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HETEROGENEITY IN BREWING YEAST POPULATIONS

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Abstract

During brewing fermentations, yeast cells are subjected to a range of stress factors including ethanol, osmotic and oxidative stress. These have an impact on population health, affecting fermentation consistency, efficiency, and quality of the product. Typically, the tolerance of a yeast culture to stress is assessed via analysis of the entire population generating mean data, assuming each cell has broadly equivalent characteristics. This can mask detail at the cellular level which can only be obtained by analysing individual cells. In this study, a range of yeast strains significant to the brewing industry were investigated for tolerance to a variety of environmental challenges. This was performed using a novel high throughput assay for investigating 'population heterogeneity', based on cell cytotoxicity. This revealed that some cells within a population were more tolerant to stresses than others. Furthermore, the dynamics of the stress response differed between strains in both the maximum tolerance and degree of heterogeneity. To identify potential sources of variation within populations, key cell organelles were analysed for variation in structural integrity. Yeast cells were also separated according to replicative age, by sorting based on bud scar material, this allowed for the physiological differences between daughter and aged cells to be investigated. Based on this analysis, we provide evidence to suggest that population variation is an innate inheritable characteristic. Subsequently, cell metabolites upregulated under stressed conditions were identified using metabolomics, this data was used this to conduct targeted single cell gene expression analysis, using a highly heterogenous and a less heterogeneous strain. From this, we were able to conclude that genes upregulated in response to stress were heterogenous in nature, matching the phenotypic data observed previously. We also demonstrate that the cause of phenotypic heterogeneity was likely a result of an accumulation of many heterogeneous gene expression events. Clusters were identified in specific groups of cells, implying the presence of population organisation, likely to be a 'division of labour' survival strategy. This data has implications for our understanding of strain specific fermentation limits and the techniques developed here may facilitate screening and prediction of the suitability of yeast strains for specific fermentation types.

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Abbreviations

°C- Degrees Centigrade

Bp- Base Pairs

CFU- Colony Forming Units

CV- Coefficient of Variance

$DiOC_6$- 3,3'-dihexyloxacarbocyanine iodide

DNA- Deoxyribonucleic acid

ER- Endoplasmic Reticulum

ESI- Electrospray Ionisation

ESR- Environmental Stress Response

FACS- Fluorescence Activated Cell Sorting

FAN- Free Amino Nitrogen

FSC- Forwards Scatter

GFP- Green Fluorescent Protein

GSR- General Stress Response

HILIC- Hydrophilic Interaction Chromatography

HOG- High Osmolarity Glycerol

HPLC- High Performance Liquid Chromatography

Hrs- Hours

ITS- Internal Transcribed Spacer

Kb- Kilobase

LCMS- Liquid Chromatography- Mass Spectrometry

m/m- Mass/mass concentration

Mins- Minutes

mM- MilliMolar

MMP- Mitochondrial Membrane Potential

MTG- MitoTracker Green FM

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

nM- NanoMolar

NR- Nile Red

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

RFLP- Restriction Fragment Length Polymorphisms

RNA- Ribonucleic Acid

ROS- Reactive Oxygen Species

RPM- Revolutions per minute

Secs- Seconds

SSC- Side Scatter

STRE- Stress Response Elements

TIC- Total Ion Chromatogram

TTC- Triphenyl Tetrazolium Chloride

UMI- Unique Molecular Identifier

UPR- Unfolded Protein Response

V/v- Volume/volume concentration

VDK- Vicinal Diketones

w/v- Mass/Volume concentration

WGA- Wheat Germ Agglutinin

µM- MicroMolar

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Chapter 1. Introduction

1.1 History of brewing

Beer brewing is thought to date back as far as ancient Mesopotamia, with it first being referred to in the 'Epic of Gilgamesh' tablets, dating 2100BC (Kovacs, 1989). In these, beer was a beverage consumed in times of celebration, relaxation and joy. The production and intoxicating affects were thought to be a work of the gods, specifically the goddess Ninkasi (the goddess of beer)(Damerow, 2012). The daily staple was brewed by priestesses of Ninkasi using a recipe found in another ancient tablet titled 'Hymn to Ninkasi' (O'Briant, 2017). The hymn contained instructions on how to make beer from twice-baked barley bread, honey, and dates mixed with water and wine. Ninkasi was not only the goddess of beer but was also believed to 'be' the beer itself. As such, priestesses brewed the beer with the finest ingredients and to the highest possible quality (Prince, 1916; O'Briant, 2017).

Due to its mystical properties and divine origin, beer very quickly became commercialised, gaining its own trading laws. Much like Mesopotamians, the Egyptians also believed brewing to be a divine talent, given to them by the god Osiris, and was also produced by women in a very similar manner, using both cooked and uncooked malt with water, often accompanied by additives such as honey (Godlaski, 2011). Beer brewing reached Europe around 800 BC and was made with similar materials, often coupled with bread making (traditionally by women), to supplement the family meals (Dornbusch, 1998). In the middle ages, brewing became a craft of Christian monks, providing shelter and sustenance to pilgrims (Nelson, 2018).

Despite these practices, the action of fermentation largely remained a mystery. In 1516 the Bavarians introduced the Reinheitsgebot (the beer purity law) stating that beer must be made from only water, hops and barley malt (White and Zainasheff, 2010; Tongeren, 2011). It should be noted that at this point there was no mention of yeast, since the microbial world remained unexplored. Around this time, hops started to be used in beer for enhancing flavour, but also for making the beverage less prone

to spoilage. This lead to the development of highly hopped products, ultimately leading to the production of the IPA (Indian Pale Ale) category of beer. This beer style was developed in the 1800's specifically for the East India Trading Company, allowing beer to be shipped from Great Britain to India without spoiling (Van Kerckhoven *et al*., 2020). While the first observation of yeast was performed in 1680 by Antione van Leeuwenhoek, using a primitive home-made microscope, it was only in the mid to late 1800s that the significance of yeast as the fermentation 'catalyst' became recognised (Pasteur, 1873, 1876, 1879; Geertman *et al.*, 2006).

