2005c; White et al., 2008), oxidative stress (Liu et al., 2013), fluctuations in temperature (Briggs et al., 2004; Gibson et al., 2007), nutrient limitation (Casey et al., 1983; Gibson, 2011) and other compositional or physical deviation from 'optimal' conditions (Mager and De Kruijff, 1995; Ruis and Schüller, 1995; Müller and Reichert, 2011; Gomar-Alba et al., 2015). In response to environmental stress factors, yeast cells initiate a series of cascade pathways that adapt cellular function in order to survive or mitigate the effects of the conditions faced (Mager and De Kruijff, 1995; Gasch et al., 2000; Hohmann, 2002; Mager and Siderius, 2002). These pathways, coordinated by specific sets of genes, result in the re-organization of the expression profile of each cell in order to alter the production levels of cellular proteins and metabolites, ultimately leading to adaptation of cellular physiology (Gasch et al., 2000).

Upon sensing an environmental challenge, yeast respond via the transcriptional activation of three protein groups: heat shock elements (HSEs), stress response elements (STREs) and AP-1 responsive elements (AREs) (Mager and De Kruijff, 1995; Piper, 1995; Alexandre et al., 2001; Hohmann, 2002; Gibson et al., 2007). The accumulation of abnormal protein aggregates in

response to stress activates HSE pathways and heat shock transcription factors (HSF), this results in the production of molecules such as trehalose which serves to protect the cell from the adverse effects of multiple stress factors (Lucero et al., 2000; Bonini et al., 2004; Bandara et al., 2009). STRE pathways are regulated by the high osmolarity glycerol (HOG) MAP kinase pathway (Section 1.5.2) which responds to extracellular osmolarity by altering the intracellular osmolarity via glycerol production (Hohmann, 2002; Dihazi et al., 2004). It should be noted that when yeast are subjected to moderate or severe levels of stress, the HSE and STRE pathways display a degree of overlap in response to temperature, osmolarity and ethanol stress, as all of these stress factors influence plasma membrane protein composition, which requires stabilisation in order for the cell to survive (Piper, 1995; Mager and De Kruijff, 1995; Hohmann, 2002). The induction of ARE pathways is distinct, and mainly associated with oxidative and toxic metal ion stress (Ruis and Schüller, 1995; Toone and Jones, 1999), the former of which is commonly associated with fermentation-derived stress factors (Gibson et al., 2007).

The overall genetic response to stress in yeast also induce a series of physiological changes to the cell, which serve to allow the population to survive (Gasch et al., 2000; Dhar et al., 2013). For example, exposure to osmotic stress causes the yeast vacuole to fragment in order to maintain cytosolic turgor pressure (Section 4.3) (Zieger and Mayer, 2012; Michaillat and Mayer, 2013), which is directly impacted by water efflux from the cell (Martinez de Maranon et al., 1996; Gervais and Beney, 2001; Hohmann, 2002; Reiser et al., 2003). The process of vacuolar fragmentation has distinct protein requirements that incite

alterations to the yeast cell transcription profile in order to produce the necessary proteins to facilitate fission of the organelle (Section 1.5.3) (Martinez de Maranon et al., 1996; Gervais and Beney, 2001). Similarly, mitochondrial dynamics are high regulated in response to stress, with the equilibrium between fused and unfused mitochondria shifting as a result of the expression of key proteins (Section 1.5.3) (Pastor et al., 2009; Müller and Reichert, 2011; Youle and van der Bliek, 2012; Knorre et al., 2013a). Consequently, during active yeast growth and in response to changing environments such as those encountered during fermentation, yeast vacuoles and mitochondria undergo dynamic changes in their morphologies (Kitagaki et al., 2007; Pratt et al., 2007; Kitagaki, 2009). As discussed in Chapter 4, both the vacuole and the mitochondria adopt a characteristically 'stressed' morphology during the initial stages of fermentation, appearing as fragmented vacuoles and unfused, discrete, mitochondria respectively. However, the impact of these physiological changes on the cell are not fully understood. Although for vacuole structures it is likely to be related to homeostasis and the need for a dynamic response, for mitochondria this change in structure may be simply be due to organelle segregation or damage (Chapter 4). Irrespective, as fermentation progresses, the morphologies of these organelles revert to a form more closely associated with the 'unstressed' state, for example similar to that observed when cells are fermenting at a lower gravity or growing under favourable laboratory conditions (Chapter 4). This suggests that fermenting yeast cells are able to recover or adapt to the stress levels exerted throughout standard fermentations. This suggests a key role of these organelles in the stress

response and in cellular adaptation during fermentation. However, in Chapter 4, it was also demonstrated that under intensive fermentation conditions such as those associated with high and very high gravity brewing, yeast were unable to recover this 'normal' organelle physiology even though cells retained viability. Consequently, it is evident that the relationship between yeast stress, organelle physiology, gene expression and fermentation conditions have yet to be fully elucidated.

Stresses encountered during high gravity (HG) and very high gravity (VHG) fermentations inevitably exacerbate the extent of stress that yeast cells are subjected to (Hackstaff, 1978; Puligundla et al., 2011; Wang et al., 2013). Indeed, it has been shown that the effects of osmotic and ethanol stress are heightened during VHG fermentations and manifest themselves in the form of a reduction in fermentation performance, impaired yeast health and cell death resulting in lower viabilities (Section 4.2.2; Section 5.2.4) (Younis and Stewart, 1999; Pratt et al., 2003; Puligundla et al., 2011; Zhuang et al., 2017). In order to alleviate stress, an alternative approach to VHG brewing fermentations was evaluated, whereby a VHG (22 ^{o}P) fermentation was initiated using 17.5 ^{o}P wort which was subsequently 'topped up' by the addition of a high-maltose adjunct at intervals during fermentation (Chapter 5). This regime successfully reduced fermentation attenuation time and improved yeast viability when compared to a standard batch VHG fermentation, without any alteration in beer flavour profile (Section 5.2.6). The preservation of yeast quality is arguably one of the most important outcomes since the reuse of yeast cultures in serial repitching remains a common practice in breweries. This provides a sustainable and cost-

effective mechanism for providing biomass and yeast quality is paramount to ensuring successful fermentation performances. In order to explore the mechanism by which yeast stress was alleviated through applying a top-up regime, the yeast culture was further analysed with the aim of elucidating the physiological and transcriptional response of yeast to these fermentation conditions. This was achieved through the application of next-generation RNA sequencing throughout fermentation in order to obtain a transcript of expressed genes at key timepoints. Vacuole and mitochondrial morphology were monitored alongside the expression analysis, to act as both an indicator of cellular stress and to assist in elucidating the pathways and mechanisms behind the physiological changes observed to these structures in stressed yeast cells.

6.2 Results

In this study, the relationship between wort gravity, fermentation regime, organelle morphology and gene expression was analysed. This was performed to elucidate the regulation of cellular events governing the physiologies of vacuole and mitochondria observed under these conditions and was conducted through next-generation RNA transcript sequencing. Additionally, the physiological and genetic implications of the top-up optimisation regime previously investigated (Chapter 5) were also explored. This was achieved by taking yeast samples throughout 2 L fermentations conducted using high gravity (HG) 17.5 and 22 ^{o}P very high gravity (VHG) worts, and a top-up regime which employed a base 17.5 ^{o}P wort that was supplemented during

fermentation to provide a total sugar input of 22 ^{o}P (VHG top-up) as applied in Chapter 5. Samples were taken at specific timepoints selected to represent the same stage in the process for each wort type. The key stages of fermentation were considered to be the beginning, midpoint and endpoint of the fermentation, with an additional samples taken between each of these as outlined in Figure 6.1. Additionally, during the top-up fermentation regime, samples were taken both before and immediately after the addition of the sugar adjunct 88 hours after pitching in order to study the effects of this treatment on yeast physiology during fermentation. To assess vacuole and mitochondrial morphology, confocal microscopy was used in conjunction with FM 4-64™ (vacuole) and rhodamine 123 (mitochondria) staining as previously reported (Chapter 4; Section 2.5.3; Section 2.5.4). Simultaneously, gene expression was analysed using mRNA sequencing to identify genes that were either positively or negatively impacted by fermentation conditions at each time point (Section 2.6.2).

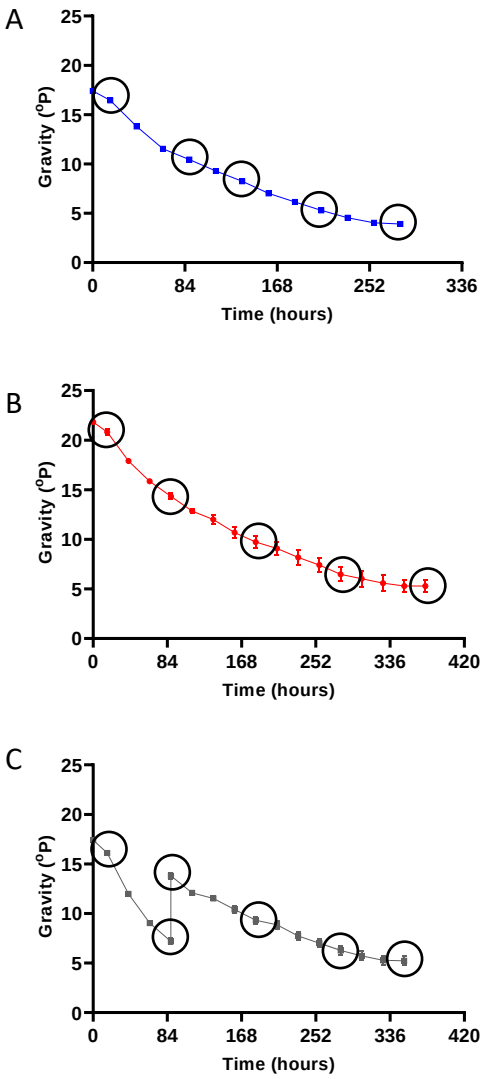


Figure 6.1. Sampling timepoints during fermentation of A – 17.5 °P HG wort, B – 22 °P VHG wort and C – a VHG with top-up regime, equal to the total gravity of a 22 °P wort.

6.2.1 The impact of wort gravity and fermentation regime on vacuole morphology

As discussed in Chapter 4, the physiological changes that occur in respect to yeast vacuolar morphology throughout fermentation are dictated by the level

of stress being exerted on the fermenting yeast population. Vacuole fragmentation was shown to occur upon pitching into both standard gravity wort and wort supplemented with an additional non-assimilable stressor (sorbitol). This indicated that the conditions in pitching wort were sufficient to evoke an osmotic stress response, even at what is considered to be 'standard' gravity (Chapter 4). Here the aim was to further investigate the additional stress exerted on a fermenting yeast population as induced by high and very high gravity worts, and the consequences of this additional stress on vacuole morphology throughout fermentation. In addition, the potential for the sugar top-up regime developed in Chapter 5 to alleviate stress was also examined.

It can be seen that upon pitching into a 17.5 ^{o}P wort, the yeast vacuoles appeared to be moderately fragmented (Figure 6.2A). Relatively small vacuoles were present which were more frequent in number when compared to the 'typical' vacuole morphology observed under favourable unstressed conditions (Chapter 4). This vacuolar state is likely to occur due to the osmotic stress exerted by the high sugar concentrations present in the pitching wort during the initial stages of fermentation, which occurs to preserve intracellular turgor pressure in response to heightened environmental osmolarity (Martinez de Maranon et al., 1996; Zieger and Mayer, 2012; Zhuang et al., 2017). Furthermore, fragmented vacuole morphology has been associated with exponentially growing yeast cells, potentially indicating that this form assists in the dynamic transfer of key enzymes responsible for growth and associated cellular activity (Vida and Emr, 1995). Therefore, the fragmentation of vacuoles that is observed at this stage under these conditions cannot be attributed solely

to stress and is likely a combination of this and the exponential growth of yeast during the initial stages following pitching.

Subsequent sample analysis indicated that yeast vacuoles continued to appear fragmented between 16 and 208 hours (Figure 6.2A; 6.2E). However, the fragmented vacuoles observed at 88 hours after pitching were significantly smaller and occupied a smaller proportion of the yeast cell than those visualised after 16 hours. Furthermore, the presence of these smaller vacuoles were also present in samples taken at 136 hours (Figure 6.2C) and 208 hours (Figure 6.2D). It is suggested that this could potentially be a result of 'dehydration' of yeast cells occurring due to the osmolarity of the fermentation medium (Zhuang et al., 2017). Furthermore, this could potentially occur due to the biogenesis of small, vesicle-like autophagosome structures important for homeostasis and repair (Journo et al., 2008; Li and Kane, 2009; Fukuda and Kanki, 2018). Upon completion of the fermentation at 280 hours, the vacuole morphology observed was no longer overtly fragmented (Figure 6.2E), supporting previous observations that cells were able to revert to a 'normal' morphology (Chapter 4), although it should be noted that vacuoles remained relatively small in proportion to the whole yeast cell when compared to samples taken between 88 and 208 hours.

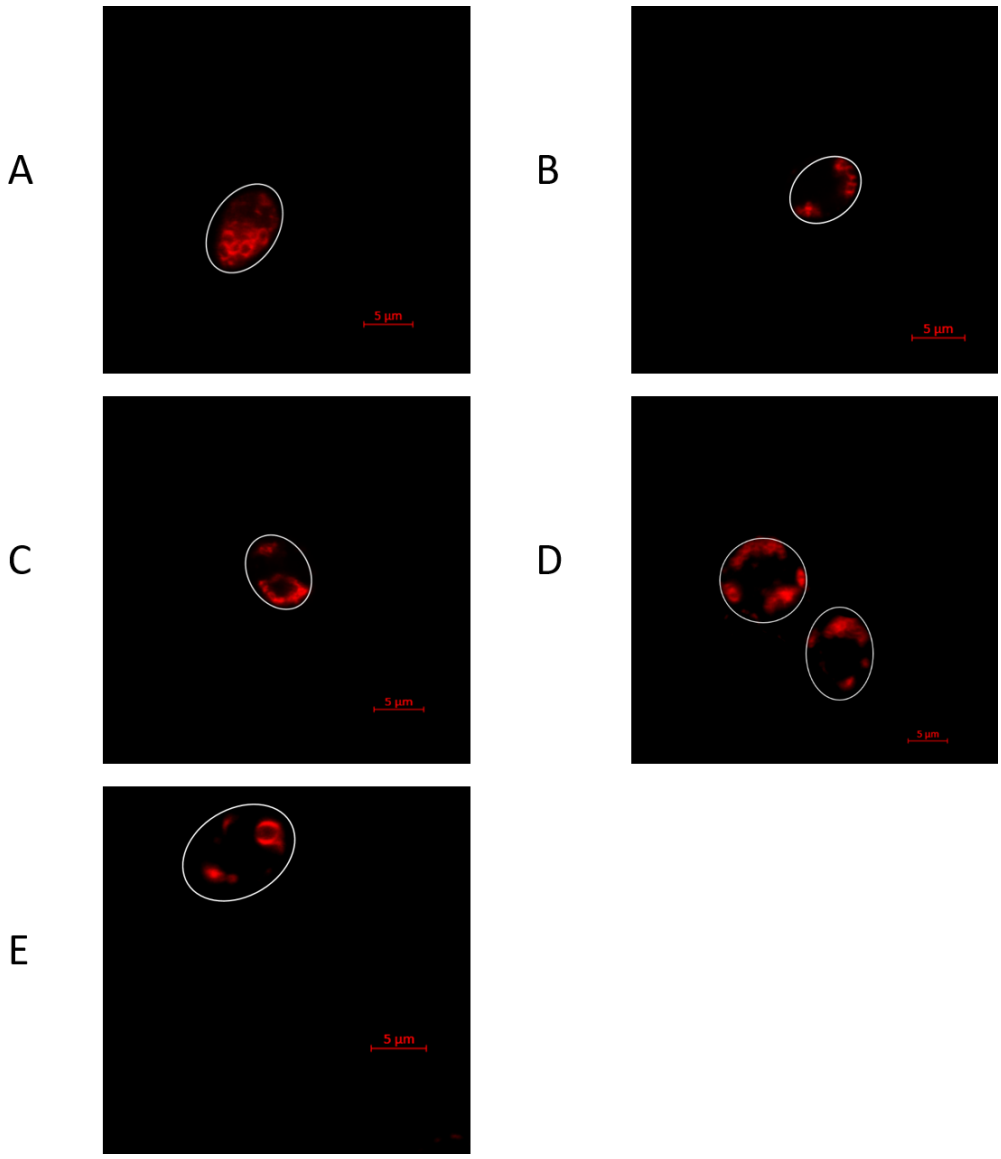


Figure 6.2. Yeast vacuole morphology after 16 (A), 88 (B), 136 (C), 208 (D) and 280 (E) hours of fermentation in 17.5 ^{o}P high gravity wort. Yeast cells were stained with FM 4-64™ and visualised by confocal microscopy.

In order to determine the impact of increasing gravity on vacuole morphology, samples were also taken from 22 ^{o}P very high gravity fermentations as described previously (Section 2.4.3; Section 6.1.1). It can be seen that vacuole fragmentation was rapidly observed as before. After 16 hours after pitching

into a 22 ^{o}P wort, vacuoles were small and frequent in number as well as being widely dispersed throughout the cell (Figure 6.3A). The morphology of vacuoles under VHG conditions at this stage in fermentation was indistinguishable from that observed at the same timepoint under HG conditions (Figure 6.2A), likely reflective of the similar stage of growth in each instance. However, it is interesting to note that despite the increase in osmotic stress associated with the use of higher gravity worts, distinct physiological differences were not visible between the VHG and HG conditions during the initial stages of fermentation.

After 88 hours of fermentation, yeast cells under VHG conditions exhibited a similar vacuole morphology to that observed after 16 hours. At this stage, vacuoles appeared extremely small and high in frequency (Figure 6.3B), indicating that fragmentation of vacuoles had occurred. However, the extremely small and numerous vacuoles observed indicate that this may have been occurring in a different fashion to that seen previously (Section 4.2.5) and to cells taken from the HG fermentation at the same timepoint (Figure 6.2). Interestingly, cells taken at 184 hours into the 22 ^{o}P fermentation showed a similar vacuolar physiology to those at 88 hours in the 17.5 ^{o}P fermentation. At these stages there was no statistical difference ($p \geq 0.1$) between the gravities of each condition; the gravity after 88 hours in the 17.5 ^{o}P wort was 10.45 ^{o}P ± 0.1 and compared to 9.72 ^{o}P ± 0. 5 after 184 hours in the 22 ^{o}P fermentation. Furthermore, viabilities of each condition at each timepoint showed no significant difference when analysed ($p \geq 0.1$); the viability after 88 hours in the 17.5 ^{o}P fermentation was 90.37 % ± 0.9 compared to 91.63% ± 0.6 in the 22 ^{o}P

fermentation. Consequently, it can be seen that morphology was related to stress and gravity rather than fermentation progression *per se*.

Yeast samples taken at 280 hours (Figure 6.3D) and 376 hours (Figure 6.3E) displayed extremely small vacuole structures, similar to that observed at 184 hours (Figure 6.3C). By the endpoint of fermentation, vacuolar structures occupied an extremely small proportion of the yeast cell, and there was no occurrence of characteristically 'healthy' or 'unstressed' vacuole morphologies. It can therefore be concluded that although broadly similar vacuolar structures were observed when comparing 17.5 and 22 ^{o}P fermentations, for the latter the key difference was morphology at the end point, supporting the data reported previously (Chapter 4) indicating that increased stress caused an irreversible change in structure.

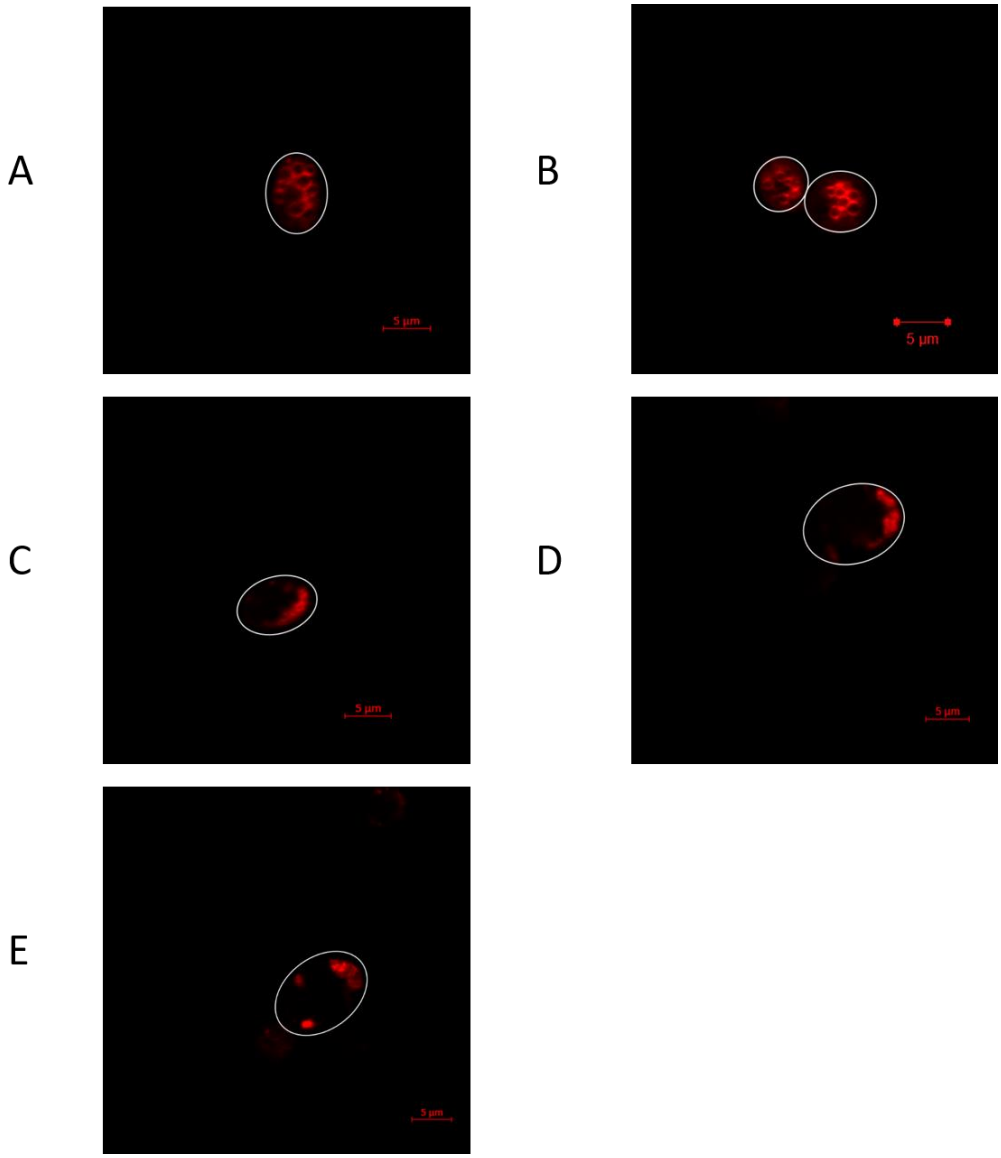


Figure 6.3. Yeast vacuole morphology after 16 (A), 88 (B), 184 (C), 280 (D) and 376 (E) hours of fermentation in 22 ^{o}P very high gravity wort. Yeast cells were stained with FM 4-64™ and visualised by confocal microscopy.

In order to assess the impact of a sugar top-up regime on vacuole morphology, samples were taken and analysed in the same way as described above. As with the other conditions analysed in this study, vacuole fragmentation occurred as yeast were pitched into the 17.5 ^{o}P base wort (Figure 6.4A). This was followed

by the appearance of extremely small vacuolar structures at 88 hours (Figure 6.4B), similar to the vacuolar morphology observed at the same timepoint during the standard 17.5 ^{o}P fermentation. Immediately after this timepoint, the sugar adjunct was added to the fermentation medium and a further sample was taken with identical results (Figure 6.4C). However, samples taken following 184 hours, at the approximate midpoint of the top-up regime fermentation, indicated that vacuoles had reverted to a highly fragmented state while occupying a greater proportion of the cell (Figure 6.3D). This morphology was similar to that observed in yeast cells taken from the first 16 hours of fermentation (Figures 6.4A), suggesting that the cells were in a similar physiological state. This was unexpected since the sugar added was maltose (rather than glucose) based and free of oxygen, both of which may have provided a rationale for the change in structure.