In 1883 Emil Hansen, employed by Carlsberg, developed a mechanism to isolate and grow pure yeast cultures to be used in beer fermentations; prior to this mixed cultures of wild yeast were used in fermentations (Samuel, 1996). This reflected advances in microbial understanding at the time and helped to establish the importance of yeast in brewing fermentations. Despite being a historical practice, recipes today are still broadly based on the same principles. As such, brewing can be defined as the process by which a sugary wort is produced from malted barley (or other cereal crops), flavoured with hops, and subsequently fermented by yeast to create beer (Lodolo *et al.*, 2008).

1.2 The Brewing Process

Beer brewing is now a more precise science that requires intensive pre-processing of raw materials and in-depth recipe development to provide a consistent and flavoursome product. The brewing process can be divided into two main stages, often referred to as 'the hot side' and 'the cold side'. The hot side refers to wort production and consists of grain milling, mashing and the boiling of sweet wort with hops (Fix, 1999). Following this the cold side reflects the cooling of wort and the addition of yeast allowing the fermentation to take place (White and Zainasheff, 2010).

In more detail, the entire brewing process begins by allowing barley to germinate. Germination is very tightly controlled by steeping the grain and subsequently drying at temperatures dependant on the requirement (Turner *et al.*, 2019). During this

process, enzymes are activated to break down reserve starch stores, although the process is halted before fermentable sugars are released (Hough and Hough, 1991). The grain is then kilned, at different temperatures and duration, to impart various flavours and colours to the beer (Lodolo *et al.*, 2008). Following this, the malted barley is combined with hot water in a process called mashing, to extract fermentable sugars, amino acids and other essential compounds for flavour, mouthfeel and yeast nutrition (He *et al.*, 2014). The resulting sugary material (referred to as mash) is separated from the spent grain, either by filtration, or by utilising the natural filtration of the grain bed in a lauter vessel (Boulton *et al*., 2013). This liquor is referred to as wort, which is then boiled. Hops are often added at this stage, principally for their alpha acids, which are isomerised during boiling. These add bitterness to the product, as well as enhancing foam stability and lending antimicrobial properties to the final product (Simpson, 1993; Smith *et al*., 1998). Boiling the wort, also provides a mechanism for sterilisation, haze precipitation, and the degradation of enzymes that may change the fermentability of the wort (Lewis, 2000; Jin *et al.*, 2009). The sweet wort is then rapidly cooled prior to fermentation, typically using a para-flow in-line during transfer to the fermentation vessel in order to enhance efficiency (Willaert, 2007).

Yeast is then pitched into the sterile wort, with the primary function being to use the fermentable sugars to produce biomass, ethanol, and flavour compounds (Iorizzo *et al.*, 2021). Typically wort is aerated prior to yeast pitching; oxygen is required by yeast for the production of lipids and sterols, essential for membrane biosynthesis (Verbelen *et al.*, 2009). The supply of carbohydrates, nitrogen, essential nutrients, and oxygen allow the culture to rapidly increase in biomass, becoming highly active (O'Connor-Cox and Ingledew, 1990; Rees and Stewart, 1999). Under these conditions yeast rapidly adopt a fermentative form of metabolism, utilising the oxygen for production of cellular components while also using the fermentable sugars to produce energy, biomass and ethanol. Despite oxygen being present, the respiratory pathway is not used in brewing yeast, due to a peculiarity known as the Crabtree effect (De Deken, 1966). This is elicited by the presence of fermentable sugars, causing pathways associated with oxidative phosphorylation to be repressed and for the fermentation pathway to be favoured (Postma *et al.*, 1989). Through these processes fermentation

conditions quickly become anaerobic and ethanolic, giving yeast a survival advantage over potential beer spoilage contaminants (Pfeiffer and Morley, 2014). While the fermentation environment is ideally suited to brewing yeasts, there are still many unwanted wild yeasts and bacteria such as *Brettanomyces sp.* and *Lactobacillus sp*. that can survive and spoil beer (Shimotsu *et al.*, 2015; Umegatani *et al.*, 2022). However these contaminants do not pose a risk to human health. As a result it is important that good brewing practices are followed in order to prevent spoilage contamination and allow the brewing yeast to ferment successfully. Uncontaminated fermentation will subsequently continue until all fermentable sugars have been consumed, thus producing beer.

1.3 Brewing Yeast

Yeast are single celled eukaryotic fungi, commonplace in many bioprocesses. Although various genera and species can be used, *Saccharomyces* yeasts are most commonly encountered in controlled, inoculated fermentations. Strains belonging to *S. cerevisiae* in particular are used for industrial bio-ethanol production, bio-pharmaceutical production and the food and drink industry (Donalies *et al.*, 2008; Edgardo *et al.*, 2008; Kim *et al*., 2015). There are many reasons for the popularity of *Saccharomyces* yeasts, predominantly due to their rapid growth, efficient yield, stress resistance and ease of genetic manipulation (Searle and Kirsop, 1979; Dequin, 2001). While budding yeast are susceptible to genetic variation, the asexual nature of reproduction gives a desirable degree of consistency and 'clonality', required to achieve predictable and uniform products (Steensels *et al.*, 2014).

1.3.1 Yeast history and origins

1.3.1.1 Yeast domestication

As described above (Section 1.1), beer was historically created using spontaneous fermentations. This practice can still be observed in farmhouse brewing (Garshol, 2020) and for the production of niche products (Lentz *et al.*, 2014). In such instances, a strategy is employed to encourage the ingress of natural flora, for example by using

a shallow open vessel known as a 'coolship' (Bokulich *et al*., 2012). This allows the inoculum for a spontaneous fermentation to be created, ultimately leading to the production of a 'wild ale' (defined as a sanitary drink with pleasant flavour), with relatively ease and reproducibility (Markowski, 2004). By transferring some of the fermented liquor to be used in subsequent fermentations the aspect of spontaneity was somewhat controlled and domesticated, resulting in a more consistent and improved final product (Lægreid, 2017). While relatively uncommon on an industrial scale at the current time, such practices have led to the domestication of yeast cultures. This development was likely triggered due to the realisation that a more consistent product was formed when a fermenter was not cleaned, as a result leaving remnants of the yeast culture at the bottom of the vessel (Preiss *et al.*, 2018). This allowed the 'best' yeast to be carried forwards and there is evidence that related practices were used to achieve a similar end point. For example, yeast cultures could be maintained on a cloth or piece of wood having been taken from the trub at the end of fermentation (Preiss *et al.*, 2018; Foster *et al.*, 2021). In this way, individual isolates showing potential for mass beer production have gradually become domesticated, with major brewing companies now closely guarding their own production strains. Although often considered a series of historical events, one recent example of this includes the group of Norwegian Kveik yeast, which originated in spontaneous fermentation processes and are gradually being adopted across the industry (Sicard and Legras, 2011; Gallone *et al.*, 2018; Preiss *et al.*, 2018).