Samples taken from 280 and 352 hours showed cells that appeared to have reverted to the extremely small structures are visible in the latter stages of both the 22 ^{o}P and 17.5 ^{o}P fermentation (Figure 6.2; Figure 6.3). The occurrence of extremely small vacuolar structures was prevalent in both the 22 ^{o}P and the top-up regime fermentation, both of which contained the same total concentration of extract and a similar gravity at these timepoints. No apparent differences between the 22 ^{o}P and top-up regime were observed in terms of vacuole physiology at the fermentation endpoint. However, the extent of vacuole fragmentation observed here was greater than that observed in previous experiments (Chapter 4). This could potentially be due to the elevated gravity of the base wort, or the inclusion of adjunct potentially reducing the

nutritional content of the wort, causing starving and evoking a physiological response.

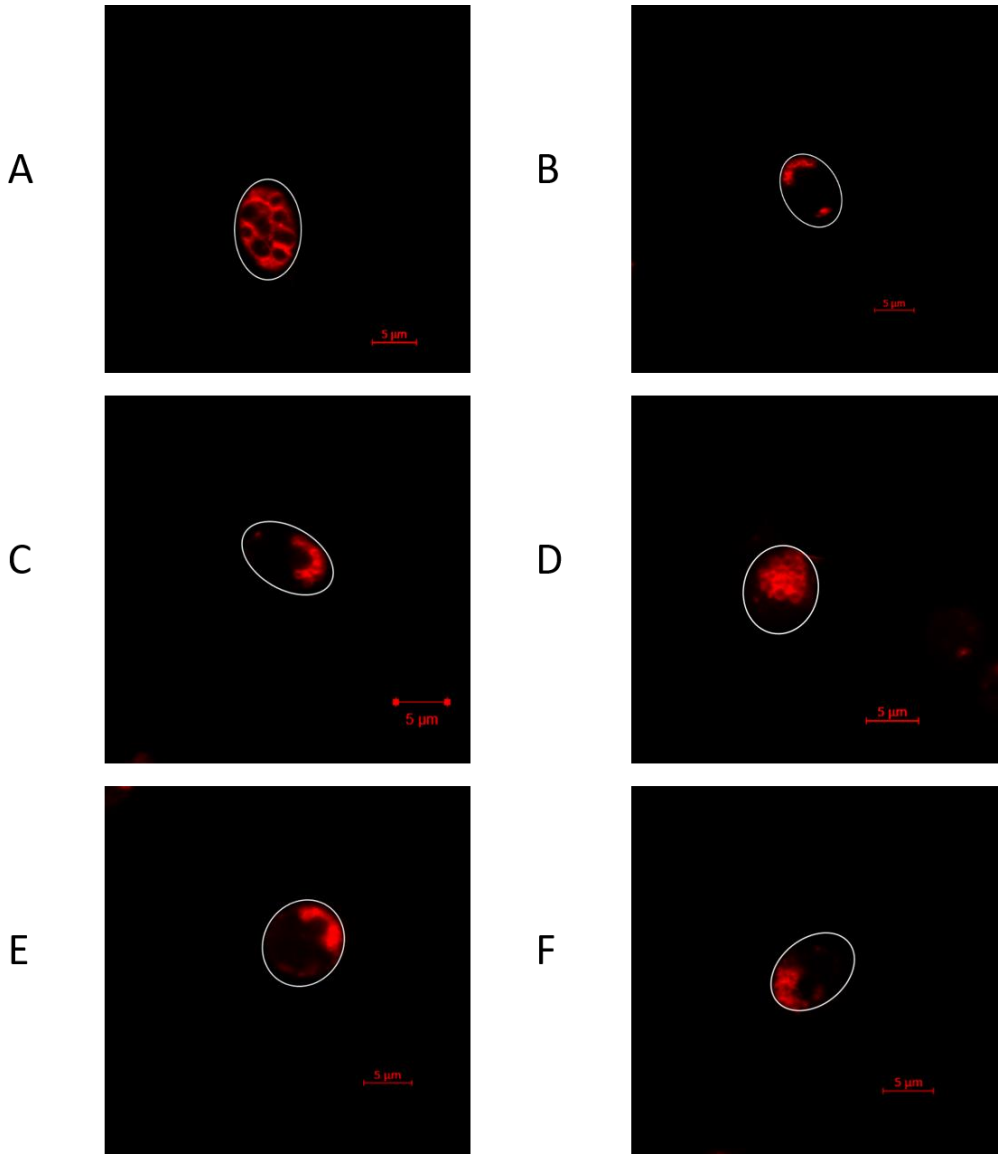


Figure 6.4. Yeast vacuole morphology after 16 (A), 88 (pre top-up) (B), 88 (post top-up) (C), 184 (D), 280 (E) and 352 (E) hours of fermentation in 22 ^{o}P high gravity wort provided using a top-up regime. Yeast cells were stained with FM 4-64™ and visualised by confocal microscopy.

6.2.2 The impact of wort gravity and fermentation regime on mitochondrial physiology

Mitochondrial dynamics, like that of the yeast vacuole, are highly influenced by environmental parameters and extracellular stress (Chapter 4). This is likely a result of the role of yeast mitochondria in fermentative metabolism, which extends beyond that of an energetic capacity. Although the precise function of mitochondria in fermenting brewing yeast have yet to be fully elucidated, it is likely that they are involved in the cellular stress response, sterol biosynthesis and flavour metabolite biosynthesis (Section 4.3). As discussed in Chapter 4, heightened stress can cause the mitochondria to undergo fission and fragmentation events, a morphological phenomenon that has also been linked to ethanol stress (Kitagaki et al., 2007), as well as apoptosis and facilitation of mitophagy (Fannjiang et al., 2004; Gomes and Scorrano, 2013). In order to investigate the impact of fermentation regimes on mitochondrial physiology, yeast samples taken from high and very high gravity worts were analysed. In addition, the potential for the sugar top-up regime developed in Chapter 5 to alleviate stress was also examined by a similar analysis of mitochondrial structure using the fluorescent stain Rhodamine 123.

Once yeast had been pitched into a 17.5 ^{o}P HG wort, mitochondria rapidly developed small, 'discrete' structures, distributed across the cell (Figure 6.5). This mitochondrial morphology is synonymous with the effects of stress (Chapter 4), which are in turn associated with the initial stages of fermentation. This morphology was observed until the fermentation midpoint (136 hours)

whereby mitochondrial fusion became apparent; mitochondria appeared as elongated structures. As discussed previously, mitochondrial fusion has been suggested to be the functional form of this organelle and is linked to the unstressed state (Chapter 4). This suggests that yeast cells between 88 hours and 136 hours, yeast were able to adapt more effectively to the external environment, even though yeast vacuoles at this stage remained small and highly fragmented, suggesting the presence of some degree of extracellular stress. This morphology continued to be observed until 280 hours whereby the fermentation had attenuated. Figures 6.5C and 6.5D show an extensive network of elongated mitochondria, with figure 6.5E displaying a single elongated mitochondria with one extremely small mitochondrion. As mitochondrial fission is associated with extracellular stress and ethanol toxicity, the morphologies visible in Figures 6.5C – 6.5E suggest that the level of extracellular stress exerted on the fermenting yeast population in the latter stages of the process were not sufficient to invoke mitochondrial fission under these conditions. As suggested in Chapter 4, mitochondrial fission could be a result of mitochondrial damage or a reduction in overall mitochondrial mass, possibly caused by the extended anaerobic conditions.

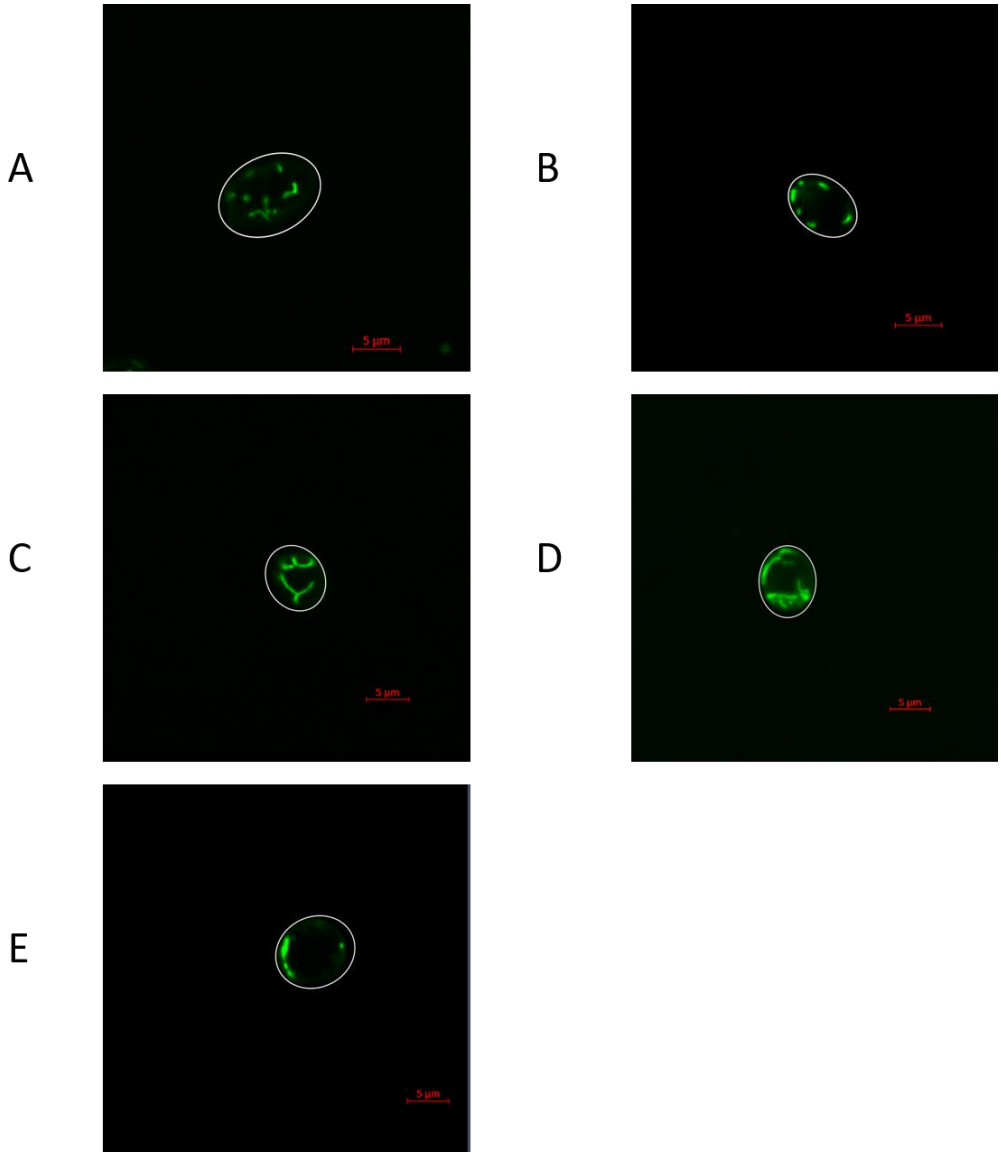


Figure 6.5. Yeast mitochondria morphology after 16 (A), 88 (B), 136 (C), 208 (D) and 280 (E) hours of fermentation in 17.5 ^{o}P high gravity wort. Yeast cells were stained with Rhodamine 123 and visualised by confocal microscopy.

As yeast were pitched into 22 ^{o}P wort, mitochondrial fragmentation was visible after 16 hours (Figure 6.6A). This morphology was also present after 88 hours (Figure 6.6B), whereby small mitochondria were present at a relatively high numeracy, suggesting that the yeast cells were stressed at both timepoints. The

occurrence of stressed cells is likely attributed to the higher concentration of sugars present in the pitching wort. Under these conditions, mitochondrial morphologies resemble those in the 17.5 ^{o}P fermentation, despite the significantly higher gravity.

Samples taken at 88 hours (Figure 6.6B), 184 hours (Figure 6.6C) and 280 hours (Figure 6.6D) and 376 hours (Figure 6.6E) all showed mitochondria present in the unfused state, occurring as smaller, more frequent structures throughout the fermenting yeast cells. In contrast to the 17.5 ^{o}P conditions, mitochondrial morphology at VHG continues to reflect that associated with stressed cells throughout the remainder of the fermentation process. This suggests that the levels of stress exerted on the fermenting yeast population at 22 ^{o}P were sufficiently high to prevent the yeast from adapting efficiently to the environment. The likely cause of this inability to adapt is the combined effect of the osmotic stress induced by the heightened concentration of sugars and the high concentration of ethanol in the fermentation medium toward the latter stages of the process and at the fermentation endpoint (Chapter 4).

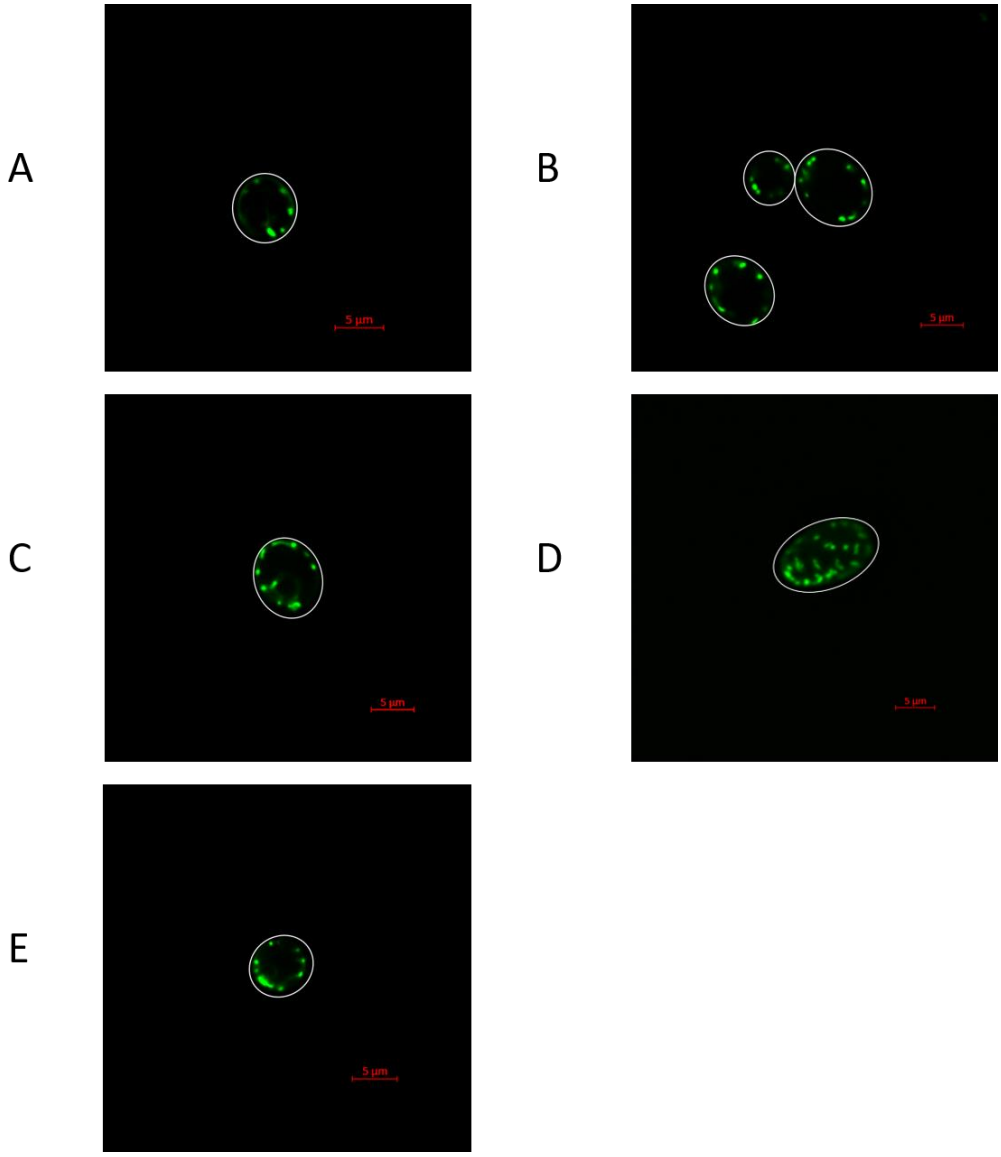


Figure 6.6. Yeast mitochondria morphology after 16 (A), 88 (B), 184 (C), 280 (D) and 376 (E) hours of fermentation in 22 °P very high gravity wort. Yeast cells were stained with Rhodamine 123 and visualised by confocal microscopy.

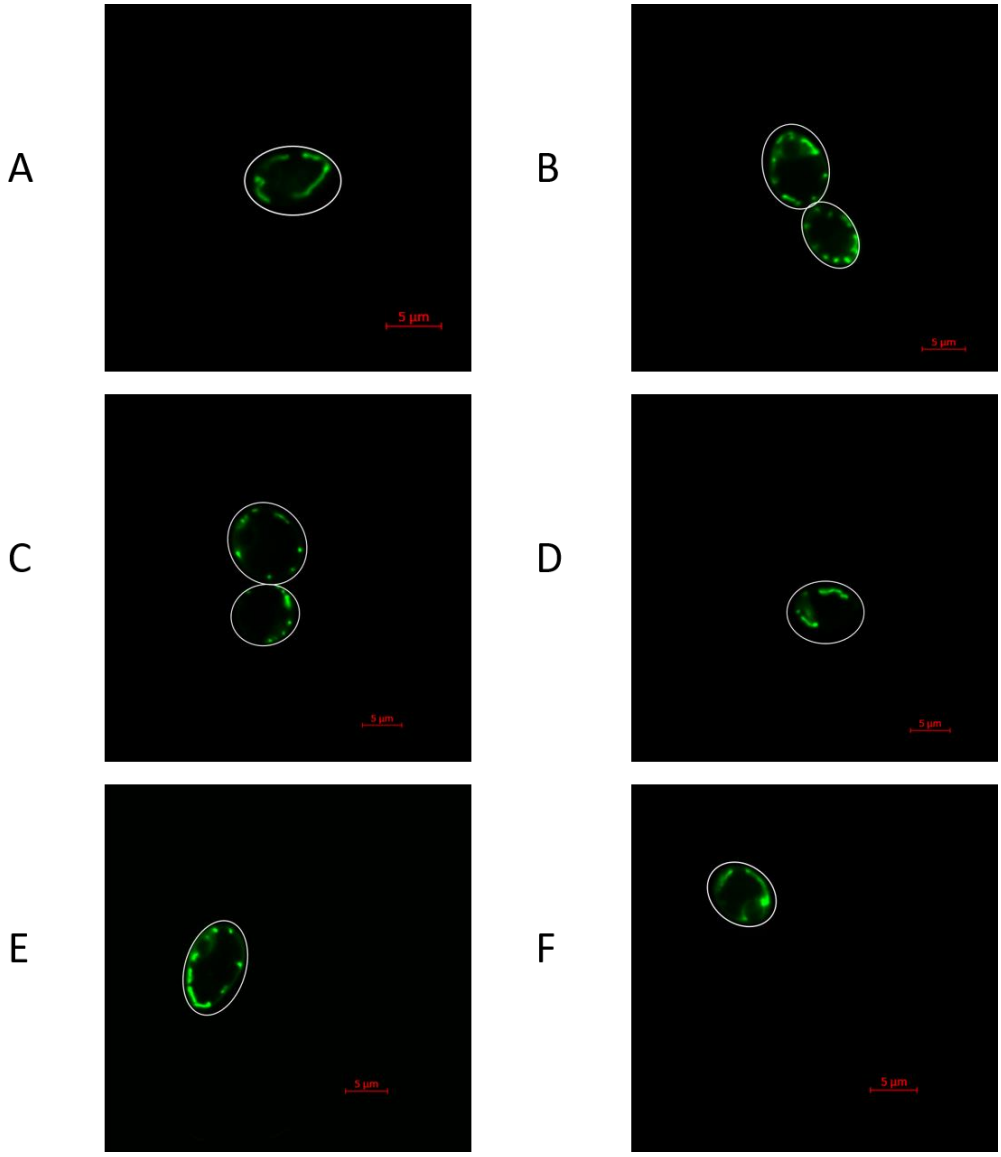


Figure 6.7. Yeast mitochondria morphology after 16 (A), 88 (pre top-up) (B), 88 (post top-up) (C), 184 (D), 280 (E) and 352 (E) hours of fermentation in 22 ^{o}P high gravity wort provided using a top-up regime. Yeast cells were stained with Rhodamine 123 and visualised by confocal microscopy.

Interestingly, in the top-up fermentation regime, mitochondria morphology after 16 hours appeared in the fused form (Figure 6.7A). This was surprising since this had not been observed previously at this stage of fermentation. This

may suggest that yeast cells were less stressed than in the other conditions, which may also be reflective of the higher pitching rate employed for the top-up regime, whereby the total number of cells inoculated into the wort was equal to that of the 22 ^{o}P fermentation. This combination resulted in a more rapid initiation of fermentation than with the other fermentations, which may have manifested itself in terms of mitochondrial morphology.

As fermentation progressed a higher degree of mitochondrial fission was observed than at equivalent time points in the other fermentations. This could potentially be a reflection of the production of ethanol, as indicated by the high rate of wort extract reduction, especially since mitochondrial fragmentation is linked to ethanol toxicity (Kitagaki et al., 2007). Immediately after the sugar supplementation at 88 hours, mitochondrial morphology continued to adopt an unfused morphology, being present as smaller structures (Figure 6.7C). However, samples taken at the approximate midpoint of fermentation (184 hours) exhibited a more fused mitochondrial structure (Figure 6.7D), although smaller mitochondria were also present in some cells. This suggested that this sample reflects a timepoint during which the yeast were partially stressed. At the fermentation endpoint in the top-up regime, yeast mitochondria appeared as elongated structures, indicating a non-stressed physiological state. Consequently, it can be seen that at the end of both the 17.5 ^{o}P and the top-up regime fermentations, mitochondria were in the normal fused form, while in the 22 ^{o}P mitochondria they exhibited a discrete unfused structure indicative of stress. This suggests that the degree of stress being exerted on the yeast

during a ‘standard batch’ VHG fermentation was alleviated using the top-up regime, at least in terms of mitochondrial physiology.

6.2.3 Expression of vacuole and mitochondria physiology-regulating genes during fermentation

The morphology of yeast vacuoles and mitochondria are regulated by an extensive network of genetic responses (Zieger and Mayer, 2012; Youle and van der Bliek, 2012). In order to elucidate and validate the morphological changes in organelles that were observed throughout fermentation under different conditions, each timepoint was subjected to mRNA sequencing in order to determine the gene expression profile of physiology-regulating genes. A selection of genes actively involved in the regulation of vacuole fragmentation and functionality, mitochondrial fusion/fission and mitochondrial dynamics were selected for targeted gene expression analysis (Table 6.1) based on previous literature and their assigned function as stated on the Saccharomyces Genome Database (2021). Sequence depth was used as a determinant of gene expression as outlined in Section 2.6.2.

Table 6.1. Genes influencing vacuole and mitochondrial morphology and functionality selected for targeted gene expression analysis.