1.3.1.2 The discovery of yeast as the origin of fermentation

It should be emphasised that the domestication of yeast largely occurred without any knowledge of its biological properties. In the 1700's and early 1800's the action of turning sweet sugary liquors into wine and beer was primarily researched by chemists, who believed there that the process was chemical in nature, rather than biological. The accepted understanding was that there were chemical reactions taking place that converted sugars into alcohol and carbon dioxide (Barnett, 1998). Aided by the discovery of microscopes in the mid 1800's, the physicist Charles Cagniard-Latour concluded that the fermentation activity in beer and wine was due to a living

organism, yeast, who proceeded to document and provide the first description of yeast cells budding as a mechanism of replication (Barnett, 1998). Physiologist Theodor Schwann also performed extensive research on yeast, concluding that yeast were responsible for utilising sugar as a carbon source and in return produced ethanol and carbon dioxide (Schwann, 1837; Thomas, 2017). It was during these experiments that Schwann also mentioned the necessity of nitrogenous substances in order for yeast to survive. He also described in detail the morphology of yeast using more modern microscopy techniques, concluding that yeast were in fact microscopic fungi (Schwann, 1837). At the time however, these findings were reputed to the extent that in 1839 the chemist J. von Liebig defiantly concluded that fermentation was a result of air mixing with plant juices containing sugar, resulting in putrefaction and decay of the vegetable matter, leading to alcohol production (Barnett, 1998). Eventually, some clarity was obtained through investigations into wine and beer fermentations, by Louis Pasteur (Fleming, 1947; Barnett, 2000; Verbelen and Delvaux, 2009). In these studies, Pasteur found that wine fermentations could not initiate if the fermentable liquor was boiled. It was also demonstrated that yeast could be found on the exterior surface of grapes. This fact, coupled with microscopy work, led to the acceptance that yeast were living organisms and needed to be alive to achieve fermentation of sugars in both beer and wine (Pasteur, 1879). This work, published in 1879, was named 'La Fermentation' and while resolving many of the mysteries of fermentation, also helped interest and acceptance into the field of microbiology (Pasteur, 1873, 1876, 1879).

In the 1880's, with further developments in microbiology techniques and the invention of solid media-poured agar plates by Robert Koch, attention began to turn to the isolation of pure yeast cultures, pioneered by Emil Hansen (Section 1.1) (Koch, 1908; Barnett and Lichtenthaler, 2001). In 1883, using microscopy and serial dilutions, Hansen was able to grow pure cultures from single cells, helping to standardise brewing practices at Carlsberg lager, for whom he was working (Barnett and Lichtenthaler, 2001). This practice gained widespread popularity very quickly and led to the rapid isolation of many different kinds of pure yeast cultures. Yeast isolates began to be collected and purified from many different types of fermentation, including sake, beer, wine and kefir (Libkind *et al.*, 2011; Ohya and Kashima, 2019).

Simultaneously, Emil Fischer led the development of enzyme research, discovering and isolating enzymes from yeast that could break down large sugars into simple fermentable sugars, and first coining the 'lock and key' principle of enzyme activity (Cramer, 1995; Barnett and Lichtenthaler, 2001).

1.4 Yeast strains used in brewing

The choice of yeast for beer fermentations largely depends on the product style and the flavour profile desired by the brewer. Currently, there are a broad spectrum of yeasts that are available for use in the brewing industry, primarily arising due to artificial selection over time for their desirable properties. However, brewing yeasts can be broadly divided into two categories: lager type and ale type strains. Both lager and ale yeasts are members of the *Saccharomyces* genus, but considerable genetic and physiological differences exist such that ale yeast are classified as *S. cerevisiae* and lager yeast belong to the species *S. pastorianus* (Vidgren *et al*., 2005; Gibson *et al.*, 2013).

1.4.1 Lager yeast strain derivation and characteristics

Lager yeasts are often referred to as 'bottom fermenting' due to their behaviour during industrial processes, and were initially classified as *Saccharomyces carlsbergensis* yeasts (Hansen, 1908). However, the true heritage of lager type yeasts has often been debated; at various stages lager yeast were believed to be hybrids result from a natural hybridization event between various species. Initial reports indicated that this may have included *S. cerevisiae* and *S. bayanus* parental strains (Martini and Martini, 1987; Nakao *et al.*, 2009). Similarly, sequence divergence in the *met2* gene, encoding a homoserine acetyltransferase, suggested a hybridization event involving the species *Saccharomyces monacensis* (Hansen and Kielland-Brandt, 1994). However, current consensus is that lager yeast have arisen as a result of a hybridization event between *S. cerevisiae* and *Saccharomyces eubayanus*, a cryotolerant strain recently discovered and isolated in Patagonia (Libkind *et al.*, 2011; Langdon *et al.*, 2019). Although some debate regarding how this actually occurred

does still remain, it is certainly agreed in most parts that lager yeast should be referred to as *S. pastorianus*.

Lager yeasts are typically used to ferment at 10-13°C and as such are often subject to a much slower rate of fermentation than their ale counterparts, requiring more time for flavour development and reduction of diacetyl (White and Zainasheff, 2010; Gibson *et al.*, 2013). Within the category of lager yeasts, there are two distinct groups based on genetic divergence, known as Saaz and Frohberg types, named after the locations in German where they were used historically. These strains differ significantly in their genetic makeup which is reflected in their physiological properties (Wendland, 2014). Saaz strains contain a significantly higher portion of the *S. eubyanus* genome than Frohberg type and have a greater cold tolerance, increased flocculation ability, lower ester production and reduced ethanol yields (Gibson *et al.*, 2013; Mertens *et al.*, 2015; Krogerus *et al.*, 2017). It is believed that the higher proportion of the cryotolerant *S. eubyanus* genome that is present in Saaz strains is the main cause of these phenotypic characteristics (Gibson *et al.*, 2013; Walther *et al.*, 2014).

1.4.2 Ale yeast strain derivation and characteristics

In contrast to lager yeasts, ale strains have emerged as a result of centuries of domestication and selection, rather than being a product of hybridization (Meier-Dörnberg *et al.*, 2017). Ale strains are often referred to as 'top cropping' due to their tendency to rise to the surface in open squared vessels, and belong to the species *S. cerevisiae*. Historically it is believed that artificial selection of these yeasts was achieved by repitching or 'backslopping', a method of using a small amount of fermented material to initiate a new fermentation (Gallone *et al.*, 2016; Spitaels *et al.*, 2017). Such practices led to the continuous growth of yeast cultures over time, with exposure to successive environments comprising a specific set of challenges or stresses. Over time this led to the gradual evolution of yeast populations, such that strains became more adapted to the beer brewing environment (Belloch *et al.*, 2008; Gallone *et al.*, 2016). Domestication, selection and forced evolution in this way has

resulted in *S. cerevisiae* ale yeast becoming a relatively diverse group of organisms. These are typically characterised as being polyploid and non-spore forming, similarities that they share with their lager counterparts (White and Zainasheff, 2010; Zhu *et al*., 2016; De Chiara *et al.*, 2022).

A further contributor to the diversity of *S. cerevisiae* yeasts, is that independent domestication events have occurred at multiple locations around the world, fuelling the emergence of a spectrum of strains with different physiological characteristics. It should be noted that the vast majority of yeasts used in biotechnology belong to this group, however, this is also true within the brewing category. Yeast used to produce ale products can be either highly flocculant or of very low flocculence, can impart fruity flavours or phenolic flavours, and can also range in the ability to ferment at high gravity and produce high alcohol beers (Lodolo *et al.*, 2008; Van Mulders *et al.*, 2010; White and Zainasheff, 2010; Mertens *et al.*, 2017). Irrespective, ale strains are commonly used to ferment at around 20°C, but can function well at up to 35°C when required (Lodolo *et al.*, 2008; White and Zainasheff, 2010; Hiralal *et al*., 2014).

1.4.3 The importance of yeast flavour production

Hop selection and the malt bill often define the overarching character of a beer. However, yeast are not only responsible for alcohol production, but also bring crucial flavour and mouthfeel attributes to a beer. Fusel alcohols (higher alcohols), esters and carbonyl compounds produced by yeasts can have a significant impact on the flavour and aroma of beer and can be detected in very small quantities, often near the flavour threshold (Kucharczyk, Żyła and Tuszyński, 2020). For example, ethyl acetate brings fruity and solvent like aromas to a beer and has a taste threshold of 25-30mg/L, while ethyl octanoate is responsible for apple and aniseed flavours and has a threshold of as little as 0.9-1mg/L (Harrison and Collins, 1968; Xu *et al.*, 2017). Other fusel alcohols such as isoamyl and amyl alcohols, responsible for alcoholic and banana flavours, have flavour thresholds of around 50mg/l (Pires *et al.*, 2014). The balance of flavours is key in achieving a palatable product and this can be used to good effect; fruity esters are often desired in ale fermentations particularly to complement the use of citrus hops (White and Zainasheff, 2010; Hiralal *et al*., 2014).

Yeast also function to remove certain aldehydes from wort leading to a reduction in 'grainy' characteristics over time. Similarly, yeast are responsible for the production and subsequent removal of the vicinal diketone known as diacetyl, a compound that imparts a butterscotch character to beer that must be eliminated prior to sale (Haukeli and Lie, 1978; Bamforth and Kanauchi, 2004). In some instances, alternative and specific flavour characteristics are desired. For example, some Belgian ale and German Weizen beers contain phenolic flavours which give them their famous character (Lentz, 2018). These are only produced by phenolic yeasts, and their presence is broadly considered to be negative in many other beer types. The volatility and potentially overpowering effect of off-flavours produced by brewing yeast mean that yeast strain selection, health and viability are of paramount importance to achieve a desired and consistent final product.

1.5 Yeast handling

1.5.1 Yeast Storage

Due to the importance of yeast to fermentation and product characteristics, it is essential that brewers maintain and store their individual yeast strains correctly. Although this is primarily to ensure the commercial protection of strains and to maintain both genetic integrity and microbiological purity, storage also forms a key part of yeast supply. Cultures can be propagated quickly from stocks in-house rather than relying on 3rd parties or open access culture banks. In order to store yeast cultures over a long term, cryoprotectants such as glycerol are used and yeast stocks are preserved in -80°C freezers or using liquid nitrogen with little to no effects on yeast health and genetic integrity (Dahmen *et al*., 1983; Bond, 2007; Gujjari *et al*., 2010; Thomasen *et al.*, 2011). This method provides an excellent way for brewers to effectively bank their yeast strains for many years. For short term storage, yeast cultures can be maintained at 4°C on nutrient agar slopes for up to six months without any major changes to yeast health and viability (Schu and Reith, 1995; Spencer and Spencer, 1996).

1.5.2 Propagation

In order to gain a sufficient quantity of healthy yeast for pitching, propagation must be performed in stages, maintaining sterility against infection throughout (Quain, 2006). Propagation profiles usually consist of first inoculating a small amount of oxygenated wort (10ml) with yeast from a stored slope. Depending on the yeast strain, this is grown for ~48 hrs before scaled up to 100ml for 48hrs, then 1L and so on until a sufficient amount of yeast is acquired for full scale fermentation. Yeast cell health and biomass are of importance during propagation and highly oxygenated (Cahill *et al*., 2000; Gibson *et al.*, 2008) and lower Plato worts are frequently used in order to promote rapid growth without exposure to stress (Cahill *et al.*, 2000). The stepwise nature of propagation also constantly provides the yeast with fresh nutrients without the buildup of harmful compounds such as ethanol, ensuring a high viability is achieved (Quain, 2006). Once enough viable yeast has been produced the pure culture can be pitched into the prepared wort and fermentation initiated.