Gene	Function
Vacuolar genes	
VAC14	Vacuole morphology and protein trafficking
FAB1	Vacuole membrane kinase involved in vacuolar sorting
VPS1	GTPase involved in vacuolar sorting
VPS55	Endosome to vacuole protein transport
BRO1	Coordination of vacuolar protein sorting
YCK3	Negative regulation of vacuole fission during hypertonic stress
Mitochondrial genes	
FIS1	Mediates mitochondrial fragmentation during ethanol stress
FZO1	Regulator of mitochondrial fusion
YSP1	Mitochondrial protein involved in programmed cell death
DNM1	GTPase that regulates mitochondrial fission and mitophagy
MDM34	Regulates mitochondrial distribution and morphology, required for mitophagy
MFB1	Maintenance of mitochondrial morphology

Vacuole morphology-influencing genes (Table 6.1) were selected in order to elucidate the mechanisms behind the vacuole fragmentation observed in Section 6.2.1 as well as in Chapter 4, and to explore any trends in their expression that could relate to the physiologies previously observed. As seen in Section 6.2.1, vacuole fragmentation events occurred in fermenting yeast cells under all conditions used in this study. Furthermore, yeast vacuoles appeared to shrink and disperse to create smaller structures in the latter stages of fermentation under all VHG conditions; a morphology that was not seen in previous experiments. The process by which a vacuole fragments depends on the activity of the Fab1 complex, which requires Vac14p multimer in order to facilitate the programmed fission of vacuoles in response to hyperosmotic shock (Zieger and Mayer, 2012; Alghamdi et al., 2013). Throughout fermentation, VAC14 expression levels increased steadily from pitching through to the fermentation midpoint for all conditions. There was no significant difference in VAC14 expression at 88 hours in the top-up regime following the sugar addition at this point. In the 17.5 ^{o}P, VAC14 expression ceased to increase after 208 hours, with both the 22 ^{o}P and top-up regime fermentations showing a similar trend with no increase in VAC14 expression observed following the fermentation midpoint for these conditions. The expression levels of FAB1 increased from 16 hours to 136 hours in the 17.5 ^{o}P fermentation, after which they remained constant until the fermentation endpoint. In the 22 ^{o}P fermentation and the top-up regime, the expression profile of the FAB1 gene was similar for both conditions, whereby an initial increase was followed by a decrease in the latter stages of fermentation. This

increase in VAC14 expression appears to coincide with the appearance of extremely small vacuolar structures throughout fermentation under the conditions used in this study.

Yeast vacuole morphology is also highly regulated by vacuole protein sorting (VPS) genes (Raymond et al., 1992; Bonangelino et al., 2002). Both VPS1 and VPS55 have been shown to affect vacuolar fragmentation (Bonangelino et al., 2002; Michaillat et al., 2012) and endosomal golgi-vacuole trafficking. Expression of VPS1 remained constant between 16 and 136 hours in the 17.5 ^{o}P fermentation, which was followed by a decline in expression as fermentation progressed beyond that point. Similarly, the 22 ^{o}P fermentation exhibited a decline in VPS1 expression between 88 hours and 376 hours. The top-up regime exhibited a sharp decrease in VPS1 expression between 16 hours and 88 hours, following an initially high level of expression. After the sugar addition, VPS1 expression increased and remained relatively high in comparison to the other conditions until 184 hours, after which expression decreased to the same level as observed at the fermentation endpoint of the 22 ^{o}P fermentation. As with VPS1, the 17.5 ^{o}P and 22 ^{o}P fermentations exhibited similar expression profiles for VPS55, indicating that the difference in gravity between these conditions had no effect on VPS55 expression. Furthermore, no difference in VPS55 expression was observed in any of the conditions applied after 16 hours. However, at 88 hours the top-up regime promoted higher levels of expression than the 17.5 ^{o}P and the 22 ^{o}P fermentations. The expression of VPS55 subsequently decreased upon addition of the sugar adjunct after 88 hours. There was no difference in the expression levels of VPS55 at the fermentation

endpoint irrespective of wort gravity or fermentation conditions. The Bro1p has previously been identified as a protein involved in vacuolar morphology dynamics, with null mutants exhibiting strong defects in vacuole fragmentation (Michaillat and Mayer, 2013).Furthermore, part of the Bro1p complex actively binds to RNS4 (VPS32) components in order to mediate the multivesicular pathway mediating secretory protein to vacuole transport (Unno et al., 2005; Galindo et al., 2007), hence why this gene was selected for analysis. However, there were no obvious trends observed in BRO1 expression that correlates with the vacuole morphologies observed in Section 6.2.1 between conditions.

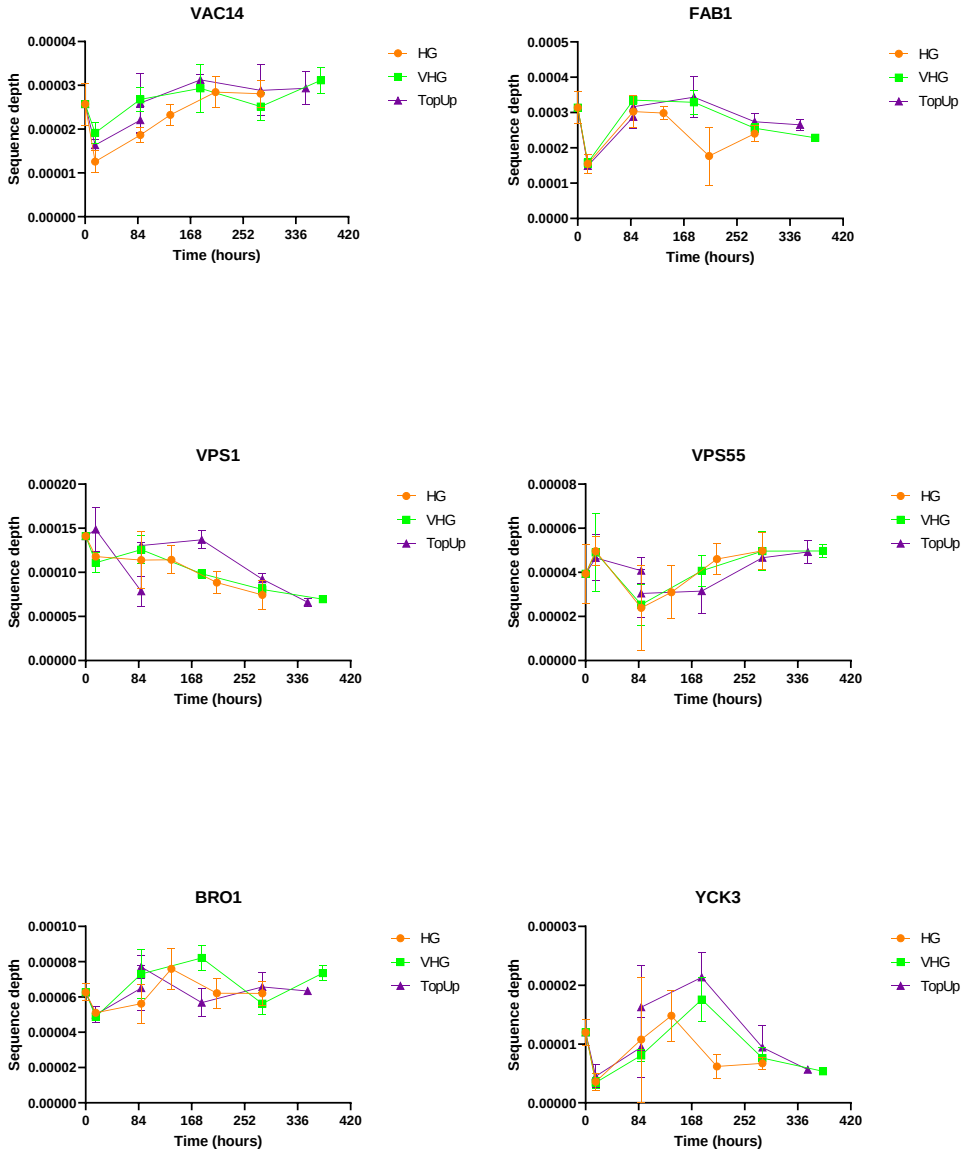


Figure 6.8. The impact of fermentation conditions on the expression profile of vacuole physiology-influencing genes. Gene expression was analysed during 17.5 ºP (HG), 22 ºP (VHG) and top-up regime (TopUp) fermentations.

Mitochondrial morphology-influencing genes were selected in order to elucidate the mechanisms behind the fusion and fission events observed in Section 6.2.2 as well as in Chapter 4, and to explore any trends in their

expression that could relate to the physiologies previously observed. As seen in Section 6.2.2, mitochondria fusion events occurred in actively fermenting yeast cells. However, in contrast, the discrete form was associated with stressed cells. It should be noted that mitochondrial morphology was able to revert to the fused (unstressed) morphology in the absence of stress in relatively low gravity fermentations, but when stress was highly elevated then these fusion events were not observed, and mitochondria remained in the discrete form. The primary genes implicated in fusion and fission events are described in Table 6.2.

Interestingly, the expression of FIS1, implicated in mitochondria fragmentation in response to stress, increased steadily throughout fermentation in all conditions (Figure 6.9), contrary to the results observed (Section 6.2.2; Chapter 4). The reason for this is unknown, however it was notable that for all conditions, expression of FZO1 (a regulator of mitochondrial fusion), although low at the 16 hour timepoint, also increased until the mid-point of fermentation after which it declined (Figure 6.9). Hence it is difficult to draw strong conclusions from this analysis in relation to the observed changes during fermentation. However, this does lend further support to the suggestion that mitochondria are highly dynamic during fermentation and that their role is likely to be more complex and perhaps more important than previously believed. As mitochondrial fusion and fission events are constantly occurring, with the role of mitochondria involved in an array of cellular functions (Section 4.3), there is a potential that monitoring the expression of the two genes solely

does not provide sufficient insights into the physiological dynamics of mitochondria.

The expression profiles of both YSP1 (programmed cell death) and DNM1 (mitophagy) exhibited a similar trend, whereby expression levels steadily increased as fermentation progressed. In the 17.5 °P conditions, MDM34 (mitochondrial distribution) expression fluctuated to a higher degree than the two VHG conditions, which remained constant throughout. It is currently unknown as to why this gene expression profile was adopted. Finally, the expression of MFB1 (maintenance of mitochondrial morphology) was higher for the top-up regime following the addition of the sugar adjunct, and remained so until 280 hours, at which point the expression in both the 17.5 ^{o}P and 22 ^{o}P fermentations had increased to a similar level to the top-up regime, suggesting a potential role of Mfb1p in maintenance of fused mitochondrial structures.

Ultimately, the expression profile of mitochondrial physiology-regulating genes did not overtly conform to the physiologies previously identified at the corresponding timepoints in Section 6.2.2. The expression of mitochondrial fission-promoting genes such as FIS1 and DNM1 were observed to increase in all conditions as each fermentation progressed, however this did not yield yeast cells with predominantly unfused mitochondria. This is possibly due to gene interaction and the involvement of other genes, as well as the complex equilibrium between mitochondria in the fused and unfused state. It is also possible that other mitochondrial physiology-regulating genes may potentially have had an influence, although these were not highlighted in the current study

through the differential expression analysis discussed below (Section 6.2.5). Furthermore, it may be that expression of genes such as DNM1, associated with mitophagy, are more indicative of what is happening inside the cell, potentially the priority here is to remove damaged or spent mitochondria (Jensen et al., 2000; Fannjiang et al., 2004).

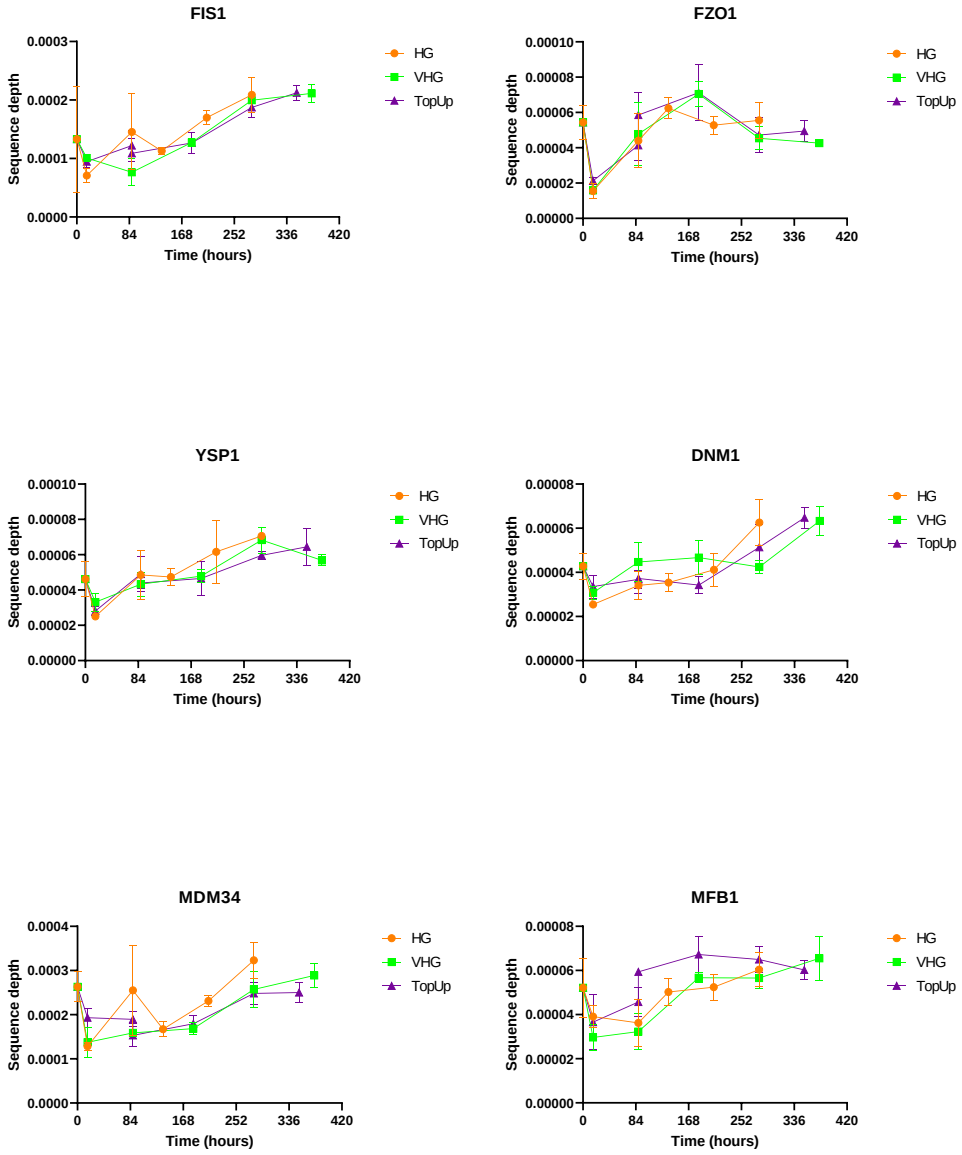


Figure 6.9. The impact of fermentation conditions on the expression profile of mitochondria physiology-influencing genes. Gene expression was analysed throughout a 17.5 ºP (HG), 22 ºP (VHG) and top-up regime (TopUp) fermentation.

6.2.4 Expression profiles of stress and autophagy-regulating genes during fermentation

In order to determine if the implementation of a top-up regime was able to alleviate stress on fermenting yeast when compared to a typical 22 ^{o}P batch-style fermentation, the expression profile of genes related to the yeast stress response was analysed. Furthermore, an assessment of the extent of cellular damage was conducted based on the expression of autophagy-related genes, due to their link between cellular damage and programmed degradation of subcellular components (Zhang, 2013). These gene groups were selected since the mechanisms driving the vacuolar and mitochondrial physiologies observed in this study could not be fully explained solely through imaging techniques, or the gene expression analysis conducted above (Section 6.2.3). Consequently, the potential for autophagy and mitophagy acting as key determinants for the observed morphologies during fermentation were investigated. A summary of the genes analysed can be found in Table 6.2.

Table 6.2. Selected cellular stress-response genes for gene expression analysis.

Gene	Function
Stress response genes	
HOG1	Osmoregulation and response to osmotic shock
HSP30	Stress-response protein involved in heat shock and ethanol toxicity
GPD1	Regulated by HOG pathway in response to osmotic stress
HOT1	Activator of Hog1p in response to osmotic stress
TPS1	Stress-induced trehalose synthase
Autophagy-regulating genes	
ATG8	Mediates vacuolar degradation and autophagosome biogenesis
ATG12	Autophagy induction involved in Cvt pathway
ATG23	Macroautophagy
ATG32	Initiation of mitophagy
PTC6	Mitochondrial protein phosphatase involved in mitophagy

The HOG pathway is heavily associated with the yeast response to osmotic stress and yeast survival under fermentation-derived osmotic extremes (Ruis and Schüller, 1995; De Nadal et al., 2004; Saito and Tatebayashi, 2004;

Gonzalez et al., 2016). Interestingly, expression levels of the HOG1 gene were not overtly influenced by the fermentation conditions applied (Figure 6.10). Furthermore, expression levels of the HOG1 gene remained relatively consistent throughout fermentation for all conditions. A potential reason for the consistent expression of the HOG1 gene observed in Figure 6.10 is the involvement of other gene activators/regulators in the mediation of the HOG pathway. As a key mediator of the HOG pathway, GPD1 was used as an indicator of osmolarity as an activator of HOG MAPK elements. In both 22 ^{o}P conditions, GPD1 expression increased steadily throughout fermentation, indicating an increase in the osmotic stress response as fermentation progresses. Expression of GPD1 fluctuated more in the 17.5 ^{o}P fermentation, increasing rapidly between 16 and 88 hours, indicating a more abrupt shift in extracellular osmolarity between these timepoints. No differences were found in GPD1 expression amongst all conditions used in this study at the fermentation endpoint. Similarly, the HOT1 gene (involved in the regulation of the HOG pathway in response to osmotic shock) expression of followed a similar trend to the expression of GPD1, in that it increased as fermentation progresses, with no significant differences observed between all conditions at their respective endpoints. This data does suggest that the accepted belief that osmotic stress reduces during fermentation due to sugar uptake should be challenged, in line with the observations of Zhuang et al., 2017, who indicated that osmotic stress increases during fermentation due primarily to the production of ethanol.

In support of this, expression of HSP30 (referred to as a 'heat shock' element, but also induced by high ethanol concentrations) increased throughout fermentation in both the 22 ^{o}P fermentation and the top-up regime, both of which resulted in ethanol yields above 10% v/v. As seen in Figure 6.10., HSP30 expression levels increased in these two conditions after 88 hours, rising steadily as fermentation progressed and resulting in similar expression levels upon completion of fermentation. In the 17.5 ^{o}P fermentation, the expression of HSP30 remained at a base level and exhibited no up-regulation under these conditions. This suggests that the ethanol generated during 17.5 ^{o}P fermentation was not intense enough to illicit an HSP genetic response, whereas the higher ethanol concentrations produced by the two VHG conditions were sufficiently stressful in terms of ethanol toxicity. It is possible that analysis of HSP30 gene expression could prove to be an effective strategy for determining the limits in terms of wort gravity and yeast fermentation performance, or for determining the suitability of yeast strains for VHG brewing.

Although there are many strategies to protect cells against stress, one of the most well recognised molecules produced by yeast cells is trehalose. This carbohydrate acts to protect membrane structures (Crowe et al., 1984; Hallsworth, 1998; Hounsa et al., 1998) and is commonly associated with the effects of fermentation-derived stress factors (Section 1.5.3) (Lucero et al., 2000; Alexandre et al., 2001; Walther et al., 2013; Saini et al., 2018). The production of trehalose in response to stress is primarily regulated by the trehalose phosphate synthase (TPS1) gene. Analysis of expression of the TPS1

gene indicated that it fluctuated throughout fermentation under all experimental conditions in a similar fashion to HOG1. The expression of TPS1 was notably higher at 16 hours and at the fermentation endpoint during 22 ^{o}P fermentations, potentially indicating higher levels of stress at these time points, especially when compared to the 17.5 ^{o}P fermentation and the top-up regime. However, in general there were few trends that could be drawn from the data, indicating that all of the conditions tested elicited a similar response.

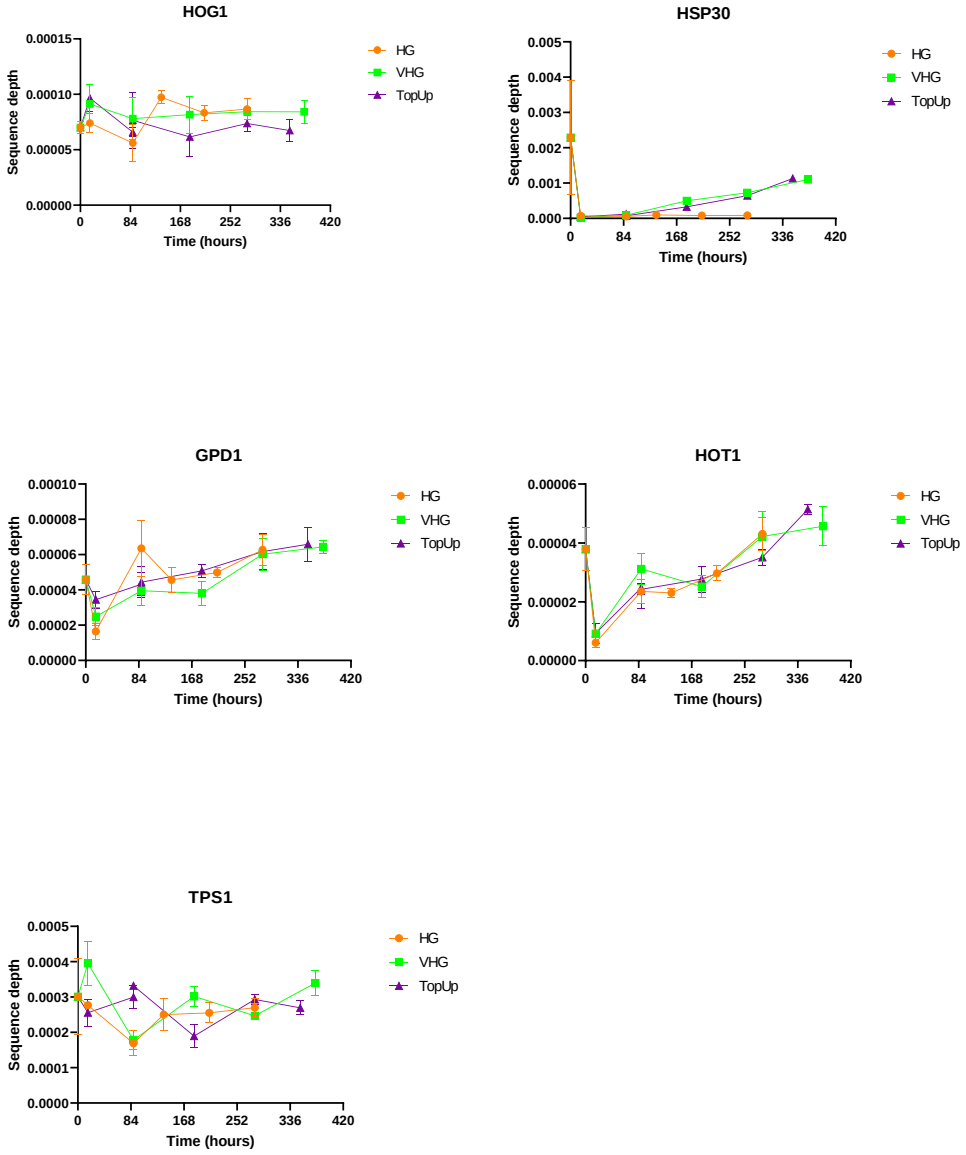


Figure 6.10. The impact of fermentation conditions on yeast stress response-mediated gene expression. Gene expression was analysed throughout a 17.5 °P (HG), 22 °P (VHG) and top-up regime (TopUp) fermentation.

As cellular components become damaged, they are removed by autophagy, the mechanism by which cells break down cellular components (Huang and Klionsky, 2002; Farré et al., 2009). This includes the breakdown of damaged

organelles, including mitochondria (mitophagy), which can occur as a result of extracellular stress (Mendl et al., 2011; Müller and Reichert, 2011; Shutt and McBride, 2013). In yeast, autophagy is mediated by the cytosol to vacuole-targeting (Cvt) pathway (Bryant and Stevens, 1998; Thumm, 2000; Lynch-Day and Klionsky, 2010; Fukuda and Kanki, 2018), which coordinates the breakdown of cellular components or organelles that require removal from the cell by the yeast lysosomic vacuole (Mechler et al., 1988; Li and Kane, 2009; Zhang, 2013). A summary of the genes analysed for expression levels can be found in Table 6.2. As shown in Figure 6.11, expression of ATG8 was observed to be highest at the midpoint of fermentation under all conditions, with expression relatively low at 16 hours followed by an up-regulation of the gene. This suggests that relatively low levels of autophagy occurred during the initial stages of fermentation irrespective of conditions. This was perhaps unsurprising given that this time period reflects the period of active growth for the yeast culture. However, it is interesting to note that the upregulation of ATG8 gene, involved in autophagosome biogenesis, coincided with the appearance of smaller vacuolar bodies as reported in Section 6.2.1. In contrast, the expression of ATG12 did not show any distinct changes throughout fermentation in any of the conditions analysed, remaining at base levels throughout. In the case of ATG23 (macroautophagy and degradation of cellular components via the vacuole or lysosomes), expression increased more dramatically between 16 and 88 hours in the 17.5 ^{o}P fermentation and the top-up regime than the 22 ^{o}P fermentation, indicating that the expression of this gene was not specifically upregulated by higher gravity conditions. However, it

does indicate that under all conditions there was a requirement for cellular breakdown at the mid-point of fermentation where yeast was at its most active and before cellular metabolism had begun to slow down.

The initiation of mitophagy is mediated by the ATG32 gene, prompting the targeted degradation of mitochondria. The expression of ATG32 was higher under all conditions at the fermentation endpoint than during the initial stages. However, expression remained low for the 17.5 ^{o}P fermentation between 16 and 88 hours, while the VHG and top-up regimes caused a rapid upregulation of ATG32 between these two timepoints. This could potentially stem from the heightened stress exerted on the yeast by the VHG conditions for the 22 ^{o}P fermentation and potentially, the rapid fermentation progression observed in the top-up regime at this stage. Furthermore, PTC6 expression, also related to mitophagy, slowly increased as fermentation progresses, with a similar trend observed across all conditions, indicating a potential influence of ethanol on the integrity of yeast mitochondria, prompting the need for removal of the damaged mitochondria. Interestingly, the addition of the sugar adjunct during the top-up regime had no influence on the expression of the autophagy-related genes analysed, with expression closely matching the VHG fermentation. The exception to this was for ATG32, where expression more closely followed that of the 17.5 ^{o}P fermentation, at least until the point of adjunct addition as alluded to above.

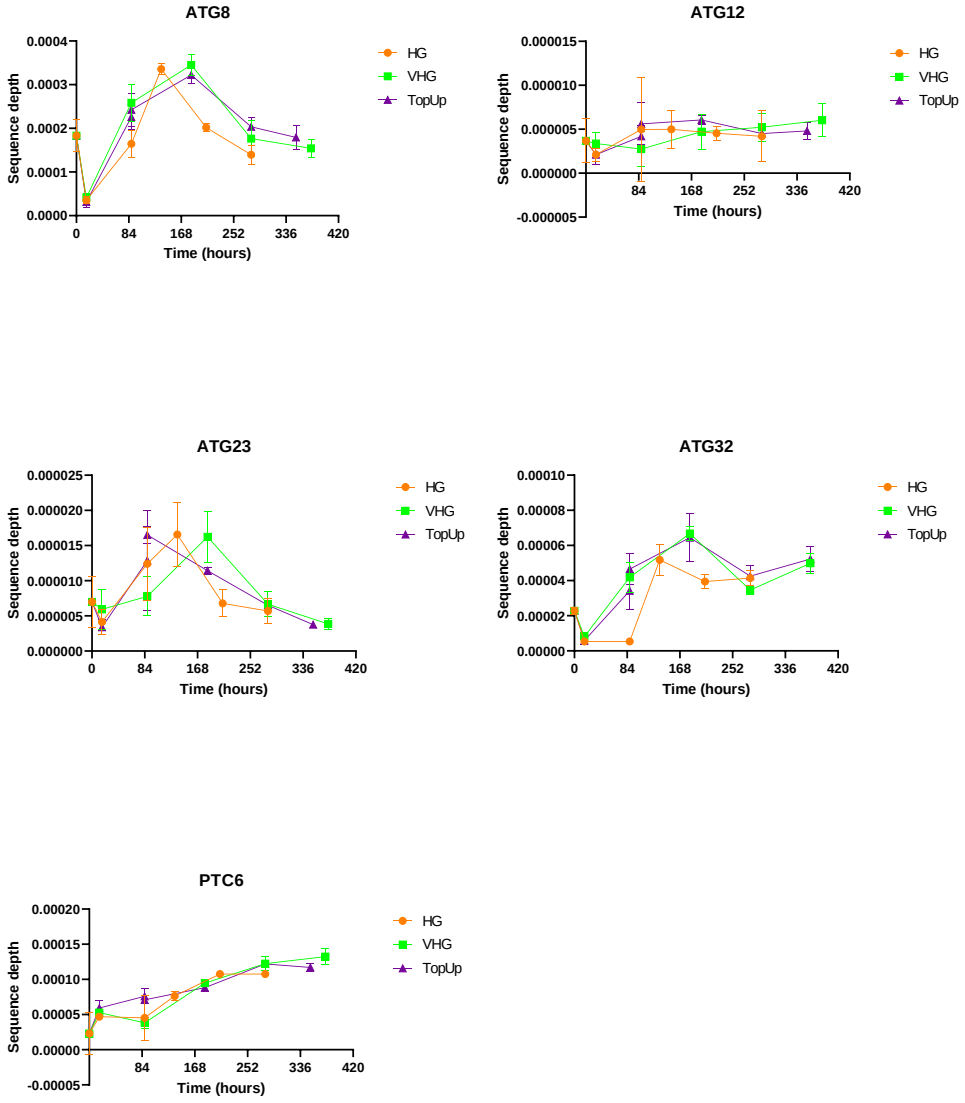
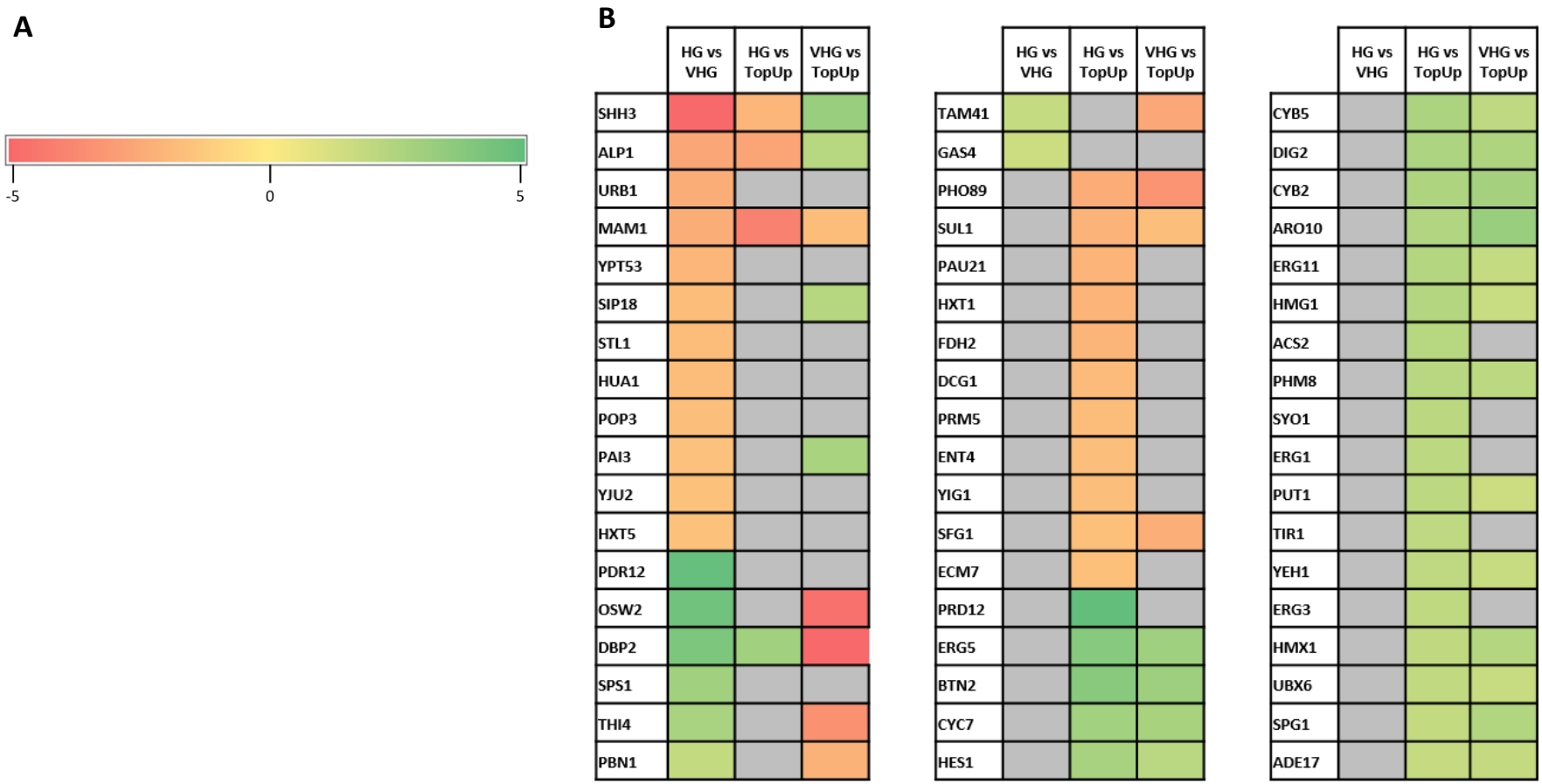


Figure 6.11. The impact of fermentation conditions on autophagy-related gene expression. Gene expression was analysed throughout a 17.5 ^{o}P (HG), 22 ^{o}P (VHG) and top-up regime (TopUp) fermentation.

6.2.5 Differential gene expression analysis dependent on fermentation conditions

As the yeast genetic response to extracellular stimuli is complex, targeted gene expression analysis such as that reported above can often overlook the impact

of certain genes effecting various pathways (Gasch et al., 2000; Gonzalez et al., 2016). Furthermore, gene activators can be employed to regulate genetic responses, with no change in gene expression observed for the target gene (Mager and Siderius, 2002; Hersen et al., 2008). Thus, the influence of the fermentation conditions in this study were subjected to conditional dynamic analysis, whereby genes that differed in their expression pattern were 'flagged' as being either up or down regulated. The condition dynamic analysis was performed as outlined in Section 2.6.3, which accounts for the gene expression at all sample timepoints under all conditions (as detailed in Section 6.2). Genes highlighted as being differentially expressed were then cross-referenced for their function using the Saccharomyces Genome Database (Saccharomyces Genome Database, 2021). Each condition was compared directly against one other variable to elucidate the influence of the different fermentation conditions on the yeast genetic response. A heat map summarising the data and the key genes identified can be found in Figure 6.12 and these are discussed further below.


A
-5
0
5
B
HG vs VHG
HG vs TopUp
VHG vs TopUp
SHH3
ALP1
URB1
MAM1
YPT53
SIP18
STL1
HUA1
POP3
PAI3
YJU2
HXT5
PDR12
OSW2
DBP2
SPS1
THI4
PBN1
TAM41
GAS4
PHO89
SUL1
PAU21
HXT1
FDH2
DCG1
PRM5
ENT4
YIG1
SFG1
ECM7
PRD12
ERG5
BTN2
CYC7
HES1
CYB5
DIG2
CYB2
ARO10
ERG11
HMG1
ACS2
PHM8
SYO1
ERG1
PUT1
TIR1
YEH1
ERG3
HMX1
UBX6
SPG1
ADE17

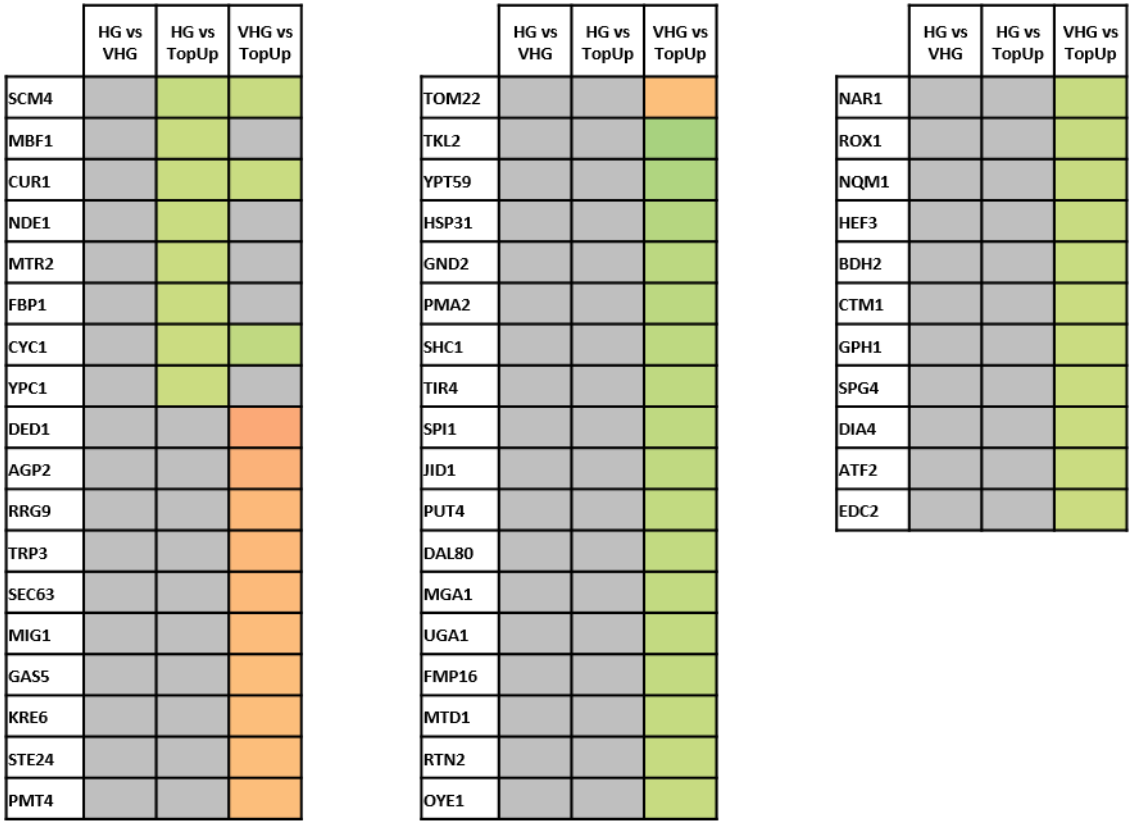

HG vs VHG
HG vs TopUp
VHG vs TopUp
SCM4
MBF1
CUR1
NDE1
MTR2
FBP1
CYC1
YPC1
DED1
AGP2
RRG9
TRP3
SEC63
MIG1
GAS5
KRE6
STE24
PMT4
TOM22
TKL2
YPT59
HSP31
GND2
PMA2
SHC1
TIR4
SPI1
JID1
PUT4
DAL80
MGA1
UGA1
FMP16
MTD1
RTN2
OYE1
NAR1
ROX1
NQM1
HEF3
BDH2
CTM1
GPH1
SPG4
DIA4
ATF2
EDC2

Figure 6.12. The influence of fermentation conditions on yeast gene expression. Differences in gene expression were assigned a colour-coded indicator based on the log_2-fold difference in gene expression (A) based on values ranging from -5 to 5, whereby lower gene expression (red) and higher gene expression (green) is indicated. Condition dynamic analysis (B) was used in order to determine differentially expressed genes for a 17.5 ^oP (HG), 22 ^oP (VHG) and a 22 ^oP top-up regime fermentation whereby genes were identified as either up or down regulated when comparing two conditions. Although only genes showing variation in expression were highlighted by the analysis, all of the potential combinations are indicated here to identify potential trends; hence no difference in expression (grey) is also indicated (i.e. these genes were not flagged under the specified conditions). The difference in gene expression refers to the primary condition set out by the comparison, therefore in X vs Y, a green result would indicate that the expression of the given gene was higher in condition X than condition Y.

Table 6.3. Summary of genes identified as having higher expression for a 17.5 °P fermentation when comparing the condition dynamics against a 22 °P fermentation.

Gene	Function
PDR12	Acid resistance
OSW2	Gene of unknown function
SPS1	SSP1 phosphorylation, cell division
DBP2	mRNA decay and rRNA processing
THI4	Thiamine biosynthesis, required for mitochondrial genome stability
PBN1	Protease post-translational processing
TAM41	Cardiolipin biosynthesis
GAS4	Spore wall assembly

Table 6.4. Summary of genes identified as having higher expression for a 22 °P fermentation when comparing the condition dynamics against a 17.5 °P fermentation.

Gene	Function
SHH3	Mitochondrial protein of unknown function
ALP1	Arginine transport
URB1	Ribosome biogenesis
MAM1	Cell division
YPT53	Stress tolerance, vacuolar protein sorting
SIP18	Induced by osmotic stress
STL1	Osmotic shock
HUA1	Actin patch assembly
POP3	Hypoxia
PAI3	Osmotic stress induction, Hog1p dependant
YJU2	Pre-mRNA splicing
HXT5	Induced by impaired growth rate

Under 17.5 ^{o}P fermentation conditions, the fermenting yeast exhibited a higher expression of cell growth-related genes DBP2, SPS1 and PBN1 (Table 6.3.), indicating a higher degree of growth executed at a cellular level than the 22 ^{o}P conditions. Furthermore, the upregulation of the TAM41 gene, involved in cardiolipin biosynthesis, suggests that mitochondrial biogenesis was also higher under these conditions. This coincides with the results found in Section 4.2.4., whereby extracellular osmotic stress resulted in reduced mitochondrial content. Additionally, the increased expression of YPT53 suggests an enhanced stress response under the VHG conditions, which actively invokes vacuolar protein sorting, a pathway commonly associated with vacuolar fragmentation (Raymond et al., 1992; Bonangelino et al., 2002). This provides a further rational explaining why vacuole fragmentation was more prominent throughout the VHG fermentation than the HG fermentation (Section 6.2.1).

Most likely due to the heightened osmotic stress associated with the 22 ^{o}P conditions, several osmotic stress-related genes were identified as being upregulated when compared to the 17.5 ^{o}P conditions (Table 6.4.). For example, genes STL1 and SIP18 were found to be overexpressed under these conditions. Furthermore, a gene regulated downstream of the HOG complex was also found to be upregulated (PAI3), potentially explaining why no change in HOG1 expression was observed when conditions with higher osmolarity were implemented. Although several growth-related genes were found to be expressed more highly in under VHG conditions, HXT5 expression was upregulated, suggesting impaired yeast growth.

Table 6.5. Summary of genes identified as having higher expression for a top-up regime fermentation when comparing the condition dynamics against a 22 ºP fermentation.

Gene	Function
OSW2	Spore wall assembly
SPS1	Spore wall assembly
THI4	Thiamine biosynthesis, required for mitochondrial genome stability
PHO89	Active in early growth phase
PRM5	Cell integrity signalling
TAM41	Cardiolipin biosynthesis
DED1	RNA helicase
SFG1	Pseudohyphal growth
DBP2	Remodelling of RNA-protein complex
AGP2	Polyamine and carnitine transport
PBN1	Autocatalytic post-translational processing of the protease B precursor Prb1p
RRG9	Unknown function
TRP3	Indole-3-glycerol phosphate synthase
SEC63	Part of secretory complex
MIG1	Glucose repression
MAM1	Expressed during first meiotic division
SUL1	Control of endogenous activated sulphate intermediates
GAS5	1,3-beta-glucanosyltransferase
KRE6	β1,6-glucan biosynthesis
STE24	ER quality control, removal of dysfunctional proteins
PMT4	Mannose transfer
TOM22	Transport of mitochondria-directed proteins

Table 6.6. Summary of genes identified as having higher expression for a 22 °P fermentation when comparing the condition dynamics against a top-up regime fermentation.

Gene	Function
ARO10	Ehrlich pathway
SHH3	Mitochondrial protein of unknown function
BTN2	Ethanol stress
ERG5	Ergosterol biosynthesis
TKL2	Synthesis of aromatic amino acids
CYC7	DNA replication stress
PAI3	Dependant on Hog1p, osmotic induction
DIG2	Inhibitor of Ste12p, regulation of invasive growth
YPT59	Autophagy
SPG1	Stationary phase gene
CYB2	Expression repressed by glucose and anaerobic conditions
SIP18	Induced by osmotic stress, role in survival of desiccation
HSP31	Oxidative stress
URB1	Ribosome biogenesis
HES1	Ergosterol biosynthesis
GND2	NADPH regeneration in pentose phosphate pathway
PHM8	Phosphate starvation and ribose salvage pathway
PMA2	Proton pump, cytoplasmic pH and plasma membrane potential regulator
CYB5	Sterol and lipid biosynthesis
SHC1	Induced by alkaline pH. Chitin synthase III activator
TIR4	Cell wall mannoprotein expressed under anaerobic conditions, induced by cold shock
SPI1	Requires Msn2p/Msn49, induced under stressful conditions
CYC1	Cellular respiration
JID1	Possible Hsp40p chaperone
PUT4	High-affinity transport of proline
DAL80	Negative regulator of nitrogen degradation pathways
MGA1	Protein similar to heat shock transcription factor
ADE17	De novo purine biosynthesis
UGA1	Required for oxidative stress tolerance and nitrogen utilization
FMP16	Potential involvement in stress response
ERG11	Ergosterol biosynthesis

MTD1	catalytic role in oxidation of cytoplasmic one-carbon units
HMX1	Oxidative stress response, regulated by ATF1
RTN2	DNA replication stress
UBX6	Repressed by inositol and choline
CYB2	Repressed by glucose and anaerobic conditions
OYE1	Geraniol reduction; involved in sterol metabolism, oxidative stress response and programmed cell death
YEH1	Sterol homeostasis
NAR1	Oxidative stress resistance
ROX1	Involved in hyperosmotic stress resistance
SCM4	Mitochondrial outer membrane protein of unknown function
NQM1	Induced during diauxic shift
HEF3	Expressed during zinc deficiency
BDH2	Alcohol dehydrogenase enzyme
HMG1	Sterol biosynthesis, DNA replication stress
CUR1	Sorting and deposition of misfolded proteins
CTM1	Not required for respiratory growth
GPH1	Regulated by HOG MAP kinase pathway
SPG4	Required for high-temperature survival during stationary phase
DIA4	Mitochondrial sery-tRNA synthetase
ATF2	Volatile ester formation
PUT1	Proline utilisation
EDC2	Heat stress

A significant number of genes were found to be differentially expressed between the 22 ^{o}P fermentation and the top-up regime, despite the two conditions incurring the same overall total gravity. A vast number of genes induced by osmotic stress were found to be more highly expressed under the 22 ^{o}P conditions (Table 6.6), which indicated that less osmotic stress was exerted on the fermenting yeast population when the top-up regime was implemented. Namely, genes SIP18, ROX1 and GPH1 were found to be upregulated under the standard batch VHG conditions, validating the implementation of the top-up regime for reducing the osmotic stress induced

on the fermenting yeast population. Although there was no statistical difference found in the ethanol yields from the two fermentation regimes (Section 5.2.6), genes involved in the ethanol stress response, or the overlapping function with the heat-induced stress response (SPI1, EDC2 and BTN2) were found to be more highly expressed in the 22 ^{o}P fermentation than in the top-up regime. This supports the observation that the role of heat-shock proteins in response to ethanol toxicity is important, as discussed in Section 6.2.4. Expression of the autophagy related gene, YPT59, was also indicated as being higher under the 22 ^{o}P conditions than in the top-up regime, again suggesting that these cells may have sustained enhanced damage causing autophagy to be promoted.

As with the comparison between HG and VHG conditions, expression data indicated that cardiolipin biosynthesis (important for mitochondrial membrane function) (Joshi et al., 2009) was increased throughout the top-up regime fermentation (Table 6.5), strengthening the argument that the environmental stress associated with 22 ^{o}P fermentations caused an inhibition of mitochondrial biosynthesis. Several ergosterol biosynthesis genes were also found to be overexpressed in the 22 ^{o}P fermentation (ERG5, ERG11, HES1, HMG1) (Table 6.5), providing a potential reason for the increased mitochondrial fragmentation (and associated increase in mitochondrial functionality) observed in this study and in Chapter 4. As mitochondrial fission can occur in order to increase mitochondrial productivity under stressful conditions, the role of mitochondria in sterol synthesis could therefore occur more efficiently in the unfused state, with fusion and fission dynamics serving

to improve mitochondrial efficiency (Viana et al., 2020). Furthermore, ergosterol has been shown to be essential for mitochondrial DNA maintenance (Cirigliano et al., 2019). Aside from mitochondrial relevance, ergosterol is also likely to be upregulated in order to fortify cellular membranes in response to the higher levels of stress exerted on the yeast population by the VHG conditions (Krantz et al., 2004; Rupcic et al., 2010; Dupont et al., 2011).