1.6 Yeast and fermentation

1.6.1 Yeast central carbon metabolism and fermentation

As discussed, the primary function of brewing yeast is to convert the fermentable sugars in wort to ethanol, carbon dioxide and flavour compounds (Section 1.2). However, in order to do this, yeast not only need sugars, but also require assimilable nitrogen and other nutrients (O'Connor-Cox and Ingledew, 1989). Fortunately all-grain malts supply all of these nutrients, including essential vitamins and minerals such as riboflavin, inositol, zinc, calcium, sulphur and copper (White and Zainasheff, 2010; He *et al.*, 2014). The metabolic pathways in yeast utilise these vitamins and minerals to begin the production of essential proteins and enzymes for growth, replication and metabolism (Boulton *et al*., 2013). During the initial stages of fermentation, simple sugars such as glucose and fructose pass into the cell via permeases without the requirement for energy (Bisson *et al.*, 1993). As a result, these two sugars are taken up and utilised first, along with sucrose which is broken down into glucose and

fructose in the periplasmic space using the enzyme invertase (D'Amore *et al.,* 1989). Following this, disaccharides such as maltose and maltotriose are assimilated and converted into glucose units, all of which enter the glycolytic pathway (Salema-Oom *et al.*, 2005). The uptake of maltose and maltotriose is primarily via active transport, which requires energy in the form of ATP (Dietvorst *et al*., 2005; Gibson *et al.*, 2008). This means that there can be a delay before the concentration of these sugars declines in the substrate (Zastrow *et al.*, 2001; Alves-Jr *et al.*, 2007). Importantly, maltose and maltotriose are the predominant sugars in wort, so the necessity to ferment these sugars rapidly is crucial to achieve the finished product expediently. The uptake and utilisation of these disaccharides are dictated by genes of the *MAL* loci, an attribute that both ale and lager yeasts possess (Vidgren *et al*., 2005). However the disaccharide melibiose, also present in brewing wort albeit in very low concentrations, can only be assimilated by lager yeast (Dellweg *et al*., 1970). This is due to the presence of *MEL* genes (Sanchez *et al*., 2012), essential for breaking down melibiose into glucose and galactose which can then be metabolised (Vincent *et al.*, 1999). It should be noted that the presence of monosaccharides such as glucose also causes repression of maltose and maltotriose assimilation, another factor in the ordered pattern of sugar uptake by yeasts (Hazell and Attfield, 1999; Verstrepen *et al.*, 2004).

Interestingly brewing yeast are not able to utilise dextrins, complex carbohydrates that make up around 20-30% of the total wort sugars (Buckee and Hargitt, 1978; Willaert, 2007). These provide body and mouthfeel as well as some sweetness to the product. However, with some modern brewing techniques and current popular beer styles, these dextrins can sometimes become problematic (Bruner *et al*., 2021). Enzymes in hops can break down these dextrins into simple fermentable sugars. Due to the current trend in heavily dry hopped beers, large quantities of these enzymes have been linked to the re-initiation of fermentation with sudden availability of fermentable sugars for yeast cells (Kirkpatrick and Shellhammer, 2018).

The utilisation of wort sugars through the glycolytic pathway is key for alcohol production (Lentini *et al.*, 2003). The glycolysis pathway allows yeast to produce ATP under anaerobic or repressed conditions (Wiebe *et al.*, 2008; Tretyak *et al.*, 2020).

From glucose, a net yield of two ATP molecules are generated, along with 2 pyruvate and the generation of 2 NADH. Pyruvate is subsequently converted into ethanol via acetaldehyde, a process which also yields 2 molecules of carbon dioxide, while the cell regenerates NAD+ for further use in glycolysis (Beltran *et al.*, 2002; Barnett, 2003; White and Zainasheff, 2010). The need for NAD+ regeneration (often referred to as redox balance) can also be achieved by the conversion of dihydroxyacetone-phosphate to glycerol. From a brewing perspective this provides body to a beer, but from a cellular perspective, glycerol acts as a compatible solute that can protect the cell from osmotic stress (Taherzadeh *et al*., 2002; Zhao *et al*., 2015). Furthermore, glycerol is also an essential component of internal and external lipid membranes, crucial for anchoring membrane proteins, cell signalling, membrane trafficking and formation of droplets of storage lipid (Klug and Daum, 2014; Tesnière, 2019).

1.6.2 Yeast cell division and biomass production

Over the course of fermentation, the yeast population increases in biomass through cell division. Due to their polyploid/aneuploid nature, brewing yeast are unable to divide sexually and use asexual budding as the sole means of replication (Hittinger, 2013). This is advantageous in industrial situations since genetic uniformity, important to ensure process consistency, is easier to maintain (Phale, 2018). Once a cell has committed to the division cycle, complex actomyosin structures, septum arrangements and a chitin wall is formed prior to growth of a new daughter cell which grows or 'buds' out of the side of a mother. The process of cytokinesis describes the separation of the newly formed daughter cell, which becomes spatially independent from its mother with its own duplicated genetic material and cellular organelles (Wloka and Bi, 2012). Following cytokinesis, scar structures rich in chitin remain on the surface of mother cells, allowing an accurate indication of replicative cell age to be determined (Cabib and Bowers, 1971; Brewer *et al*., 1984; Powell et al., 2003a, 2003b). Although physiological changes have been linked to the ageing process in yeast, fundamentally daughter cells are 'reset' and are capable of living a full lifespan (Steffen *et al*., 2009).

The production of new cells (virgin/daughter) is important for the survival and growth of a cell culture, but the nature of the budding process means that population will comprise cells of a range of ages. Theoretically, populations should consist of 50% virgin cells, 25% first generation, 12.5% second generation and so on (Powell *et al*., 2003b). Given that division is asymmetric and that daughter cells are capable of exhibiting a lifespan typical of the yeast strain, the age distribution and frequency of budding within a population should theoretically mean that a yeast cell culture is immortal. From a practical brewing perspective this means that a culture can be used indefinitely. While this is certainly true in some instances, the presence of significant process related stress factors sometimes renders this impossible, due to a gradual deterioration in yeast quality as discussed below (Powell *et al*., 2003b).