Table 6.7. Summary of genes identified as having higher expression for a top-up regime fermentation when comparing the condition dynamics against a 17.5 ^{o}P fermentation.

Gene	Function
MAM1	Cell division
ALP1	Arginine transporter
PHO89	Plasma membrane phosphate and sodium ion transport
SUL1	Sulphate transport
PAU21	Unknown function
HXT1	Low-affinity glucose transport
FDH2	Formate dehydrogenase
SHH3	mitochondrial inner membrane protein of unknown function
DCG1	Nitrogen catabolite repression
PRM5	Cell integrity signalling
ENT4	Unknown function
YIG1	Anaerobic glycerol production
SFG1	Superficial pseudohyphae
ECM7	Zinc deficiency

Table 6.8. Summary of genes identified as having higher expression for a 17.5 ^{o}P fermentation when comparing the condition dynamics against a top-up regime fermentation.

Gene	Function
PRD12	Degradation of mitochondrial proteins
ERG5	Ergosterol biosynthesis
BTN2	Ethanol stress, pH homeostasis
ERG5	Ergosterol biosynthesis
DBP2	rRNA processing
CYC7	Cellular respiration DNA replication stress
HES1	Ergosterol biosynthesis
CYB5	Lipid biosynthesis
DIG2	Cellular growth
CYB2	Lipid biosynthesis
ARO10	Decarboxylation of phenylpyruvate to phenylacetaldehyde (Erlich pathway)
ERG11	Ergosterol biosynthesis
HMG1	Sterol biosynthesis, DNA replication stress
ACS2	Growth on glucose under anaerobic conditions
HES1	Ergosterol biosynthesis
PHM8	Phosphate starvation
SYO1	Transport adapter
CYC7	Hypoxia
ERG1	Ergosterol biosynthesis
PUT1	Proline oxidase
TIR1	Cell wall mannoprotein, cold shock, anaerobiosis, acidic pH
YEH1	Ester hydrolase
ERG3	Ergosterol biosynthesis
HMX1	Oxidative stress response
UBX6	Ubiquitin regulation
SPG1	Stationary phase gene
BTN2	Possible role in pH homeostasis
ADE17	Purine biosynthesis
SCM4	Paralog of ATG33 (mitophagy-specific protein)
DBP2	mRNA decay and rRNA processing

MBF1	DNA replication stress
CUR1	Paralog of BTN2 - expressed during severe ethanol stress
YEH1	Sterol biosynthesis
NDE1	Mitochondrial respiration
MTR2	mRNA transport
FBP1	Autophagy-mediated degradation and glucose metabolism
CYC1	Respiration
YPC1	Alkaline ceramidase
MBF1	DNA replication stress

The initial stages of the top-up regime fermentation occur under the same conditions as the 17.5 ^{o}P fermentation, with the exception that a higher yeast cell concentration (pitching rate) was applied in the top-up regime, prompting the rapid initiation of fermentation as discussed previously (Section 5.2.5). Despite this similarity, a significant number of genes were differentially expressed between the two conditions throughout the process. Notably, ergosterol and lipid biosynthesis-regulating gene expression levels were found to be higher for the 17.5 ^{o}P fermentation (Table 6.8), potentially related to the greater number of cells able to divide at a given time. Several stress-related genes (HMX1, TIR1, BTN2) and autophagy-related genes (FBP1, SCM4, PRD12) were also found to be more highly expressed throughout the 17.5 ^{o}P fermentation, indicating that the implementation of a top-up regime fermentation exerts relatively less stress on the fermenting yeast, despite the higher total gravity. This was also reflected in the higher yeast viability at fermentation endpoint in the top-up regime conditions than in the 17.5 ^{o}P conditions (Section 5.2.5). However, it is unlikely that the addition of sugar adjunct induces a lower degree of stress on the fermenting yeast. This

phenomenon could potentially stem from the higher cell concentration and subsequent rapid initiation of fermentation which reduces the length of time yeast are exposed to the pitching wort conditions and associated high sugar concentration.

6.3 Discussion

The aim of this study was to rationalise results from the physiological analysis of cells under various conditions with the underpinning genetic response, in order to elucidate the influence of fermentation conditions on yeast organelle morphology, and to identify potential mechanisms behind their regulation. To achieve this yeast cells were used to conduct three fermentation types: a high gravity fermentation at 17.5 ^{o}P, a very high gravity fermentation at 22 ^{o}P and a combined approach where a 17.5 ^{o}P fermentation was adjusted midway via a top-up strategy whereby a total complement of 22 ^{o}P wort was provided. As expected, the presence of characteristically 'stressed' morphologies were present under all conditions; both vacuoles and mitochondria appeared as fragmented structures during the initial stages of fermentation. However, at the endpoint of fermentation, the influence of stress on mitochondrial morphology was more apparent in 22 ^{o}P VHG fermentations than either the 17.5 ^{o}P or top-up regimes. In contrast, differences in vacuole morphology were less discriminatory when comparing cells obtained post-fermentation. This suggests that the use of mitochondrial physiological analysis as a determinant of cellular stress is arguably a better indication of the cellular state than vacuolar analysis.

Interestingly, irrespective of these observations, observed changes to the structure of the vacuole or the mitochondria were not always accompanied by an increase in expression of genes primarily associated with vacuole fragmentation or mitochondrial fission/fusion. In fact, the expression of these genes generally tended to increase as fermentation progressed, irrespective of the fermentation format and organelle morphology. Similarly, the expression of genes related to autophagy and mitophagy increased for all conditions as fermentation progressed, with several autophagy-regulating genes reaching maximum expression levels at the midpoint of fermentation. At this stage, vacuoles adopted a different morphology whereby they were significantly smaller than observed in previous studies. This alludes to a potentially higher degree of cellular dehydration caused by the fermentation conditions, potentially resulting in a demand for the breakdown of damaged materials (Thumm, 2000; Li and Kane, 2009). Another potential reason is an alternative function of the yeast vacuoles at this point, with these smaller vacuoles potentially adopting autophagosomic or lysosomic roles (Li and Kane, 2009; Michaillat et al., 2012), especially since their appearance coincided with the heightened expression of autophagy-inducing genes.

The expression levels of stress-related genes generally increased as fermentation progressed, supporting previous observations that fermentations are inherently stressful (Alexandre et al., 2001; Hewitt and Nebe-Von-Caron, 2001; Gibson et al., 2007; Mendes-Ferreira et al., 2007; White et al., 2008; Piddocke et al., 2009; Puligundla et al., 2011; Liu et al., 2013). Furthermore, VHG fermentations procured a greater response than HG fermentations which,

while not unexpected, does clearly demonstrate that higher gravity fermentations are more stressful than those conducted at lower gravities. This was partially supported by expression patterns of the related genes such as the trehalose production-regulating gene, TPS1, which showed elevated activity at the beginning of 22 ^{o}P fermentations specifically. Related to this, it was interesting to note that the top-up regime appeared to alleviate stress, since many additional genes involved in the yeast stress response were observed to be upregulated in the comparative VHG fermentation. However, despite preconceptions of brewing fermentations, which predominantly identify the initial stages of the process as incurring the greatest degree of osmotic stress due to the high concentration of sugars in the pitching wort, the results of this study show that a greater degree of osmotic shock was exerted on the yeast population during the latter stages of fermentation, irrespective of format. These findings are synonymous with previous studies which have discussed the positive influence of ethanol on the degree of osmolarity of the environment, and the subsequent activation of osmotic stress pathways in yeast during fermentation (Hallsworth (1998); Zhuang et al. (2017).

Based on the initial gene analysis, this study also highlighted that a 'one dimensional' approach to interpreting the effects of fermentation conditions on gene expression can be ineffective, as key pathway-regulating genes were omitted from the initial analysis where only specific genes are selected. By investigating the condition dynamics in response to the different fermentation conditions, some interesting findings were uncovered. For example, the analysis indicated that ergosterol synthesis was significantly upregulated in

both the 17.5 ^{o}P and 22 ^{o}P conditions when compared to the top-up regime. This was particularly insightful, since we previously demonstrated that the major benefits observed in fermentation efficiency and yeast health at high gravity were induced through supplementation with ergosterol (Chapter 5), as well as the detrimental effects of heightened osmotic stress on the yeast plasma membrane (Chapter 4). Furthermore, the evidence of cross-talk between mitochondria and ergosterol synthesis pathways could be a potential requirement for increased mitochondrial efficiency (Leber et al., 1998; Jordá and Puig, 2020) as well as the role of ergosterol in protecting creating a clear trend between the genetic analysis and the mitochondrial morphologies observed throughout this body of work. This indicates that perhaps the greatest strategy towards alleviating stress at high gravity is to ensure membrane health and functionality, and this remains an important avenue for brewing yeast research in the future, all the while utilising organelle morphology analysis as a platform to assess cellular stress.

CHAPTER 7: CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

As the landscape of the brewing industry changes towards more cost-effective and sustainable brewing methods, the application of very high gravity (VHG) brewing methods offer an attractive solution to reduce brewery costs and energy input. This can be achieved through the production of high-alcohol content beers that can be diluted to produce greater volumes of standard-alcohol content through the application of fermentation processes incurring a higher concentration of fermentable sugars. This elevated sugar content increases the potential for ethanol production by yeast during fermentation. The process, by its very nature, creates a hostile environment for brewing yeast due to heightened stress factors caused by high levels of sugar exerting osmotic pressure on the yeast and high ethanol concentrations having toxic effects on the fermenting yeast population (Devantier et al., 2005a; Puligundla et al., 2011; Wang et al., 2013), amongst other problematic aspects of the process. It is because of this hostile environment that the full potential of very high gravity fermentation approaches have yet to be fully realised by the brewing industry, and thus the potential economic and environmental benefits are not being harnessed.

A vast array of brewing yeast strains exist and are used within the industry, each with unique qualities. The work carried out in Chapter 3 was not only to assess the unique characteristics of a selection of brewing yeast strains, but also to test these strains in their ability to perform under VHG conditions. Of the five strains used in this study, two of these were ale-type strains. The two

strains exhibited relatively good stress tolerance and performance at VHG. The other three were found to be lager strains, of which only one exhibited promising results in regards to VHG performance, which was strain SMCC 100. At this stage, a decision was required as to which strain would be selected for further analysis. Due to its enhanced performance under more stressful conditions than the other strains assessed in this study, as well as its application for lager-style brewing (which accounts for the majority of beer brewed and consumed globally), SMCC 100 was selected to progress for further analysis. Although SMCC 100 was considered to be the best performing strain at VHG, issues with severely elongated fermentation times and impaired yeast-derived flavour production were still observed, confirming that more work is needed in order to understand how these conditions influence the yeast cell, and use this information to conceptualise a method to improve the VHG fermentation process.

As discussed, the hostile environment associated with VHG worts are experienced by the yeast throughout the entirety of the fermentation process, as yeast are immediately exposed to heightened osmotic stress as they are inoculated to the wort. The issue of osmotic stress (which in this case is caused by the high sugar concentration present in the VHG wort) arises as the water potential of the environment is lower than that of the yeast cytosol, causing water efflux from the cell, causing a dehydration effect (Albertyn et al., 1994; Simonin et al., 2007). This exerts physical pressure on the cell, causing damage to the plasma membrane and other sub-cellular components (Simonin et al., 2007). The yeast, in response to this pressure, alters its physiology through the

coordination and alteration to gene expression profiles resulting in pro-survival physiological changes that allow the yeast cell to cope with the environmental stress (Attfield, 1997; Hohmann, 2002; Gomar-Alba et al., 2015). This body of work aimed to elucidate how yeast cells alter their physiology within a brewing fermentation environment, and if the excess stress associated with higher-gravity brewing will have any influence on these physiological changes.

In Chapter 4, it was confirmed that osmotic stress (induced by sorbitol) caused a decrease in yeast quality, manifesting in impaired membrane integrity and an increase in the level of dead cells within a population. In the living portion of the yeast population exposed to this stress, changes in organelle morphology were observed when compared to cells that were not considered to be stressed. The physiological phenomenon that were observed in response to stress were namely vacuole fragmentation (the dissociation of large vacuoles into multiple smaller ones) and mitochondrial fission (the process whereby elongated, of 'fused' mitochondria break apart into many smaller mitochondria), which was also accompanied by an increase in mitochondrial activity (thought to be the reason behind mitochondria fission in order to improve mitochondrial efficiency) and a reduction in cellular mitochondrial content. These morphological events have been previously addressed as a result of the yeast stress response (Fannjiang et al., 2004; Berman et al., 2008; Müller and Reichert, 2011; Zieger and Mayer, 2012; Michaillat and Mayer, 2013; Kanki et al., 2015). Within a fermentation environment, these changes occurred even in a standard gravity fermentation, however physiology returned to 'normal' upon the completion of fermentation, suggesting that

although standard gravity fermentations are somewhat stressful, yeast are able to adapt to the environment and maintain a healthy morphology. When excess osmotic stress was applied, the physiology of the yeast cells did not revert to normal. These changes were also accompanied by an inability of the yeast to perform adequately in terms of fermentation progression. Whereas this confirmed that the presence of osmotic stress caused unwanted physiological changes to the yeast cell, the full story behind the reasons and mechanisms behind these changes were not fully understood at this stage.

The next steps were to address how to improve yeast performance by optimising the fermentation process at VHG, with the aim of studying the effect that improved performance would have on cellular physiology. Two methods of optimisation were assessed for their efficacy in improving fermentation performance. The first of which was supplementing the wort with nutrients deemed to be beneficial for yeast, including zinc, magnesium, nitrogen, biotin (vitamin B_7), pyridoxine (vitamin B_1), thiamine (vitamin B_7) and ergosterol. These were screened individually for their ability to reduce fermentation time and improve yeast quality, after which a 'nutrient mix' was created which included the optimum concentration of each nutrient. This nutrient mix was extremely successful at reducing fermentation time and improving yeast quality, however issues with flavour meant that the applications of this optimisation procedure to the brewing industry are somewhat limited.

The other method that was assessed was a sugar top-up regime. This method utilised a system whereby wort gravity was increased mid-fermentation rather

than before the addition of yeast (the 'usual' approach to a VHG fermentation process) in order to prevent yeast exposure to unduly high levels of osmotic stress at this stage. Although not quite as effective as the nutrient supplementation approach, implementing a sugar top was successful in Improving yeast performance and quality, without any negative impacts of beer flavour. Thus, this method was selected to progress with for further experimentation.

As the top-up regime was successful in improving yeast performance, it was still unclear as to whether or not this was successful in reducing the stress exerted on yeast during fermentation. This question remained, along with the absence of an understanding of vacuole and mitochondrial physiology in response to stressful fermentation conditions. To elucidate this, the morphology of these organelles were monitored throughout high and very high gravity fermentations, as well as the top-up VHG regime. This analysis was performed alongside gene expression analysis to not only confirm if the top-up regime successfully reduced the stress exerted on the fermenting yeast, but to shed some light on why the changes in vacuole and mitochondria occur and the involvement of these organelles in yeast survival during stress.

The analysis performed on these fermentation conditions further confirmed that VHG conditions invoke stress-related changes to both vacuole and mitochondria, however utilisation of a top-up regime reduced the occurrence of these events suggesting an alleviation of stress. This was further supported by the gene expression analysis that showed a higher expression of stress-

related genes in the VHG conditions than the top-up regime conditions. Finally, insights into the involvement of vacuoles and mitochondria in the response of yeast cells to stressful conditions were provided by this analysis. This evidence highlighted the activities of vacuoles in removal of damaged cellular components, providing a novel insight into the physiological occurrences observed regarding yeast vacuoles in response to fermentation-related stresses. Several insights into the reasoning behind mitochondrial morphological changes in response to stress were also established. From the evidence provided by the results in Chapter 6, the role of mitochondria in ergosterol synthesis (Flis and Daum, 2013; Cirigliano et al., 2019), a mechanism whereby the yeast cell fortifies the plasma membrane to protect itself from stress (Dupont et al., 2011; Jordá and Puig, 2020), was highlighted to be expressed to a higher degree in the typical approach to VHG fermentation when compared to the top-up regime. Evidence from these results also eluded to impaired cardiolipin biosynthesis, potentially causing impaired mitochondria biogenesis. Hence, mitochondrial fission occurs to improve efficiency of the organelle, which is required to support the increased need for membrane protection.

The practical applications of this project aim to support the brewing industry in producing beer (and other fermented beverage) more sustainably by utilising VHG processes to increase production capacities while reducing energy input. This also has the added benefit of reducing production costs, further improving the attractiveness of this practice to brewers. The top-up regime applied in this body of work offers brewers the ability to not only achieve the benefits of VHG

fermentation in terms of yields and costs, but to apply this method to existing products (due to the absence of negative impacts on beer flavour). The elucidation of the physiological and genetic events that occur when yeast are exposed to stress offers a novel platform whereby yeast health can be assessed and further understood. The results from this body of work also provides valuable insights into the functioning of a yeast cell in response to environmental stress, which not only has applications to the brewing and beverage industries, but also to biotechnology and biological sciences as a whole.

7.2 Future Work

The work completed in this thesis could be improved and/or built upon by addressing the following;

i. Increasing the scope of the assessment of strain-specific responses to stress. As each strain in this study was shown to exhibit different tolerance levels in regards to stress factors, studying their physiological response to stresses in order to assess their regulation of organelle morphologies in order to assess if these physiological regulation mechanisms can dictate a strain's ability to tolerate VHG stress factors more adequately than others. Increased analysis surrounding the work conducted in Chapter 3 to include organelle morphology analysis, plasma membrane depolarisation assessment and viabilities of each strain under different fermentation

conditions would provide beneficial insights into strain specificity in stress tolerance.

ii. Following the insights into VHG stress and ergosterol biosynthesis provided from the results of Chapter 6, further understanding could be built around these findings. It could be insightful to ascertain which fermentation-derived stresses have the greatest influence on sterol synthesis pathways, and if the cumulative effect of stresses such as osmotic stress and ethanol toxicity behave similarly due to the importance of sterols and plasma membrane fortification in survival under these conditions (Alexandre et al., 1994; Dupont et al., 2011; Jordá and Puig, 2020). Furthermore, increased understanding between mitochondria, mitochondrial DNA and the ergosterol synthesis pathway would also be beneficial to understanding the physiological effects observed throughout this thesis and understanding their importance in regards to protection of the yeast plasma membrane.

iii. A large portion of the work conducted in this thesis focussed on osmotic stress. However, other stresses such as ethanol toxicity (Alexandre et al., 2001; Stanley et al., 2010; Saini et al., 2018), oxidative stress (Liu et al., 2013; Kitagaki and Takagi, 2014) and pH extremes (Guo and Olsson, 2016) can also prove problematic in regards to yeast health. By applying the same techniques developed throughout this body of work while assessing the influence of the above on yeast physiology would provide further insights into

regulation of yeast survival mechanism in response to different cellular targets of stress.

iv. Although the utilisation of nutritional supplements as a method of VHG fermentation optimisation was omitted from consideration due to issues regarding VDK levels and flavour imbalances, these issues could be addressed through alteration of fermentation parameters and process alterations to mitigate these problems. Fermentation temperature and pitching rate, amongst other parameters, could be altered to address differences in higher alcohol and ester production (Erten et al., 2007; Saerens et al., 2008). Alternatively, the use of each nutrient in the final 'nutrient mix' was inclusive of the optimal concentration of each nutrient individually. However, the combined nutrient effect may surpass the maximum rate of extract uptake, meaning that concentrations of certain nutrients may be present in excess amounts. Although the top-up regime was selected for further analysis, future work could potentially explore the physiological implications of this optimisation method despite the limitations of its use for brewing applications.

References

ABE, F. & HIRAKI, T. 2009. Mechanistic role of ergosterol in membrane rigidity and cycloheximide resistance in Saccharomyces cerevisiae. *Biochim Biophys Acta,* 1788**,** 743-52.

ADAMS, C. & VUUREN, H. 2010. *Effect of Timing of Diammonium Phosphate Addition to Fermenting Grape Must on the Production of Ethyl Carbamate in Wine*.

ADYA, A. K., CANETTA, E. & WALKER, G. M. 2006. Atomic force microscopic study of the influence of physical stresses on Saccharomyces cerevisiae and Schizosaccharomyces pombe. *FEMS yeast research,* 6**,** 120-128.

ALBERTYN, J., HOHMANN, S., THEVELEIN, J. M. & PRIOR, B. A. 1994. GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol Cell Biol,* 14.

ALEXANDRE, H., ANSANAY-GALEOTE, V., DEQUIN, S. & BLONDIN, B. 2001. Global gene expression during short-term ethanol stress in Saccharomyces cerevisiae. *FEBS Lett,* 498**,** 98-103.

ALEXANDRE, H., ROUSSEAUX, I. & CHARPENTIER, C. 1994. Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in Saccharomyces cerevisiae and Kloeckera apiculata. *FEMS Microbiology Letters,* 124**,** 17-22.

ALFENORE, S., MOLINA-JOUVE, C., GUILLOUET, S. E., URIBELARREA, J. L., GOMA, G. & BENBADIS, L. 2002. Improving ethanol production and viability of Saccharomyces cerevisiae by a vitamin feeding strategy during fed-batch process. *Appl Microbiol Biotechnol,* 60.

ALGHAMDI, T. A., HO, C. Y., MRAKOVIC, A., TAYLOR, D., MAO, D. & BOTELHO, R. J. 2013. Vac14 protein multimerization is a prerequisite step for Fab1 protein complex assembly and function. *The Journal of biological chemistry,* 288**,** 9363-9372.

ANDERSON, R. G. & KIRSOP, B. H. 1974. The control of volatile ester synthesis during the fermentation of wort of high specific gravity. *Journal of the Institute of Brewing,* 80**,** 48-55.

ATTFIELD, P. V. 1997. Stress tolerance: The key to effective strains of industrial baker's yeast. *Nature Biotechnology,* 15**,** 1351-1357.

AULD, D. S. & BERGMAN, T. 2008. Medium- and short-chain dehydrogenase/reductase gene and protein families. *Cellular and Molecular Life Sciences,* 65**,** 3961.

AUSTRIACO, N. 2012. Endoplasmic reticulum involvement in yeast cell death. *Frontiers in Oncology,* 2.

AVONCE, N., MENDOZA-VARGAS, A., MORETT, E. & ITURRIAGA, G. 2006. Insights on the evolution of trehalose biosynthesis. *BMC Evolutionary Biology,* 6**,** 109.

BAFRNCOVÁ, P., ŠMOGROVIČOVÁ, D., SLÁVIKOVÁ, I., PÁTKOVÁ, J. & DÖMÉNY, Z. 1999. Improvement of very high gravity ethanol fermentation by media supplementation using Saccharomyces cerevisiae. *Biotechnology Letters,* 21**,** 337-341.

BAKER, E., WANG, B., BELLORA, N., PERIS, D., HULFACHOR, A. B., KOSHALEK, J. A., ADAMS, M., LIBKIND, D. & HITTINGER, C. T. 2015. The Genome Sequence of Saccharomyces eubayanus and the Domestication of Lager-Brewing Yeasts. *Mol Biol Evol,* 32**,** 2818-31.