1.7 Yeast viability and vitality

The quality of yeast used in industry is important, not only to achieve a complete fermentation, but also to maintain consistency between brews. To ensure the highest quality yeast are used, measurements of viability and vitality are commonly undertaken. Viability refers to the number of live cells in a population, usually expressed as a percentage. Conversely, vitality is a measure of health and metabolic ability, and is harder to quantify (Smart *et al.*, 1999; White and Zainasheff, 2010). Because of this, viability is often preferred as a simpler means of determining the fermentation capacity of yeast cells. In order for a population to function effectively, yeast viability should be maintained at above 90%; a high proportion of dead cells can cause sluggish fermentations, undesirable flavour development and foam collapse (Layfield and Sheppard, 2015; Thomson *et al*., 2015). It should be noted that, albeit more difficult to assess, cell populations with a low vitality may also cause issues as performance and consistency can be hampered even though viability may appear to be high (Guido *et al.*, 2004).

Determining population health (viability and vitality) is therefore important from a practical perspective. Rapid viability assays based on visualisation of cells are common

within the brewing industry. In particular a stain known as methylene blue is used, which is able to rapidly stain dead cells such that a ratio of live : dead individuals can be enumerated using a microscope and haemocytometer (Lee *et al.*, 1981). However below a viability of 90% the efficacy of this assay has been questioned and may be inaccurate (Smart *et al.*, 1999). Operator subjectivity has also led to the development of alternative stains (both brightfield and fluorescent) that can be used in automated systems. These give accurate and reproducible viability measurements, but at a much greater financial cost (Van Zandycke *et al.*, 2003; Thomson et al., 2015). Vitality measurements are more difficult to conduct and often more time consuming since they frequently involve analysis of traits directly related to fermentation performance. Furthermore, measuring 'health' is a more ambiguous proposition; reduced health can be caused by many different factors, which may or may not ultimately reflect performance. Typically to quantify vitality a measurement of metabolic ability is used, such as acidification-based tests, where the ability of a yeast population to acidify fresh media is used (Gabriel *et al.*, 2008). There have also been developments in fluorescent stains to measure vitality, but these often require expensive equipment and are rarely used in industry (Stewart, 2017). Recent developments have been made with high-throughput methods designed to monitor CO_2 production which may offer alternative strategies in the future (Michel *et al.*, 2020).

It should also be considered that key factors affect both viability and vitality including the conditions associated with fermentation and yeast storage. Brewing fermentations are intrinsically stressful, with factors that fluctuate and change throughout the fermentation. These environments put pressure on the yeast, which can result in reduced cell health and a loss in population viability (Jenkins *et al.*, 2003; Bleoanca *et al.*, 2013). Subsequently, it is evident that this can have a major impact on fermentation performance and reproducibility. The constant flux in environmental conditions means that yeast cell health and viability need to be maintained in order to allow fermentation to complete.

1.8 Stress Imposed on Yeast in Industrial Fermentations

Industrial brewing fermentations, similar to those producing ethanol, wine and distilled spirits, all comprise complex and fluctuating environments for yeast cells. In beer brewing fermentations, at the beginning of fermentation, yeast are exposed to high gravity wort, containing high concentrations of sugar (Casey and Ingledew, 1983). Oxygenation at this stage can also result in the generation of reactive oxygen species such as hydrogen peroxide and the superoxide anion (Verbelen *et al.*, 2009). During the course of fermentation, yeast can be challenged by fluctuations in temperature (O' Connor *et al.*, 2002), gradual changes (reductions) in nutrient concentration (Yoshida and Yoshimoto, 2015), and an increase in ethanol stress (Gibson *et al.*, 2007). Depending on the degree of mixing and/or the presence of agitation within the vessel, yeast may also have to overcome sub-environments exhbiting different intensities of each of these stresses (García *et al.*, 1994). In addition, when yeast cells flocculate and collect in the cone of the fermenter, a whole array of new stressful challenges are imposed (Powell and Smart, 2004). A summary of the different stresses that yeast are exposed to during the course of yeast handling can be seen in Figure 1.1. This image shows the profile of a typical lager fermentation, and the resulting increase and/or decrease of fermentation related stresses.

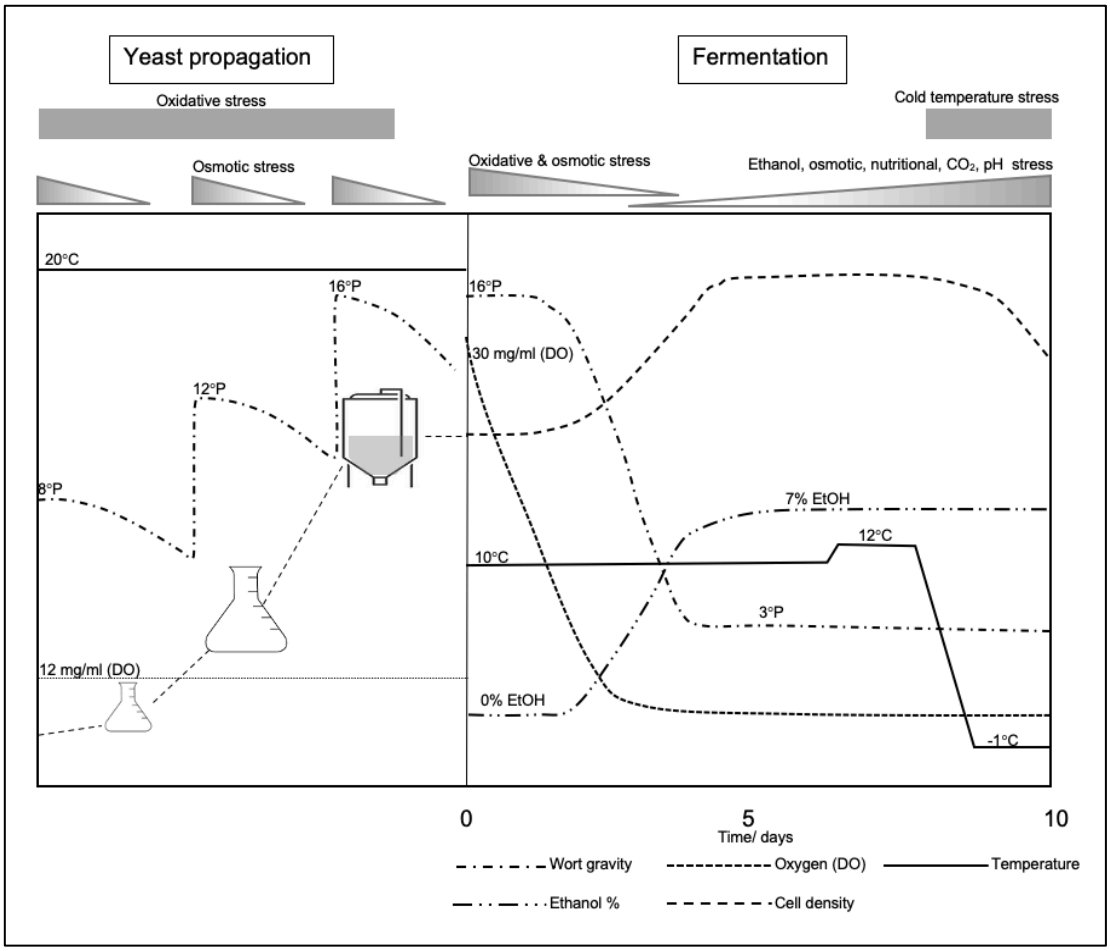

Figure 1.1. Lager fermentation stress fluctuation map. This diagram represents the fluctuations in stress factors that yeast are exposed to in a traditional lager yeast production, including propagation and fermentation stages. Adapted from Gibson et al., (2007), this diagram shows the multiple rounds of propagation and the relative osmotic and oxidative pressures that accompany each step. Furthermore the typical lager fermentation profile shows stressors such as those arising from DO, high gravities, ethanol etc. with example values for each stress to help visualise and quantify the fluctuation of intensities over time **(White and Zainasheff, 2010).** ***Note that yeast is usually stored for a period of time at cold temperatures, prior to being reused and hence these stress factors are experience multiple times by a typical brewing yeast slurry.***

1.8.1 Oxidative Stress

At the beginning of fermentation, wort oxygenation is essential for fast yeast growth but also for the production of sterols and fatty acids (David and Kirsop, 1973). However, oxygenation of wort can result in the generation of reactive oxygen species (ROS) such as hydrogen peroxide and $O_2^{\bullet-}$, increasing the amount of oxidative stress exerted on the cells (Martin *et al*., 2003; Verbelen *et al.*, 2009). These harmful ROS primarily originate from the electron transport chain and from activity associated with P450 enzymes, which can be found associated with internal membrane structures including the endoplasmic reticulum and the mitochondria in yeast (Costa and Moradas-Ferreira, 2001; Drăgan *et al*., 2006). The resultant oxidative stress can have adverse affects on yeast lipid biosynthesis, leading to oxidation of lipids and initiating a chain reaction, where radical attack on fatty acids produces lipid peroxides such as 13-hydroperoxylinoleic acid (Girotti, 1998). These lipid peroxides themselves break down to lipid hydroperoxides and further toxic compounds. Together these compounds affect lipid dependant structures such as membranes, cause damage to DNA, initiate cell cycle arrest and decrease coenzyme Q and glutathione content; the very compounds used to protect the cell from oxidative stress (Girotti, 1998; Costa and Moradas-Ferreira, 2001; Alic *et al.*, 2004). In addition, oxidative stress can also affect protein strucure; stressors such as hydrogen peroxide generate reactive carbonyl groups by reacting with amino acid residues and consequently damaging proteins (Costa and Moradas-Ferreira, 2001). ROS also oxidise 4Fe-4S clusters found in proteins, releasing free iron and leading to its accumulation that can cause damage to vacuoles (Corson *et al.*, 1999; Gomez *et al.*, 2014). This occurs in proteins such as pruvate decarboxyase and gylceraldehyde-3-phosphate dehydrogenase, essential for alcoholic fermentation and glycolysis respectively (Cabiscol *et al.*, 2000; Cyrne *et al.*, 2010). Finally oxidative stress can also cause damage to yeast DNA by base oxidation, resulting in strand breaks and intrachromosomal recombination, this is a particular problem in yeast mitochondria, which are especially susceptible due to the close priximity of radical generation (Bleoanca & Bahrim, 2013a; Brennan et al., 1994; Doudican et al., 2005; Farrugia & Balzan, 2012; Gibson et al., 2007).

1.8.2 Osmotic Stress

Osmotic stress occurs as a result of different concentrations of solutes across the cell membrane, often caused by high concentrations of sugars and nutrients. This scenario is inevitable in matrices such as brewing worts, where high solute concentrations put osmotic pressure on the cell, resulting in intracellular water diffusing out of the cell in order to achieve osmotic balance (Pátková *et al.*, 2000; Hohmann, 2002). This affect is exacerbated in many brewing fermentations, since the use of high gravity worts with elevated sugar concentrations reflects a growing trend due to economical benefits. Due to high concentrations of maltose, maltotriose and free amino nitrogen (FAN) (Saerens *et al.*, 2008) in these worts, osmotic pressures can become intense. The resulting osmotic stress induces changes within the yeast cell, leading to alterations in cell morphology, growth rate and often a decline in viability (Pratt *et al*., 2003). Yeast cells have to respond effectively in order to maintain turgor pressure and limit water efflux/influx in high gravity worts. This needs to be achieved to prevent the cell from shrinkage, bursting, cell envelope crenation, invagination and ultimately cell death (Casey and Ingledew, 1983; Kempf and Bremer, 1998; Pratt *et al*., 2003). The osmotic stress at the beginning of high gravity wort fermentations can also cause cells to acumulate intracellular ethanol, further damaging the cell and leading to more intensely hampered growth and fermentation rates (D'Amore *et al*., 1987). Furthermore, osmotic stress imposed on yeast can also affect the ability for the cell to take up and ferment sugars, impeding fermentation performance (Pátková *et al.*, 2000).

1.8.3 Carbon Dioxide Stress

As a fermentation proceeds, different challenges are imposed on yeast cells, including those associated with CO_2 stress, where increasing levels of carbon dioxide can cause inhibition of cell division (Norton and Krauss, 1972). Further to this, yeast fermentations are often operated under constant CO_2 pressure which, over a number of generations, could potentially have an impact on cell viability (Devantier *et al.*, 2005; Bleoanca and Bahrim, 2013). It has been suggested that stress derived from a

pressurised CO_2 environment can affect beer flavour balance, with evidence showing these conditions can cause slower decline/cellular uptake of the undesired vicinal diketones (Arcay-Ledezma and Slaughter, 1984). This may be due to the affect carbon dioxide has on cell lipid and fatty acid concentrations and so membrane composition (Castelli *et al*., 1969), potentially leading to an inability of cells to take up free diacteyl from solution through the membrane (Arcay-Ledezma and Slaughter, 1984).