BAKER, E. P., PERIS, D., MORIARTY, R. V., LI, X. C., FAY, J. C. & HITTINGER, C. T. 2019. Mitochondrial DNA and temperature tolerance in lager yeasts. *Science Advances,* 5**,** eaav1869.
BAMFORTH, C. W. 2003. Wort Composition and Beer Quality. *Brewing Yeast Fermentation Performance.*
BAMFORTH, C. W. 2006. *Scientific Principles of Malting and Brewing*, American Society of Brewing Chemists.
BANDARA, A., FRASER, S., CHAMBERS, P. J. & STANLEY, G. A. 2009. Trehalose promotes the survival of Saccharomyces cerevisiae during lethal ethanol stress, but does not influence growth under sublethal ethanol stress. *FEMS Yeast Research,* 9**,** 1208-1216.
BARNETT, J. A. 1992. The taxonomy of the genus Saccharomyces meyen ex reess: A short review for non-taxonomists. *Yeast,* 8**,** 1-23.
BARNETT, J. A. 2000. A history of research on yeasts 2: Louis Pasteur and his contemporaries, 1850–1880. *Yeast,* 16**,** 755-771.
BERMAN, S. B., PINEDA, F. J. & HARDWICK, J. M. 2008. Mitochondrial fission and fusion dynamics: the long and short of it. *Cell death and differentiation,* 15**,** 1147-1152.
BIENZ, M. & PELHAM, H. R. 1986. Heat shock regulatory elements function as an inducible enhancer in the Xenopus hsp70 gene and when linked to a heterologous promoter. *Cell,* 45**,** 753-60.
BLANK, H. M., GAJJAR, S., BELYANIN, A. & POLYMENIS, M. 2009. Sulfur Metabolism Actively Promotes Initiation of Cell Division in Yeast. *PLOS ONE,* 4**,** e8018.
BLATEYRON, L. & SABLAYROLLES, J. M. 2001. Stuck and slow fermentations in enology: statistical study of causes and effectiveness of combined additions of oxygen and diammonium phosphate. *J Biosci Bioeng,* 91**,** 184-9.
BLIECK, L., TOYE, G., DUMORTIER, F., VERSTREPEN, K. J., DELVAUX, F. R., THEVELEIN, J. M. & VAN DIJCK, P. 2007. Isolation and characterization of brewer's yeast variants with improved fermentation performance under high-gravity conditions. *Appl Environ Microbiol,* 73**,** 815-24.
BOLDOGH, I. R., YANG, H. C. & PON, L. A. 2001. Mitochondrial inheritance in budding yeast. *Traffic,* 2**,** 368-374.
BONANGELINO, C. J., CHAVEZ, E. M. & BONIFACINO, J. S. 2002. Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae. *Mol Biol Cell,* 13**,** 2486-501.
BONINI, B. M., VAN DIJCK, P. & THEVELEIN, J. M. 2004. Trehalose Metabolism: Enzymatic Pathways and Physiological Functions. *In:* BRAMBL, R. & MARZLUF, G. A. (eds.) *Biochemistry and Molecular Biology.* Berlin, Heidelberg: Springer Berlin Heidelberg.
BORZANI, W. & VAIRO, M. L. 1958. Quantitative adsorption of methylene blue by dead yeast cells. *Journal of bacteriology,* 76**,** 251-255.
BOTSTEIN, D., CHERVITZ, S. A. & CHERRY, J. M. 1997. Yeast as a model organism. *Science (New York, N.Y.),* 277**,** 1259-1260.
BOTSTEIN, D. & FINK, G. R. 2011. Yeast: an experimental organism for 21st Century biology. *Genetics,* 189**,** 695-704.
BOULTON, C. & QUAIN, D. 2001. Brewing yeast and fermentation.
BOX, W., BENDIAK, D., CASTONGUAY, L., FELICIANO, S., FISCHBORN, T., GIBSON, B., KUENKER, M., LODOLO, B., MILLER, M., NICHOLLS, S., THIELE, F., WHITE, L. & POWELL, C. 2012. Differentiation of ale and lager yeast strains by rapid X-α-gal analysis. *Journal of the American Society of Brewing Chemists,* 70**,** 313-315.
BRIGGS, D. E., BOULTON, C. A. & BROOKES, P. A. 2004. *Brewing: Science and Practice*, Taylor & Francis.

BRYANT, N. J. & STEVENS, T. H. 1998. Vacuole Biogenesis in Saccharomyces cerevisiae Protein Transport Pathways to the Yeast Vacuole. *Microbiology and Molecular Biology Reviews,* 62**,** 230-247.
BUBNOVÁ, M., ZEMANČÍKOVÁ, J. & SYCHROVÁ, H. 2014. Osmotolerant yeast species differ in basic physiological parameters and in tolerance of non-osmotic stresses. *Yeast,* 31**,** 309-321.
CALLEJO, M. J., TESFAYE, W., GONZÁLEZ, M. C. & MORATA, A. 2019. Craft beers: Current situation and future trends. *New Advances on Fermentation Processes*.
CAPUSONI, C., ARIOLI, S., DONZELLA, S., GUIDI, B., SERRA, I. & COMPAGNO, C. 2019. Hyper-Osmotic Stress Elicits Membrane Depolarization and Decreased Permeability in Halotolerant Marine Debaryomyces hansenii Strains and in Saccharomyces cerevisiae. *Frontiers in microbiology,* 10**,** 64-64.
CASEY, G. P., MAGNUS, C. A. & INGLEDEW, W. M. 1983. High gravity brewing: Nutrient enhanced production of high concentrations of ethanol by brewing yeast. *Biotechnology Letters,* 5**,** 429-434.
CASEY, G. P., MAGNUS, C. A. & INGLEDEW, W. M. 1984. High-gravity brewing: effects of nutrition on yeast composition, fermentative ability, and alcohol production. *Applied and environmental microbiology,* 48**,** 639-646.
CAUSTON, H. C., REN, B., KOH, S. S., HARBISON, C. T., KANIN, E., JENNINGS, E. G., LEE, T. I., TRUE, H. L., LANDER, E. S. & YOUNG, R. A. 2001. Remodeling of yeast genome expression in response to environmental changes. *Molecular biology of the cell,* 12**,** 323-337.
CAVALIER-SMITH, T. 1975. The origin of nuclei and of eukaryotic cells. *Nature,* 256**,** 463-468.
CEBOLLERO, E. & GONZALEZ, R. 2006. Induction of autophagy by second-fermentation yeasts during elaboration of sparkling wines. *Applied and environmental microbiology,* 72**,** 4121-4127.
CHAZOTTE, B. 2011. Labeling mitochondria with MitoTracker dyes. *Cold Spring Harbor protocols* [Online], 2011. Available: http://europepmc.org/abstract/MED/21807856

https://doi.org/10.1101/pdb.prot5648 [Accessed 2011/08//].
CHUANG, L. F. & COLLINS, E. B. 1968. Biosynthesis of diacetyl in bacteria and yeast. *Journal of bacteriology,* 95**,** 2083-2089.
CIRIGLIANO, A., MACONE, A., BIANCHI, M. M., OLIARO-BOSSO, S., BALLIANO, G., NEGRI, R. & RINALDI, T. 2019. Ergosterol reduction impairs mitochondrial DNA maintenance in S. cerevisiae. *Biochim Biophys Acta Mol Cell Biol Lipids,* 1864**,** 290-303.
COGHE, S., GHEERAERT, B., MICHIELS, A. & DELVAUX, F. R. 2006. Development of Maillard Reaction Related Characteristics During Malt Roasting. *Journal of the Institute of Brewing,* 112**,** 148-156.
COURNIA, Z., ULLMANN, G. M. & SMITH, J. C. 2007. Differential effects of cholesterol, ergosterol and lanosterol on a dipalmitoyl phosphatidylcholine membrane: a molecular dynamics simulation study. *J Phys Chem B,* 111**,** 1786-801.
CREGG, J. M. & HIGGINS, D. R. 1995. Production of foreign proteins in the yeast Pichia pastoris. *Canadian journal of botany,* 73**,** 891-897.
CROWE, J. H., CROWE, L. M. & CHAPMAN, D. 1984. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science,* 223**,** 701-3.
CYERT, M. S. 2003. Calcineurin signaling in Saccharomyces cerevisiae: how yeast go crazy in response to stress. *Biochem Biophys Res Commun,* 311**,** 1143-50.

D'AMORE, T., PANCHAL, C. J., RUSSEIL, I. & STEWART, G. G. 1988. Osmotic pressure effects and intracellular accumulation of ethanol in yeast during fermentation. *Journal of Industrial Microbiology,* 2**,** 365-372.
D'AMORE, T., RUSSELL, I. & STEWART, G. G. 1989. Sugar utilization by yeast during fermentation. *Journal of Industrial Microbiology,* 4**,** 315-323.
DANIELSEN, G. 1992. Biotechnology of insulin therapy: edited by John C. Pickup, Blackwell Scientific Publications, 1991. UK£ 49.50 (x+ 186 pages) ISBN 0 632 03038 0. *Trends in Biotechnology,* 10**,** 455.
DATABASE, S. G. 2021. *Saccharomyces Genome Database* [Online]. Available: yeastgenome.org [Accessed 2021].
DAVID, M. H. & KIRSOP, B. H. 1973. Yeast growth in relation to the dissolved oxygen and sterol content of wort. *Journal of the Institute of Brewing,* 79**,** 20-25.
DE NADAL, E., ZAPATER, M., ALEPUZ, P. M., SUMOY, L., MAS, G. & POSAS, F. 2004. The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. *Nature,* 427**,** 370-4.
DE NICOLA, R. & WALKER, G. M. 2011. Zinc Interactions with Brewing Yeast: Impact on Fermentation Performance. *Journal of the American Society of Brewing Chemists,* 69**,** 214-219.
DEERE, D., SHEN, J., VESEY, G., BELL, P., BISSINGER, P. & VEAL, D. 1998. Flow cytometry and cell sorting for yeast viability assessment and cell selection. *Yeast,* 14**,** 147-60.
DEKONINCK, T. M. L., VERBELEN, P. J., DELVAUX, F., VAN MULDERS, S. E. & DELVAUX, F. R. 2013. The Importance of Wort Composition for Yeast Metabolism During Accelerated Brewery Fermentations. *Cerevisia,* 38**,** 57.
DENGIS, P. B., NÉLISSEN, L. R. & ROUXHET, P. G. 1995. Mechanisms of yeast flocculation: comparison of top- and bottom-fermenting strains. *Applied and Environmental Microbiology,* 61**,** 718-728.
DENIS, V. & CYERT, M. S. 2002. Internal Ca(2+) release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. *J Cell Biol,* 156**,** 29-34.
DENOTH LIPPUNER, A., JULOU, T. & BARRAL, Y. 2014. Budding yeast as a model organism to study the effects of age. *FEMS Microbiology Reviews,* 38**,** 300-325.
DEQUIN, S. & BARRE, P. 1994. Mixed Lactic Acid–Alcoholic Fermentation by Saccharomyes cerevisiae Expressing the Lactobacillus casei L(+)–LDH. *Bio/Technology,* 12**,** 173-177.
DEVANTIER, R., PEDERSEN, S. & OLSSON, L. 2005a. Transcription analysis of S. cerevisiae in VHG fermentation: The genome-wide transcriptional response of Saccharomyces cerevisiae during very high gravity ethanol fermentations is highly affected by the stationary phase. *Industrial Biotechnology,* 1**,** 51-63.
DEVANTIER, R., SCHEITHAUER, B., VILLAS-BOAS, S. G., PEDERSEN, S. & OLSSON, L. 2005b. Metabolite profiling for analysis of yeast stress response during very high gravity ethanol fermentations. *Biotechnol Bioeng,* 90.
DEVANTIER, R., SCHEITHAUER, B., VILLAS-BÔAS, S. G., PEDERSEN, S. & OLSSON, L. 2005c. Metabolite profiling for analysis of yeast stress response during very high gravity ethanol fermentations. *Biotechnology and Bioengineering,* 90**,** 703-714.
DHAR, R., SÄGESSER, R., WEIKERT, C. & WAGNER, A. 2013. Yeast Adapts to a Changing Stressful Environment by Evolving Cross-Protection and Anticipatory Gene Regulation. *Molecular Biology and Evolution,* 30**,** 573-588.

DIAKOV, T. T., TARSIO, M. & KANE, P. M. 2013. Measurement of vacuolar and cytosolic pH in vivo in yeast cell suspensions. *Journal of visualized experiments : JoVE***,** 50261.

DIHAZI, H., KESSLER, R. & ESCHRICH, K. 2004. High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. *J Biol Chem,* 279.

DINSDALE, M. G., LLOYD, D. & JARVIS, B. 1995. Yeast vitality during cider fermentation: two approaches to the measurement of membrane potential. *Journal of the Institute of Brewing,* 101**,** 453-458.

DRAGONE, G., MUSSATTO, S. I. & ALMEIDA E SILVA, J. B. 2007. High Gravity Brewing by Continuous Process Using Immobilised Yeast: Effect of Wort Original Gravity on Fermentation Performance. *Journal of the Institute of Brewing,* 113**,** 391-398.

DUINA, A. A., MILLER, M. E. & KEENEY, J. B. 2014. Budding yeast for budding geneticists: a primer on the Saccharomyces cerevisiae model system. *Genetics,* 197**,** 33-48.

DUNN, B. & SHERLOCK, G. 2008. Reconstruction of the genome origins and evolution of the hybrid lager yeast Saccharomyces pastorianus. *Genome Res,* 18**,** 1610-23.

DUPONT, S., BENEY, L., FERREIRA, T. & GERVAIS, P. 2011. Nature of sterols affects plasma membrane behavior and yeast survival during dehydration. *Biochim Biophys Acta,* 1808**,** 1520-8.

EPPS, D. E., WOLFE, M. L. & GROPPI, V. 1994. Characterization of the steady-state and dynamic fluorescence properties of the potential-sensitive dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol (Dibac4(3)) in model systems and cells. *Chem Phys Lipids,* 69**,** 137-50.

ERNANDES, J. R., WILLIAMS, J. W., RUSSELL, I. & STEWART, G. G. 1993. Respiratory deficiency in brewing yeast strains—effects on fermentation, flocculation, and beer flavor components. *Journal of the American Society of Brewing Chemists,* 51**,** 16-20.

ERTEN, H., TANGULER, H. & CAKIROZ, H. 2007. The Effect of Pitching Rate on Fermentation and Flavour Compounds in High Gravity Brewing. *Journal of the Institute of Brewing,* 113**,** 75-79.

FANNJIANG, Y., CHENG, W.-C., LEE, S. J., QI, B., PEVSNER, J., MCCAFFERY, J. M., HILL, R. B., BASAÑEZ, G. & HARDWICK, J. M. 2004. Mitochondrial fission proteins regulate programmed cell death in yeast. *Genes & development,* 18**,** 2785-2797.

FARAG, M. A., ELMASSRY, M. M., BABA, M. & FRIEDMAN, R. 2019. Revealing the constituents of Egypt's oldest beer using infrared and mass spectrometry. *Scientific reports,* 9**,** 16199-16199.

FARRÉ, J.-C., KRICK, R., SUBRAMANI, S. & THUMM, M. 2009. Turnover of organelles by autophagy in yeast. *Current opinion in cell biology,* 21**,** 522-530.

FEHRMANN, S., PAOLETTI, C., GOULEV, Y., UNGUREANU, A., AGUILANIU, H. & CHARVIN, G. 2013. Aging yeast cells undergo a sharp entry into senescence unrelated to the loss of mitochondrial membrane potential. *Cell reports,* 5**,** 1589-1599.

FELDMANN, H. 2012. Yeast Cell Architecture and Functions. *Yeast.* Wiley-Blackwell.

FLEET, G. H. 2007. Yeasts in foods and beverages: impact on product quality and safety. *Current Opinion in Biotechnology,* 18**,** 170-175.

FLEET, G. H. 2011. Chapter 5 - Yeast Spoilage of Foods and Beverages. *In:* KURTZMAN, C. P., FELL, J. W. & BOEKHOUT, T. (eds.) *The Yeasts (Fifth Edition).* London: Elsevier.

FLIS, V. V. & DAUM, G. 2013. Lipid transport between the endoplasmic reticulum and mitochondria. *Cold Spring Harbor perspectives in biology,* 5**,** a013235.
FOURY, F., ROGANTI, T., LECRENIER, N. & PURNELLE, B. 1998. The complete sequence of the mitochondrial genome of Saccharomyces cerevisiae. *FEBS Lett,* 440**,** 325-31.
FUKUDA, T. & KANKI, T. 2018. Mechanisms and Physiological Roles of Mitophagy in Yeast. *Molecules and cells,* 41**,** 35-44.
GALINDO, A., HERVÁS-AGUILAR, A., RODRÍGUEZ-GALÁN, O., VINCENT, O., ARST JR, H. N., TILBURN, J. & PEÑALVA, M. A. 2007. PalC, One of Two Bro1 Domain Proteins in the Fungal pH Signalling Pathway, Localizes to Cortical Structures and Binds Vps32. *Traffic,* 8**,** 1346-1364.
GALLONE, B., MERTENS, S., GORDON, J. L., MAERE, S., VERSTREPEN, K. J. & STEENSELS, J. 2018. Origins, evolution, domestication and diversity of Saccharomyces beer yeasts. *Curr Opin Biotechnol,* 49**,** 148-155.
GANCEDO, C. & FLORES, C. L. 2004. The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. *FEMS Yeast Res,* 4**,** 351-9.
GANZHORN, A. J. & PLAPP, B. V. 1988. Carboxyl groups near the active site zinc contribute to catalysis in yeast alcohol dehydrogenase. *J Biol Chem,* 263**,** 5446-54.
GARRETT, J. M. 2008. Amino acid transport through the Saccharomyces cerevisiae Gap1 permease is controlled by the Ras/cAMP pathway. *The international journal of biochemistry & cell biology,* 40**,** 496-502.
GASCH, A. P., SPELLMAN, P. T., KAO, C. M., CARMEL-HAREL, O., EISEN, M. B., STORZ, G., BOTSTEIN, D. & BROWN, P. O. 2000. Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. *Molecular Biology of the Cell,* 11**,** 4241-4257.
GERVAIS, P. & BENEY, L. 2001. Osmotic mass transfer in the yeast Saccharomyces cerevisiae. *Cell Mol Biol (Noisy-le-grand),* 47**,** 831-9.
GIBSON, B. R. 2011. 125th Anniversary Review: Improvement of Higher Gravity Brewery Fermentation via Wort Enrichment and Supplementation. *Journal of the Institute of Brewing,* 117**,** 268-284.
GIBSON, B. R., LAWRENCE, S. J., LECLAIRE, J. P., POWELL, C. D. & SMART, K. A. 2007. Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiol Rev,* 31**,** 535-69.
GIBSON, B. R., STORGÅRDS, E., KROGERUS, K. & VIDGREN, V. 2013. Comparative physiology and fermentation performance of Saaz and Frohberg lager yeast strains and the parental species Saccharomyces eubayanus. *Yeast,* 30**,** 255-266.
GIBSON, R. P., TURKENBURG, J. P., CHARNOCK, S. J., LLOYD, R. & DAVIES, G. J. 2002. Insights into Trehalose Synthesis Provided by the Structure of the Retaining Glucosyltransferase OtsA. *Chemistry & Biology,* 9**,** 1337-1346.
GILLILAND, R. B. 1962. YEAST REPRODUCTION DURING FERMENTATION. *Journal of the Institute of Brewing,* 68**,** 271-275.
GILLILAND, R. B. & HARRISON, G. A. 1966. Flavour in beer. *J Appl Bacteriol,* 29**,** 244-52.
GIMENO-ALCAÑIZ, J. V., PÉREZ-ORTÍN, J. E. & MATALLANA, E. 1999. Differential pattern of trehalose accumulation in wine yeast strains during the microvinification process. *Biotechnology Letters,* 21**,** 271-274.
GOFFEAU, A. 2000. Four years of post-genomic life with 6,000 yeast genes. *FEBS Lett,* 480**,** 37-41.

GOFFEAU, A., BARRELL, B. G., BUSSEY, H., DAVIS, R. W., DUJON, B., FELDMANN, H., GALIBERT, F., HOHEISEL, J. D., JACQ, C., JOHNSTON, M., LOUIS, E. J., MEWES, H. W., MURAKAMI, Y., PHILIPPSEN, P., TETTELIN, H. & OLIVER, S. G. 1996. Life with 6000 Genes. *Science,* 274**,** 546-567.

GOMAR-ALBA, M., MORCILLO-PARRA, M. A. & OLMO, M. L. 2015. Response of yeast cells to high glucose involves molecular and physiological differences when compared to other osmostress conditions. *FEMS Yeast Res,* 15**,** fov039.

GOMES, L. C. & SCORRANO, L. 2013. Mitochondrial morphology in mitophagy and macroautophagy. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1833**,** 205-212.

GONZALEZ, R., MORALES, P., TRONCHONI, J., CORDERO-BUESO, G., VAUDANO, E., QUIRÓS, M., NOVO, M., TORRES-PÉREZ, R. & VALERO, E. 2016. New Genes Involved in Osmotic Stress Tolerance in Saccharomyces cerevisiae. *Frontiers in microbiology,* 7**,** 1545-1545.

GOUNALAKI, N. & THIREOS, G. 1994. Yap1p, a yeast transcriptional activator that mediates multidrug resistance, regulates the metabolic stress response. *The EMBO Journal,* 13**,** 4036-4041.

GUILLAMON, J., SABATE, J. J., BARRIO, E., CANO, J. & QUEROL, A. 1998. Rapid identification of yeast species based on RFLP analysis of the ribosomal ITS regions. *Archives of microbiology,* 169**,** 387-92.

GUO, Z.-P. & OLSSON, L. 2016. Physiological responses to acid stress by Saccharomyces cerevisiae when applying high initial cell density. *FEMS Yeast Research,* 16**,** fow072.

HACKSTAFF, B. 1978. Various aspects of high gravity brewing. *Technical Quarterly Master Brewers Association of America*.

HALLSWORTH, J. E. 1998. Ethanol-induced water stress in yeast. *Journal of Fermentation and Bioengineering,* 85**,** 125-137.

HARRIS, F. M., BEST, K. B. & BELL, J. D. 2002. Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1565**,** 123-128.

HARRISON, G. 1970. The flavour of beer—a review. *Journal of the Institute of Brewing,* 76**,** 486-495.

HAZELWOOD, L. A., DARAN, J.-M., VAN MARIS, A. J. A., PRONK, J. T. & DICKINSON, J. R. 2008. The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on Saccharomyces cerevisiae Metabolism. *Applied and Environmental Microbiology,* 74**,** 2259.

HE, Y., DONG, J., YIN, H., CHEN, P., LIN, H. & CHEN, L. 2014a. Monitoring of the production of flavour compounds by analysis of the gene transcription involved in higher alcohol and ester formation by the brewer's yeast Saccharomyces pastorianus using a multiplex RT-qPCR assay. *Journal of the Institute of Brewing,* 120**,** 119-126.

HE, Y., DONG, J., YIN, H., ZHAO, Y., CHEN, R., WAN, X., CHEN, P., HOU, X., LIU, J. & CHEN, L. 2014b. Wort composition and its impact on the flavour-active higher alcohol and ester formation of beer – a review. *Journal of the Institute of Brewing,* 120**,** 157-163.

HECHT, K. A., O'DONNELL, A. F. & BRODSKY, J. L. 2014. The proteolytic landscape of the yeast vacuole. *Cellular logistics,* 4**,** e28023-e28023.

HERNLEM, B. & HUA, S.-S. 2010. Dual Fluorochrome Flow Cytometric Assessment of Yeast Viability. *Current Microbiology,* 61**,** 57-63.

HERRMANN, J. M., WOELLHAF, M. W. & BONNEFOY, N. 2013. Control of protein synthesis in yeast mitochondria: The concept of translational activators. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1833**,** 286-294.

HERSEN, P., MCCLEAN, M. N., MAHADEVAN, L. & RAMANATHAN, S. 2008. Signal processing by the HOG MAP kinase pathway. *Proc Natl Acad Sci U S A,* 105**,** 7165-70.
HERSKOWITZ, I. 1988. Life cycle of the budding yeast Saccharomyces cerevisiae. *Microbiological reviews,* 52**,** 536.
HEWITT, C. J. & NEBE-VON-CARON, G. 2001. An industrial application of multiparameter flow cytometry: assessment of cell physiological state and its application to the study of microbial fermentations. *Cytometry,* 44**,** 179-87.
HILL, A. E. & STEWART, G. G. 2019. Free Amino Nitrogen in Brewing. *Fermentation,* 5**,** 22.
HIRASAWA, T., ASHITANI, K., YOSHIKAWA, K., NAGAHISA, K., FURUSAWA, C., KATAKURA, Y., SHIMIZU, H. & SHIOYA, S. 2006. Comparison of transcriptional responses to osmotic stresses induced by NaCl and sorbitol additions in Saccharomyces cerevisiae using DNA microarray. *J Biosci Bioeng,* 102**,** 568-71.
HOHMANN, S. 2002. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev,* 66**,** 300-72.
HOHMANN, S. 2016. Nobel Yeast Research. *FEMS Yeast Research,* 16.
HOUNSA, C. G., BRANDT, E. V., THEVELEIN, J., HOHMANN, S. & PRIOR, B. A. 1998. Role of trehalose in survival of Saccharomyces cerevisiae under osmotic stress. *Microbiology,* 144 (Pt 3)**,** 671-80.
HUANG, W.-P. & KLIONSKY, D. J. 2002. Autophagy in yeast: a review of the molecular machinery. *Cell structure and function,* 27**,** 409-420.
HUCKER, B., WAKELING, L. & VRIESEKOOP, F. 2016. Vitamins in brewing: presence and influence of thiamine and riboflavin on wort fermentation. *Journal of the Institute of Brewing,* 122**,** 126-137.
HURT, E. C., MUTVEI, A. & CARMO-FONSECA, M. 1992. The Nuclear Envelope of the Yeast Saccharomyces cerevisiae. *In:* JEON, K. W. & FRIEDLANDER, M. (eds.) *International Review of Cytology.* Academic Press.
HUUSKONEN, A., MARKKULA, T., VIDGREN, V., LIMA, L., MULDER, L., GEURTS, W., WALSH, M. & LONDESBOROUGH, J. 2010. Selection from Industrial Lager Yeast Strains of Variants with Improved Fermentation Performance in Very-High-Gravity Worts. *Applied and Environmental Microbiology,* 76**,** 1563.
ISHMAYANA, S., KENNEDY, U. J. & LEARMONTH, R. P. 2017. Further investigation of relationships between membrane fluidity and ethanol tolerance in Saccharomyces cerevisiae. *World Journal of Microbiology and Biotechnology,* 33**,** 218.
IWAHASHI, H., OBUCHI, K., FUJII, S. & KOMATSU, Y. 1995. The correlative evidence suggesting that trehalose stabilizes membrane structure in the yeast Saccharomyces cerevisiae. *Cell Mol Biol (Noisy-le-grand),* 41**,** 763-9.
IZAWA, S., IKEDA, K., MIKI, T., WAKAI, Y. & INOUE, Y. 2010. Vacuolar morphology of Saccharomyces cerevisiae during the process of wine making and Japanese sake brewing. *Applied Microbiology and Biotechnology,* 88**,** 277-282.
JENSEN, R. E., HOBBS, A. E., CERVENY, K. L. & SESAKI, H. 2000. Yeast mitochondrial dynamics: fusion, division, segregation, and shape. *Microsc Res Tech,* 51**,** 573-83.
JONES, R. P. & GREENFIELD, P. F. 1987. Ethanol and the fluidity of the yeast plasma membrane. *Yeast,* 3**,** 223-32.
JORDÁ, T. & PUIG, S. 2020. Regulation of Ergosterol Biosynthesis in Saccharomyces cerevisiae. *Genes,* 11**,** 795.
JOSEPA, S., GUILLAMON, J. M. & CANO, J. 2000. PCR differentiation of Saccharomyces cerevisiae from Saccharomyces

bayanus/Saccharomyces pastorianus using specific primers. *FEMS Microbiol Lett,* 193**,** 255-9.
JOSEY, M., MASKELL, D. L. & ALEX SPEERS, R. 2019. Impact of Artificially Induced Respiratory Deficient Yeast on Beer Flavor and Fermentation. *Journal of the American Society of Brewing Chemists,* 77**,** 21-29.
JOSHI, A. S., ZHOU, J., GOHIL, V. M., CHEN, S. & GREENBERG, M. L. 2009. Cellular functions of cardiolipin in yeast. *Biochim Biophys Acta,* 1793**,** 212-8.
JOURNO, D., WINTER, G. & ABELIOVICH, H. 2008. Monitoring autophagy in yeast using FM 4-64 fluorescence. *Methods Enzymol,* 451**,** 79-88.
KACHROO, A. H., LAURENT, J. M., YELLMAN, C. M., MEYER, A. G., WILKE, C. O. & MARCOTTE, E. M. 2015. Evolution. Systematic humanization of yeast genes reveals conserved functions and genetic modularity. *Science,* 348**,** 921-5.
KANKI, T., FURUKAWA, K. & YAMASHITA, S.-I. 2015. Mitophagy in yeast: Molecular mechanisms and physiological role. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1853**,** 2756-2765.
KANLAYAVATTANAKUL, M. & LOURITH, N. 2009. Carboxymethylglucan in cosmetics. *Thai Pharmaceutical and Health Science Journal-วารสาร ไทยเภสัชศาสตร์ และ วิทยาการ สุขภาพ,* 3.
KIMATA, Y., ISHIWATA-KIMATA, Y., YAMADA, S. & KOHNO, K. 2006. Yeast unfolded protein response pathway regulates expression of genes for anti-oxidative stress and for cell surface proteins. *Genes Cells,* 11**,** 59-69.
KIRSOP, B. H. 1974. Oxygen in brewery fermentation. *Journal of the Institute of Brewing,* 80**,** 252-259.
KITAGAKI, H. 2009. Mitochondrial-morphology-targeted breeding of industrial yeast strains for alcohol fermentation. *Biotechnology and Applied Biochemistry,* 53**,** 145-153.
KITAGAKI, H., ARAKI, Y., FUNATO, K. & SHIMOI, H. 2007. Ethanol-induced death in yeast exhibits features of apoptosis mediated by mitochondrial fission pathway. *FEBS Lett,* 581**,** 2935-42.
KITAGAKI, H. & TAKAGI, H. 2014. Mitochondrial metabolism and stress response of yeast: Applications in fermentation technologies. *Journal of Bioscience and Bioengineering,* 117**,** 383-393.
KJELDSEN, T. 2000. Yeast secretory expression of insulin precursors. *Appl Microbiol Biotechnol,* 54**,** 277-86.
KLIONSKY, D. J., HERMAN, P. K. & EMR, S. D. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiol Rev,* 54**,** 266-92.
KLIS, F. M., MOL, P., HELLINGWERF, K. & BRUL, S. 2002. Dynamics of cell wall structure in Saccharomyces cerevisiae. *FEMS Microbiol Rev,* 26**,** 239-56.
KNORRE, D., POPADIN, K., SOKOLOV, S. & SEVERIN, F. 2013a. Roles of Mitochondrial Dynamics under Stressful and Normal Conditions in Yeast Cells. *Oxidative medicine and cellular longevity,* 2013**,** 139491.
KNORRE, D. A., POPADIN, K. Y., SOKOLOV, S. S. & SEVERIN, F. F. 2013b. Roles of mitochondrial dynamics under stressful and normal conditions in yeast cells. *Oxidative medicine and cellular longevity,* 2013**,** 139491-139491.
KOK, Y. J., YE, L., MULLER, J., OW, D. S.-W. & BI, X. 2019. Brewing with malted barley or raw barley: what makes the difference in the processes? *Applied microbiology and biotechnology,* 103**,** 1059-1067.
KONO, K., AL-ZAIN, A., SCHROEDER, L., NAKANISHI, M. & IKUI, A. E. 2016. Plasma membrane/cell wall perturbation activates a novel cell cycle checkpoint during G1 in Saccharomyces cerevisiae. *Proceedings of*

the National Academy of Sciences of the United States of America, 113**,** 6910-6915.
KRANTZ, M., NORDLANDER, B., VALADI, H., JOHANSSON, M., GUSTAFSSON, L. & HOHMANN, S. 2004. Anaerobicity prepares Saccharomyces cerevisiae cells for faster adaptation to osmotic shock. *Eukaryot Cell,* 3**,** 1381-90.
KROGERUS, K. & GIBSON, B. R. 2013. 125th Anniversary Review: Diacetyl and its control during brewery fermentation. *Journal of the Institute of Brewing,* 119**,** 86-97.
KÜHLBRANDT, W. 2015. Structure and function of mitochondrial membrane protein complexes. *BMC Biology,* 13**,** 89.
KWAST, K. E., BURKE, P. V. & POYTON, R. O. 1998. Oxygen sensing and the transcriptional regulation of oxygen-responsive genes in yeast. *The Journal of experimental biology,* 201**,** 1177-1195.
LARDY, H. A., POTTER, R. L. & HARRIS, R. H. 1949. Metabolic functions of biotin; the role of biotin in bicarbonate utilization by Lactobacillus arabinosus studied with C14. *J Biol Chem,* 179**,** 721-31.
LAVALLIÉE, F., SALVAS, Y., LAMY, S., THOMAS, D. Y., DEGRÉ, R. & DULAU, L. 1994. PCR and DNA Fingerprinting Used as Quality Control in the Production of Wine Yeast Strains. *American Journal of Enology and Viticulture,* 45**,** 86.
LEARMONTH, R. P. 2012. Membrane Fluidity in Yeast Adaptation: Insights from Fluorescence Spectroscopy and Microscopy. *In:* GEDDES, C. D. (ed.) *Reviews in Fluorescence 2010.* New York, NY: Springer New York.
LEARMONTH, R. P. & GRATTON, E. 2002. Assessment of Membrane Fluidity in Individual Yeast Cells by Laurdan Generalised Polarisation and Multi-photon Scanning Fluorescence Microscopy. *In:* KRAAYENHOF, R., VISSER, A. J. W. G. & GERRITSEN, H. C. (eds.) *Fluorescence Spectroscopy, Imaging and Probes: New Tools in Chemical, Physical and Life Sciences.* Berlin, Heidelberg: Springer Berlin Heidelberg.
LEBER, R., LANDL, K., ZINSER, E., AHORN, H., SPÖK, A., KOHLWEIN, S. D., TURNOWSKY, F. & DAUM, G. 1998. Dual localization of squalene epoxidase, Erg1p, in yeast reflects a relationship between the endoplasmic reticulum and lipid particles. *Molecular biology of the cell,* 9**,** 375-386.
LEE, S. Y., KNUDSEN, F. B. & POYTON, R. O. 1985. Differentiation of brewery yeast strains by restriction endonuclease analysis of their mitochondrial DNA. *Journal of the Institute of Brewing,* 91**,** 169-173.
LEGRAS, J.-L. & KARST, F. 2003. Optimisation of interdelta analysis for Saccharomyces cerevisiae strain characterisation. *FEMS Microbiology Letters,* 221**,** 249-255.
LEKKAS, C., STEWART, G. G., HILL, A. E., TAIDI, B. & HODGSON, J. 2007. Elucidation of the Role of Nitrogenous Wort Components in Yeast Fermentation. *Journal of the Institute of Brewing,* 113**,** 3-8.
LEWIS, M. J. & YOUNG, T. W. 2001. Hop chemistry and wort boiling. *Brewing.* Springer.
LI, S. C., DIAKOV, T. T., RIZZO, J. M. & KANE, P. M. 2012. Vacuolar H+-ATPase works in parallel with the HOG pathway to adapt Saccharomyces cerevisiae cells to osmotic stress. *Eukaryotic cell,* 11**,** 282-291.
LI, S. C. & KANE, P. M. 2009. The Yeast Lysosome-like Vacuole: Endpoint and Crossroads. *Biochimica et biophysica acta,* 1793**,** 650-663.
LIBKIND, D., HITTINGER, C. T., VALÉRIO, E., GONÇALVES, C., DOVER, J., JOHNSTON, M., GONÇALVES, P. & SAMPAIO, J. P. 2011. Microbe domestication and the identification of the wild genetic stock of lager-

brewing yeast. *Proceedings of the National Academy of Sciences,* 108**,** 14539.
LIN, Y.-J., CHANG, J.-J., LIN, H.-Y., THIA, C., KAO, Y.-Y., HUANG, C.-C. & LI, W.-H. 2017. Metabolic engineering a yeast to produce astaxanthin. *Bioresource Technology,* 245**,** 899-905.
LINKO, M., HAIKARA, A., RITALA, A. & PENTTILÄ, M. 1998. Recent advances in the malting and brewing industry. *Journal of Biotechnology,* 65**,** 85-98.
LIPKE, P. N. & OVALLE, R. 1998. Cell wall architecture in yeast: new structure and new challenges. *J Bacteriol,* 180**,** 3735-40.
LIU, C. G., LIN, Y. H. & BAI, F. W. 2013. Global gene expression analysis of Saccharomyces cerevisiae grown under redox potential-controlled very-high-gravity conditions. *Biotechnol J,* 8**,** 1332-40.
LIU, W., LI, L., YE, H., CHEN, H., SHEN, W., ZHONG, Y., TIAN, T. & HE, H. 2017. From Saccharomyces cerevisiae to human: The important gene co-expression modules. *Biomedical reports,* 7**,** 153-158.
LLOYD, W. J. W. 1986. Adjuncts. *Journal of the Institute of Brewing,* 92**,** 336-345.
LODOLO, E. J., KOCK, J. L. F., AXCELL, B. C. & BROOKS, M. 2008. The yeast Saccharomyces cerevisiae– the main character in beer brewing. *FEMS Yeast Research,* 8**,** 1018-1036.
LUCERO, P., PENALVER, E., MORENO, E. & LAGUNAS, R. 2000. Internal trehalose protects endocytosis from inhibition by ethanol in Saccharomyces cerevisiae. *Appl Environ Microbiol,* 66**,** 4456-61.
LUDOVICO, P., SANSONETTY, F. & CORTE-REAL, M. 2001. Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. *Microbiology,* 147**,** 3335-43.
LYNCH-DAY, M. A. & KLIONSKY, D. J. 2010. The Cvt pathway as a model for selective autophagy. *FEBS letters,* 584**,** 1359-1366.
MACWILLIAM, I. C. 1968. Wort composition - a review. *Journal of the Institute of Brewing,* 74**,** 38-54.
MAGER, W. H. & DE KRUIJFF, A. J. 1995. Stress-induced transcriptional activation. *Microbiological reviews,* 59**,** 506-531.
MAGER, W. H. & SIDERIUS, M. 2002. Novel insights into the osmotic stress response of yeast. *FEMS Yeast Research,* 2**,** 251-257.
MAJARA, M., O'CONNOR-COX, E. S. C. & AXCELL, B. C. 1996. Trehalose--an osmoprotectant and stress indicator compound in high and very high gravity brewing. *Journal of the American Society of Brewing Chemists,* v. 54.
MALAKAR, S., PAUL, S. K. & JOLVIS POU, K. R. 2020. Biotechnological Interventions in Beverage Production. *In:* GRUMEZESCU, A. M. & HOLBAN, A. M. (eds.) *Biotechnological Progress and Beverage Consumption.* Academic Press.
MALCORPS, P., CHEVAL, J. M., JAMIL, S. & DUFOUR, J. P. 1991. A New Model for the Regulation of Ester Synthesis by Alcohol Acetyltransferase in Saccharomyces Cerevisiae during Fermentation. *Journal of the American Society of Brewing Chemists,* 49**,** 47-53.
MALEK, J. 2001. Ancient Egyptian Materials and Technology. *American Journal of Archaeology,* 105**,** 338-340.
MALLETT, J. R., STUART, M. S. & QUAIN, D. E. 2018. Draught beer hygiene: a forcing test to assess quality. *Journal of the Institute of Brewing,* 124**,** 31-37.
MANGINOT, C., ROUSTAN, J. & SABLAYROLLES, J.-M. 1998. Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. *Enzyme and microbial technology,* 23**,** 511-517.

MANSURE, J. J. C., PANEK, A. D., CROWE, L. M. & CROWE, J. H. 1994. Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1191**,** 309-316.

MARTINEZ DE MARANON, I., MARECHAL, P. A. & GERVAIS, P. 1996. Passive response of Saccharomyces cerevisiae to osmotic shifts: cell volume variations depending on the physiological state. *Biochem Biophys Res Commun,* 227**,** 519-23.

MASON, A. B. & DUFOUR, J. P. 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast,* 16**,** 1287-1298.

MATTANOVICH, D., SAUER, M. & GASSER, B. 2014. Yeast biotechnology: teaching the old dog new tricks. *Microbial cell factories,* 13**,** 34-34.

MECHLER, B., HIRSCH, H. H., MULLER, H. & WOLF, D. H. 1988. Biogenesis of the yeast lysosome (vacuole): biosynthesis and maturation of proteinase yscB. *Embo j,* 7**,** 1705-10.

MEILGAARD, M. C. 1975. Flavor chemistry of beer : part ii : flavour and threshold of 239 aroma volatiles. *Tech. Q. Master Brew. Assoc. Am.,* 12**,** 151-168.

MENDES-FERREIRA, A., DEL OLMO, M., GARCÍA-MARTÍNEZ, J., JIMÉNEZ-MARTÍ, E., MENDES-FAIA, A., PÉREZ-ORTÍN, J. E. & LEÃO, C. 2007. Transcriptional response of Saccharomyces cerevisiae to different nitrogen concentrations during alcoholic fermentation. *Applied and environmental microbiology,* 73**,** 3049-3060.

MENDL, N., OCCHIPINTI, A., MÜLLER, M., WILD, P., DIKIC, I. & REICHERT, A. S. 2011. Mitophagy in yeast is independent of mitochondrial fission and requires the stress response gene WHI2. *Journal of Cell Science,* 124**,** 1339-1350.

MENESES, F. J., HENSCHKE, P. A. & JIRANEK, V. 2002. A Survey of Industrial Strains of Saccharomyces cerevisiae Reveals Numerous Altered Patterns of Maltose and Sucrose Utilisation. *Journal of the Institute of Brewing,* 108**,** 310-321.

MICHAILLAT, L., BAARS, T. L. & MAYER, A. 2012. Cell-free reconstitution of vacuole membrane fragmentation reveals regulation of vacuole size and number by TORC1. *Molecular Biology of the Cell,* 23**,** 881-895.

MICHAILLAT, L. & MAYER, A. 2013. Identification of Genes Affecting Vacuole Membrane Fragmentation in Saccharomyces cerevisiae. *PLoS ONE,* 8**,** e54160.

MITRA, K. & LIPPINCOTT-SCHWARTZ, J. 2010. Analysis of mitochondrial dynamics and functions using imaging approaches. *Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.],* CHAPTER**,** Unit-4.2521.

MIYATA, N., GODA, N., MATSUO, K., HOKETSU, T. & KUGE, O. 2017. Cooperative function of Fmp30, Mdm31, and Mdm32 in Ups1-independent cardiolipin accumulation in the yeast Saccharomyces cerevisiae. *Scientific Reports,* 7**,** 16447.

MIZOGUCHI, H. & HARA, S. 1998. Permeability barrier of the yeast plasma membrane induced by ethanol. *Journal of Fermentation and Bioengineering,* 85**,** 25-29.

MOAT, A. G. & LICHSTEIN, H. C. 1954. The role of biotin in carbohydrate metabolism of Saccharomyces cerevisiae. *Archives of Biochemistry and Biophysics,* 48**,** 300-309.

MOHAMMADI, S., SABERIDOKHT, B., SUBRAMANIAM, S. & GRAMA, A. 2015. Scope and limitations of yeast as a model organism for studying human tissue-specific pathways. *BMC Systems Biology,* 9**,** 96.

MOHD AZHAR, S. H., ABDULLA, R., JAMBO, S. A., MARBAWI, H., GANSAU, J. A., MOHD FAIK, A. A. & RODRIGUES, K. F. 2017. Yeasts in

sustainable bioethanol production: A review. *Biochemistry and Biophysics Reports,* 10**,** 52-61.
MORIMOTO, H., OKUNO, M., KAJITANI, R., RYUSUI, R., ITOH, T. & KODAMA, Y. 2016. Next-generation sequencing analysis of lager brewing yeast strains reveals the evolutionary history of interspecies hybridization. *DNA Research,* 23**,** 67-80.
MORITA, T., FUKUOKA, T., IMURA, T. & KITAMOTO, D. 2013. Production of mannosylerythritol lipids and their application in cosmetics. *Applied microbiology and biotechnology,* 97**,** 4691-4700.
MOSELEY, J. B. & GOODE, B. L. 2006. The yeast actin cytoskeleton: from cellular function to biochemical mechanism. *Microbiology and molecular biology reviews : MMBR,* 70**,** 605-645.
MOSER, M. J., GEISER, J. R. & DAVIS, T. N. 1996. Ca2+-calmodulin promotes survival of pheromone-induced growth arrest by activation of calcineurin and Ca2+-calmodulin-dependent protein kinase. *Molecular and cellular biology,* 16**,** 4824-4831.
MOUSIA, Z., BALKIN, R., PANDIELLA, S. & WEBB, C. 2004. The effect of milling parameters on starch hydrolysis of milled malt in the brewing process. *Process Biochemistry,* 39**,** 2213-2219.
MUKHERJEE, A., VERMA, J. P., GAURAV, A. K., CHOUHAN, G. K., PATEL, J. S. & HESHAM, A. E.-L. 2020. Yeast a potential bio-agent: future for plant growth and postharvest disease management for sustainable agriculture. *Applied Microbiology and Biotechnology,* 104**,** 1497-1510.
MÜLLER, M. & REICHERT, A. S. 2011. Mitophagy, mitochondrial dynamics and the general stress response in yeast. *Biochem Soc Trans,* 39**,** 1514-9.
NAVARRO-TAPIA, E., QUEROL, A. & PÉREZ-TORRADO, R. 2018. Membrane fluidification by ethanol stress activates unfolded protein response in yeasts. *Microbial Biotechnology,* 11**,** 465-475.
NESS, F., LAVALLÉE, F., DUBOURDIEU, D., AIGLE, M. & DULAU, L. 1993. Identification of yeast strains using the polymerase chain reaction. *Journal of the Science of Food and Agriculture,* 62**,** 89-94.
NIELSEN, J. 2013. Production of biopharmaceutical proteins by yeast: advances through metabolic engineering. *Bioengineered,* 4**,** 207-211.
NURSE, P. 2017. A Journey in Science: Cell-Cycle Control. *Molecular medicine (Cambridge, Mass.),* 22**,** 112-119.
NURSE, P., THURIAUX, P. & NASMYTH, K. 1976. Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. *Mol Gen Genet,* 146**,** 167-78.
O'CONNOR-COX, E. S. C. & INGLEDEW, W. M. 1989. Wort Nitrogenous Sources—Their Use by Brewing Yeasts: A Review. *Journal of the American Society of Brewing Chemists,* 47**,** 102-108.
O'CONNOR-COX, E. S. C. & INGLEDEW, W. M. 1990. Effect of the Timing of Oxygenation on Very High Gravity Brewing Fermentations. *Journal of the American Society of Brewing Chemists,* 48**,** 26-32.
O'CONNOR-COX, E. S. C., LODOLO, E. J. & AXCELL, B. C. 1996. Mitochondrial relevance to yeast fermentative performance: A Review. *Journal of the Institute of Brewing,* 102**,** 19-25.
OGBONNA, A. C. 1992. Developments in the malting and brewing trials with sorghum. *World J Microbiol Biotechnol,* 8**,** 87-91.
OHNO, T. & TAKAHASHI, R. 1986. Role of wort aeration in the brewing process part I: Oxygen uptake and the biosynthesis of lipids by the final yeast. *Journal of the Institute of Brewing,* 92**,** 84-87.
OLANIRAN, A. O., HIRALAL, L., MOKOENA, M. P. & PILLAY, B. 2017. Flavour-active volatile compounds in beer: production, regulation and control. *Journal of the Institute of Brewing,* 123**,** 13-23.

ORTS, W. J., HOLTMAN, K. M. & SEIBER, J. N. 2008. Agricultural chemistry and bioenergy. *J Agric Food Chem,* 56**,** 3892-9.
OSUMI, M. 1998. The ultrastructure of yeast: cell wall structure and formation. *Micron,* 29**,** 207-233.
OUGH, C. S., DAVENPORT, M. & JOSEPH, K. 1989. Effects of Certain Vitamins on Growth and Fermentation Rate of Several Commercial Active Dry Wine Yeasts. *American Journal of Enology and Viticulture,* 40**,** 208.
PACHECO, A., TALAIA, G., SÁ-PESSOA, J., BESSA, D., GONÇALVES, M. J., MOREIRA, R., PAIVA, S., CASAL, M. & QUEIRÓS, O. 2012. Lactic acid production in Saccharomyces cerevisiae is modulated by expression of the monocarboxylate transporters Jen1 and Ady2. *FEMS Yeast Research,* 12**,** 375-381.
PANCHAL, C. J. & STEWART, G. G. 1980. The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast. *Journal of the Institute of Brewing,* 86**,** 207-210.
PAPAZIAN, C. 2006. Beer styles: their origins and classification. *Handbook of brewing.* CRC Press.
PARASASSI, T., DE STASIO, G., D'UBALDO, A. & GRATTON, E. 1990. Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophys J,* 57**,** 1179-86.
PASTOR, M. M., PROFT, M. & PASCUAL-AHUIR, A. 2009. Mitochondrial function is an inducible determinant of osmotic stress adaptation in yeast. *J Biol Chem,* 284**,** 30307-17.
PATKOVA, J., SMOGROVICOVA, D., BAFRNCOVA, P. & DOMENY, Z. 2000. Changes in the yeast metabolism at very high-gravity wort fermentation. *Folia Microbiol (Praha),* 45**,** 335-8.
PERLI, T., WRONSKA, A. K., ORTIZ-MERINO, R. A., PRONK, J. T. & DARAN, J.-M. 2020. Vitamin requirements and biosynthesis in Saccharomyces cerevisiae. *Yeast,* 37**,** 283-304.
PHAM, T., WIMALASENA, T., BOX, W. G., KOIVURANTA, K., STORGÅRDS, E., SMART, K. A. & GIBSON, B. R. 2011. Evaluation of ITS PCR and RFLP for Differentiation and Identification of Brewing Yeast and Brewery 'Wild' Yeast Contaminants. *Journal of the Institute of Brewing,* 117**,** 556-568.
PIDDOCKE, M. P., KREISZ, S., HELDT-HANSEN, H. P., NIELSEN, K. F. & OLSSON, L. 2009. Physiological characterization of brewer's yeast in high-gravity beer fermentations with glucose or maltose syrups as adjuncts. *Applied Microbiology and Biotechnology,* 84**,** 453.
PIERCE, J. S. 1970. Institute of Brewing: Analysis committee measurement of yeast viability. *Journal of the Institute of Brewing,* 76**,** 442-443.
PIPER, P. W. 1995. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiol Lett,* 134**,** 121-7.
PIRES, E., TEIXEIRA, J., BRÁNYIK, T. & VICENTE, A. 2014. Yeast: The soul of beer's aroma - A review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied microbiology and biotechnology,* 98.
PISKUR, J., ROZPEDOWSKA, E., POLAKOVA, S., MERICO, A. & COMPAGNO, C. 2006. How did Saccharomyces evolve to become a good brewer? *Trends Genet,* 22**,** 183-6.
PLAPP, B. V., SAVARIMUTHU, B. R., FERRARO, D. J., RUBACH, J. K., BROWN, E. N. & RAMASWAMY, S. 2017. Horse Liver Alcohol Dehydrogenase: Zinc Coordination and Catalysis. *Biochemistry,* 56**,** 3632-3646.
POLLOCK, J. R. A. & WEIR, M. J. 1973. Chemical Aspects of the Adjunct Fermentation Process. *Proceedings. Annual meeting - American Society of Brewing Chemists,* 31**,** 1-6.

POREDA, A., CZARNIK, A., ZDANIEWICZ, M., JAKUBOWSKI, M. & ANTKIEWICZ, P. 2014. Corn grist adjunct–application and influence on the brewing process and beer quality. *Journal of the Institute of Brewing,* 120**,** 77-81.

POWELL, C. & DIACETIS, A. 2007. Long Term Serial Repitching and the Genetic and Phenotypic Stability of Brewer's Yeast. *Journal of the Institute of Brewing,* 113.

PRATT, P. L., BRYCE, J. H. & STEWART, G. G. 2003. The Effects of Osmotic Pressure and Ethanol on Yeast Viability and Morphology. *Journal of the Institute of Brewing,* 109**,** 218-228.

PRATT, P. L., BRYCE, J. H. & STEWART, G. G. 2007. The Yeast Vacuole -A Scanning Electron Microscopy Study During High Gravity Wort Fermentations. *Journal of the Institute of Brewing,* 113**,** 55-60.

PRICK, T., THUMM, M., KÖHRER, K., HÄUSSINGER, D. & VOM DAHL, S. 2006. In yeast, loss of Hog1 leads to osmosensitivity of autophagy. *The Biochemical journal,* 394**,** 153-161.

PULIGUNDLA, P., SMOGROVICOVA, D., OBULAM, V. S. & KO, S. 2011. Very high gravity (VHG) ethanolic brewing and fermentation: a research update. *J Ind Microbiol Biotechnol,* 38**,** 1133-44.

QUAIN, D. E. 1986. Differentiation of brewing yeast. *Journal of the Institute of Brewing,* 92**,** 435-438.

QUAIN, D. E. 2015. Assuring the microbiological quality of draught beer. *In:* HILL, A. E. (ed.) *Brewing Microbiology.* Oxford: Woodhead Publishing.

RAJ, S. B., RAMASWAMY, S. & PLAPP, B. V. 2014. Yeast alcohol dehydrogenase structure and catalysis. *Biochemistry,* 53**,** 5791-5803.

RAPOPORT, A., MARKOVSKIĬ, A. & BEKER, M. 1982. Increased permeability of the intracellular membranes in the dehydration and rehydration of Saccharomyces cerevisiae yeasts. *Mikrobiologiia,* 51**,** 901-904.

RAYMOND, C. K., HOWALD-STEVENSON, I., VATER, C. A. & STEVENS, T. H. 1992. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. *Mol Biol Cell,* 3**,** 1389-402.

REES, E. M. K. & STEWART, G. G. 1998. Strain specific response of brewer's yeast strains to zinc concentrations in conventional and high gravity worts. *Journal of the Institute of Brewing,* 104**,** 221-228.

REES, E. M. R. & STEWART, G. G. 1997. The effects of increased magnesium and calcium concentrations on yeast fermentation performance in high gravity worts. *Journal of the Institute of Brewing,* 103**,** 287-291.

REISER, V., RAITT, D. C. & SAITO, H. 2003. Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure. *J Cell Biol,* 161**,** 1035-40.

RIEZMAN, H. 1985. Endocytosis in yeast: Several of the yeast secretory mutants are defective in endocytosis. *Cell,* 40**,** 1001-1009.

RINALDI, T., DALLABONA, C., FERRERO, I., FRONTALI, L. & BOLOTIN-FUKUHARA, M. 2010. Mitochondrial diseases and the role of the yeast models. *FEMS Yeast Research,* 10**,** 1006-1022.

ROYA, D., MOHAMMAD, R., HOSSEIN, N. & GHOLAMREZA, G. 2013. Isolation of two riboflavin producer yeasts from environment and optimization of vitamin production. *J. Appl. Environ. Biol. Sci,* 3**,** 23-29.

RUIS, H. & SCHÜLLER, C. 1995. Stress signaling in yeast. *Bioessays,* 17**,** 959-65.

RUPCIC, J., JURESIC, G. C. & BLAGOVIC, B. 2010. Influence of stressful fermentation conditions on neutral lipids of a Saccharomyces cerevisiae brewing strain. *World J Microbiol Biotechnol,* 26**,** 1331-6.

RYAN, E. & KOHLHAW, G. 1974. Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast. *Journal of bacteriology,* 120**,** 631-637.
SAERENS, S. M., VERBELEN, P. J., VANBENEDEN, N., THEVELEIN, J. M. & DELVAUX, F. R. 2008. Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. *Appl Microbiol Biotechnol,* 80**,** 1039-51.
SAINI, P., BENIWAL, A., KOKKILIGADDA, A. & VIJ, S. 2018. Response and tolerance of yeast to changing environmental stress during ethanol fermentation. *Process Biochemistry,* 72**,** 1-12.
SAITO, H. & POSAS, F. 2012. Response to hyperosmotic stress. *Genetics,* 192**,** 289-318.
SAITO, H. & TATEBAYASHI, K. 2004. Regulation of the Osmoregulatory HOG MAPK Cascade in Yeast. *The Journal of Biochemistry,* 136**,** 267-272.
SAKAMOTO, K. & KONINGS, W. N. 2003. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology,* 89**,** 105-124.
SALEMA-OOM, M., VALADÃO PINTO, V., GONÇALVES, P. & SPENCER-MARTINS, I. 2005. Maltotriose utilization by industrial Saccharomyces strains: characterization of a new member of the alpha-glucoside transporter family. *Applied and environmental microbiology,* 71**,** 5044-5049.
SCHOCH, C. L., SEIFERT, K. A., HUHNDORF, S., ROBERT, V., SPOUGE, J. L., LEVESQUE, C. A. & CHEN, W. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci U S A,* 109**,** 6241-6.
SCHÖNBERGER, C. & KOSTELECKY, T. 2011. 125th Anniversary Review: The Role of Hops in Brewing. *Journal of the Institute of Brewing,* 117**,** 259-267.
SCHWEIZER, E. & HOFMANN, J. R. 2004. Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. *Microbiology and molecular biology reviews,* 68**,** 501-517.
SERRANO, R. 1996. Salt tolerance in plants and microorganisms: toxicity targets and defense responses. *International review of cytology.* Elsevier.
SHEN, B., HOHMANN, S., JENSEN, R. G., BOHNERT & HANS, J. 1999. Roles of Sugar Alcohols in Osmotic Stress Adaptation. Replacement of Glycerol by Mannitol and Sorbitol in Yeast. *Plant Physiology,* 121**,** 45-52.
SHUTT, T. E. & MCBRIDE, H. M. 2013. Staying cool in difficult times: Mitochondrial dynamics, quality control and the stress response. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1833**,** 417-424.
SIMONIN, H., BENEY, L. & GERVAIS, P. 2007. Sequence of occurring damages in yeast plasma membrane during dehydration and rehydration: Mechanisms of cell death. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1768**,** 1600-1610.
SINGER, S. J. & NICOLSON, G. L. 1972. The fluid mosaic model of the structure of cell membranes. *Science,* 175**,** 720-31.
SKINNER, L. R. 1927. Wort boiling. *Journal of the Institute of Brewing,* 33**,** 219-222.
SMART, K. 2008. *Brewing Yeast Fermentation Performance*, Wiley-Blackwell.
SMART, K. A. 2007. Brewing yeast genomes and genome-wide expression and proteome profiling during fermentation. *Yeast,* 24**,** 993-1013.
SMART, K. A. & WHISKER, S. 1996. Effect of Serial Repitching on the Fermentation Properties and Condition of Brewing Yeast. *Journal of the American Society of Brewing Chemists,* 54**,** 41-44.

SMIT, G., STRAVER, M. H., LUGTENBERG, B. J. & KIJNE, J. W. 1992. Flocculence of Saccharomyces cerevisiae cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. *Applied and environmental microbiology,* 58**,** 3709-3714.

SOUSTRE, I., DUPUY, P.-H., SILVE, S., KARST, F. & LOISON, G. 2000. Sterol metabolism and ERG2 gene regulation in the yeast Saccharomyces cerevisiae. *FEBS Letters,* 470**,** 102-106.

STAMBUK, B. U., DUNN, B., ALVES, S. L., JR., DUVAL, E. H. & SHERLOCK, G. 2009. Industrial fuel ethanol yeasts contain adaptive copy number changes in genes involved in vitamin B1 and B6 biosynthesis. *Genome research,* 19**,** 2271-2278.

STANLEY, D., BANDARA, A., FRASER, S., CHAMBERS, P. J. & STANLEY, G. A. 2010. The ethanol stress response and ethanol tolerance of Saccharomyces cerevisiae. *Journal of Applied Microbiology,* 109**,** 13-24.

STEENSELS, J., GALLONE, B., VOORDECKERS, K. & VERSTREPEN, K. J. 2019. Domestication of Industrial Microbes. *Current Biology,* 29**,** R381-R393.

STEWART, G. G. 2010. High-gravity brewing and distilling—past experiences and future prospects. *Journal of the American Society of Brewing Chemists,* 68**,** 1-9.

STEWART, G. G. 2017. The Production of Secondary Metabolites with Flavour Potential during Brewing and Distilling Wort Fermentations. *Fermentation,* 3**,** 63.

SUDA, Y. & NAKANO, A. 2012. The Yeast Golgi Apparatus. *Traffic,* 13**,** 505-510.

SUOMALAINEN, H. & LEHTONEN, M. 1979. The production of aroma compounds by yeast. *Journal of the Institute of Brewing,* 85**,** 149-156.

TADDEI, A. & GASSER, S. M. 2012. Structure and function in the budding yeast nucleus. *Genetics,* 192**,** 107-129.

TATEBAYASHI, K., YAMAMOTO, K., NAGOYA, M., TAKAYAMA, T., NISHIMURA, A., SAKURAI, M., MOMMA, T. & SAITO, H. 2015. Osmosensing and scaffolding functions of the oligomeric four-transmembrane domain osmosensor Sho1. *Nat Commun,* 6**,** 6975.

TEHLIVETS, O., SCHEURINGER, K. & KOHLWEIN, S. D. 2007. Fatty acid synthesis and elongation in yeast. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1771**,** 255-270.

THOMAS, D. S., HOSSACK, J. A. & ROSE, A. H. 1978. Plasma-membrane lipid composition and ethanol tolerance in Saccharomyces cerevisiae. *Arch Microbiol,* 117**,** 239-45.

THOMAS, K. C., DHAS, A., ROSSNAGEL, B. & INGLEDEW, W. M. 1995. Production of fuel alcohol from hull-less barley by very high gravity technology. *Cereal Chemistry,* 72**,** 360-364.

THUMM, M. 2000. Structure and function of the yeast vacuole and its role in autophagy. *Microsc Res Tech,* 51**,** 563-72.

TODA, T., BROEK, D., FIELD, J., MICHAELI, T., CAMERON, S., NIKAWA, J., SASS, P., BIRCHMEIER, C., POWERS, S. & WIGLER, M. 1986. Exploring the function of RAS oncogenes by studying the yeast Saccharomyces cerevisiae. *Princess Takamatsu Symp,* 17**,** 253-60.

TOONE, W. M. & JONES, N. 1999. AP-1 transcription factors in yeast. *Curr Opin Genet Dev,* 9**,** 55-61.

TUBB, R. S. & LILJESTROM, P. L. 1986. A colony-colour method which differentiates alpha-galactosidase-positive strains of yeast. *Journal of the Institute of Brewing. Institute of Brewing (Great Britain),* 92**,** 588-590.

TURAKAINEN, H., KRISTO, P. & KORHOLA, M. 1994. Consideration of the evolution of the Saccharomyces cerevisiae MEL gene family on the basis of the nucleotide sequences of the genes and their flanking regions. *Yeast,* 10**,** 1559-68.

UDEH, H. O. 2014. Effect of Mineral Ion Addition on Yeast Performance during Very High Gravity Wort Fermentation. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering,* 8**,** 1207-1216.

UNNO, K., JUVVADI, P. R., NAKAJIMA, H., SHIRAHIGE, K. & KITAMOTO, K. 2005. Identification and characterization of rns4/vps32 mutation in the RNase T1 expression-sensitive strain of Saccharomyces cerevisiae: Evidence for altered ambient response resulting in transportation of the secretory protein to vacuoles. *FEMS Yeast Research,* 5**,** 801-812.

VAN AELST, L., HOHMANN, S., BULAYA, B., DE KONING, W., SIERKSTRA, L., NEVES, M. J., LUYTEN, K., ALIJO, R., RAMOS, J. & COCCETTI, P. 1993. Molecular cloning of a gene involved in glucose sensing in the yeast Saccharomyces cerevisiae. *Mol Microbiol,* 8**,** 927-43.

VAN DER AA KÜHLE, A. & JESPERSEN, L. 1998. Detection and identification of wild yeasts in lager breweries. *Int J Food Microbiol,* 43**,** 205-13.

VAN DER REST, M. E., KAMMINGA, A. H., NAKANO, A., ANRAKU, Y., POOLMAN, B. & KONINGS, W. N. 1995. The plasma membrane of Saccharomyces cerevisiae: structure, function, and biogenesis. *Microbiological reviews,* 59**,** 304-322.

VAN URK, H., VOLL, W. L., SCHEFFERS, W. A. & VAN DIJKEN, J. P. 1990. Transient-state analysis of metabolic fluxes in Crabtree-positive and Crabtree-negative yeasts. *Applied and environmental microbiology,* 56**,** 281-287.

VAYSSIER-TAUSSAT, M., KREPS, S. E., ADRIE, C., DALL'AVA, J., CHRISTIANI, D. & POLLA, B. S. 2002. Mitochondrial membrane potential: a novel biomarker of oxidative environmental stress. *Environ Health Perspect,* 110**,** 301-5.

VELLAI, T. & VIDA, G. 1999. The origin of eukaryotes: the difference between prokaryotic and eukaryotic cells. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 266**,** 1571-1577.

VERSTREPEN, K. J., DERDELINCKX, G., DUFOUR, J. P., WINDERICKX, J., THEVELEIN, J. M., PRETORIUS, I. S. & DELVAUX, F. R. 2003a. Flavor-active esters: adding fruitiness to beer. *J Biosci Bioeng,* 96**,** 110-8.

VERSTREPEN, K. J., VAN LAERE, S. D. M., VANDERHAEGEN, B. M. P., DERDELINCKX, G., DUFOUR, J.-P., PRETORIUS, I. S., WINDERICKX, J., THEVELEIN, J. M. & DELVAUX, F. R. 2003b. Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. *Applied and environmental microbiology,* 69**,** 5228-5237.

VEVEA, J. D., SWAYNE, T. C., BOLDOGH, I. R. & PON, L. A. 2014. Inheritance of the fittest mitochondria in yeast. *Trends in cell biology,* 24**,** 53-60.

VIANA, M. P., BROWN, A. I., MUELLER, I. A., GOUL, C., KOSLOVER, E. F. & RAFELSKI, S. M. 2020. Mitochondrial Fission and Fusion Dynamics Generate Efficient, Robust, and Evenly Distributed Network Topologies in Budding Yeast Cells. *Cell Systems,* 10**,** 287-297.e5.

VIDA, T. A. & EMR, S. D. 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J Cell Biol,* 128**,** 779-92.

WALKER, G., NICOLA, R., ANTHONY, S. & LEARMONTH, R. 2006. Yeast-metal interactions: impact on brewing and distilling fermentations.

WALKER, G. M. 1998. Magnesium as a Stress-Protectant for Industrial Strains of Saccharomyces Cerevisiae. *Journal of the American Society of Brewing Chemists,* 56**,** 109-113.
WALKER, G. M. 2004. Metals in yeast fermentation processes. *Adv Appl Microbiol,* 54**,** 197-229.
WALKER, G. M. & DUFFUS, J. H. 1980. Magnesium ions and the control of the cell cycle in yeast. *J Cell Sci,* 42**,** 329-56.
WALKER, M. E., NGUYEN, T. D., LICCIOLI, T., SCHMID, F., KALATZIS, N., SUNDSTROM, J. F., GARDNER, J. M. & JIRANEK, V. 2014. Genome-wide identification of the Fermentome; genes required for successful and timely completion of wine-like fermentation by Saccharomyces cerevisiae. *BMC Genomics,* 15**,** 552.
WALSH, R. M. & MARTIN, P. A. 1977. Growth of Saccharomyces cerevisiae and Saccharomyces uvarum in a temperature gradient incubator. . *Journal of the Institute of Brewing,* 83**,** 169-172.
WALTHER, A., HESSELBART, A. & WENDLAND, J. 2014. Genome sequence of Saccharomyces carlsbergensis, the world's first pure culture lager yeast. *G3 (Bethesda),* 4**,** 783-93.
WALTHER, T., MTIMET, N., ALKIM, C., VAX, A., LORET, M. O., ULLAH, A., GANCEDO, C., SMITS, G. J. & FRANCOIS, J. M. 2013. Metabolic phenotypes of Saccharomyces cerevisiae mutants with altered trehalose 6-phosphate dynamics. *Biochem J,* 454**,** 227-37.
WANG, L., ZHAO, X. Q., XUE, C. & BAI, F. W. 2013. Impact of osmotic stress and ethanol inhibition in yeast cells on process oscillation associated with continuous very-high-gravity ethanol fermentation. *Biotechnol Biofuels,* 6**,** 133.
WEISMAN, L. S. 2003. Yeast Vacuole Inheritance and Dynamics. *Annual Review of Genetics,* 37**,** 435-460.
WEST, M., ZUREK, N., HOENGER, A. & VOELTZ, G. K. 2011. A 3D analysis of yeast ER structure reveals how ER domains are organized by membrane curvature. *The Journal of Cell Biology,* 193**,** 333.
WESTERMANN, B. 2012. Bioenergetic role of mitochondrial fusion and fission. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1817**,** 1833-1838.
WHITE, P. A., KENNEDY, A. I. & SMART, K. A. 2008. The Osmotic Stress Response of Ale and Lager Brewing Yeast Strains. *Brewing Yeast Fermentation Performance.* Blackwell Science.
WIEMKEN, A. 1990. Trehalose in yeast, stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek,* 58**,** 209-217.
WIGHTMAN, P., QUAIN, D. & MEADEN, P. 1996. Analysis of production brewing strains of yeast by DNA fingerprinting. *Letters in applied microbiology,* 22**,** 90-94.
WILLAERT, R. G. & BARON, G. V. 2001. Wort boiling today-boiling systems with low thermal stress in combination with volatile stripping. *Cerevisia,* 26**,** 217-230.
WOODING, S. & PELHAM, H. R. 1998. The dynamics of golgi protein traffic visualized in living yeast cells. *Molecular biology of the cell,* 9**,** 2667-2680.
YORKE, J., COOK, D. & FORD, R. 2021. Brewing with Unmalted Cereal Adjuncts: Sensory and Analytical Impacts on Beer Quality. *Beverages,* 7**,** 4.
YOU, K. M., ROSENFIELD, C.-L. & KNIPPLE, D. C. 2003. Ethanol tolerance in the yeast Saccharomyces cerevisiae is dependent on cellular oleic acid content. *Applied and environmental microbiology,* 69**,** 1499-1503.
YOULE, R. J. & VAN DER BLIEK, A. M. 2012. Mitochondrial Fission, Fusion, and Stress. *Science (New York, N.Y.),* 337**,** 1062-1065.

YOUNIS, O. S. & STEWART, G. G. 1999. Effect of Malt Wort, Very-High-Gravity Malt Wort, and Very-High-Gravity Adjunct Wort on Volatile Production in Saccharomyces Cerevisiae. *Journal of the American Society of Brewing Chemists,* 57**,** 39-45.

ZAKRZEWSKA, A., VAN EIKENHORST, G., BURGGRAAFF, J. E. C., VIS, D. J., HOEFSLOOT, H., DELNERI, D., OLIVER, S. G., BRUL, S. & SMITS, G. J. 2011. Genome-wide analysis of yeast stress survival and tolerance acquisition to analyze the central trade-off between growth rate and cellular robustness. *Molecular biology of the cell,* 22**,** 4435-4446.

ZANDYCKE, S., BERTRAND, D., DANIEL, H., DOUGLAS, P., HELBER, J., JENKINS, D., KANDA, H., PAWLOWSKI, K., RAINIERI, S., ROSTI, J., SUZUKI, K., TOGAMI, K., WIMALASENA, T. & POWELL, C. 2008. PCR Applications to Brewing: Differentiation of Brewing Yeast Strains by PCR Fingerprinting. *Journal of the American Society of Brewing Chemists,* 66**,** 266-270.

ZANDYCKE, S., SIMAL, O., GUALDONI, S. & SMART, K. 2003. Determination of Yeast Viability Using Fluorophores. *Journal of the American Society of Brewing Chemists,* 61**,** 15-22.

ZHANG, J. 2013. Autophagy and mitophagy in cellular damage control. *Redox Biology,* 1**,** 19-23.

ZHUANG, S., SMART, K. & POWELL, C. 2017. Impact of Extracellular Osmolality on Saccharomyces Yeast Populations during Brewing Fermentations. *Journal of the American Society of Brewing Chemists,* 75**,** 244-254.

ZIEGER, M. & MAYER, A. 2012. Yeast vacuoles fragment in an asymmetrical two-phase process with distinct protein requirements. *Molecular Biology of the Cell,* 23**,** 3438-3449.

ZIMMERMANN, A., KAINZ, K., ANDRYUSHKOVA, A., HOFER, S., MADEO, F. & CARMONA-GUTIERREZ, D. 2016. Autophagy: one more Nobel Prize for yeast. *Microbial cell (Graz, Austria),* 3**,** 579-581.

ZINSER, E., PALTAUF, F. & DAUM, G. 1993. Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J Bacteriol,* 175**,** 2853-8.