1.8.4 Ethanol Stress

Traditional brewing fermentations expose yeast to ethanol concentrations of between 3-7%, which cells can withstand with relative ease (D'Amore and Stewart, 1987). However with intensification processes such as high gravity brewing now widely used, it is not uncommon for contemporary fermentations to finish with in excess of 10% ethanol (Odumeru *et al.*, 1992). Even though yeast produce ethanol during alcoholic fermentation, this product itself can be toxic to the cell. Ethanol stress can cause changes to the plasma membrane, induce a general 'heat shock stress' response (Piper, 1995), and can have widespread effects on cell growth rate, viability, and cellular constituents (Gibson et al., 2007; Odumeru et al., 1992). Furthermore, ethanol stress can also cause reduced cell volume and increased roughness of yeast cells (Canetta *et al*., 2006). Membrane permeability can also be increased due to lipid alterations (Marza *et al*., 2002), resulting in structural changes to plasma membrane fluidity. This can potentially allow the diffusion of undesirable compounds through the membrane, eventually leading to reduced growth and viability (Piper, 1995; Canetta *et al*., 2006). In addition, ethanol has been found to damage mitochondrial DNA, resulting in mitochondrial membrane alterations, and leading to the emergence of respiratory deficient (petite) cells (Castrejón et al., 2002; Gibson et al., 2008; Gibson et al., 2007). Interestingly, it has been found that strains with improved resistance to ethanol (and hence reduced occurrence of petite mutants) exhibit a high ergosterol/phospholipid ratio and an elevated long chain fatty acid content. It is believed that this helps improve the fluidity of the cell membrane, demonstrating the importance of membrane structure and integrity to resisting ethanol stress (Chi and Arneborg, 1999; Gibson *et al.*, 2007).

1.8.5 Other fermentation stresses

During fermentation, yeast are also challenged with a range of nutrient based stresses, such as carbohydrate limitation and amino acid/assimable nitrogen limitation (Gibson et al., 2007). Nutrient stress can cause the inhibition of ribosomal RNA synthesis, and sluggish fermentations (Gibson *et al.*, 2007; Zhao and Bai, 2012; Bleoanca and Bahrim, 2013a). In addition, yeast cells exposed to a depletion of fermentable sugars and FAN can enter an arrested proliferative state with increased incidents of autophagy (Abeliovich and Klionsky, 2001; Bleoanca and Bahrim, 2013a). As a fermentation proceeds and the cell population assimilates the main carbon source, it is not uncommon for the organism to begin utilising a secondary carbon source (Walker and Stewart, 2016). In brewing yeast fermentations this is expected and it is accepted that maltose and glucose are readily used as primary carbon sources, followed by maltotriose which requires slightly different cellular machinery (Zheng *et al.*, 1994; White and Zainasheff, 2010). This shift in metabolism can potentially cause stress and, furthermore, once all fermentable carbon sources are consumed, brewing yeast can potentially metabolise ethanol and glycerol via a diauxic shift, although this is undesirable from a production perspective and can lead to further cell damage (Orlandi *et al.*, 2013).

During fermentation, the pH of the medium reduces due to the generation of organic acids and proton efflux by yeast during sugar uptake. A typical final pH of between 3.5-4.8 is an environment that healthy or adapted yeast are able to survive within. However, while a low pH conveniently makes beer a hostile environment to most contaminants, it can also be stressful for some yeast cells within a population (Suzuki, 2011). Low pH can cause alterations to yeast cell growth rate and can have a sizeable effect on gene expression, causing increased expression in genes for cell wall synthesis, carbohydrate metabolism and redox metabolism (Gibson et al., 2007; Kapteyn et al., 2001). Finally, cold temperatures are frequently encountered during yeast handling, especially when yeast is stored prior to re-use. Under storage conditions, yeast cultures are maintained in an inactive state at ~2-4°C to minimise viability loss. Lager yeast may also be exposed to relatively low temperatures (~10-

14°C) during production itself (Gibson et al., 2013). These temperatures may lead to stress that can cause an array of membrane, lipid and protein modifications, and can increase the quantity of storage solutes such as trehalose (D'Amore *et al*., 1991; Diniz-Mendes *et al.*, 1999; Bleoanca and Bahrim, 2013a).

1.9 The yeast stress response

The way in which yeast cells respond to brewing-related stresses can dictate the strain selected for a particular type of fermentation. Domestication has resulted in highly resistant strains capable of withstanding conditions that many other microorganisms cannot (Diezmann and Dietrich, 2009; Gallone *et al.*, 2016). The ability to do this is not only important to allow fermentation to come to completion, but analysis of the mechanisms that cells use to respond to stress can allow us to predict the efficiency and consistency of a yeast strain.

1.9.1 Genotypic stress response

In response to brewing fermentation stress, yeast are able to initiate a range of genetic responses. One such response is the expression of genes that encode heat shock proteins, of which there are more than 10 in the yeast genome, all with vastly different functions (Brosnan *et al.*, 2000). Hsp70, Hsp90, Hsp104 and Hsp26 are all essential in responding to stress, however there are others with functions that are not completely understood, but are likely to be of equal importance such as Hsp32 and Hsp33 (Verghese *et al.*, 2012). Many of these heat shock proteins are molecular chaperones (Mager and Ferreira, 1993), responsible for maintaining a balance in the required proteins inside the cell, aiding in protein folding, translocation and cytoplasmic anchoring (Mager and Ferreira, 1993; Verghese *et al.*, 2012). These attributes are important in times of stress, when protein misfolding, aggregation and protein translocation are all hampered (Miyagawa *et al.*, 2014). Hsp70, the highly conserved 70kDa family of heat shock proteins aids in the re-folding of misfolded proteins (Verghese *et al.*, 2012; Lotz *et al.*, 2019). Hsp90 proteins specialise in the

assembly of macromolecular structures and maturation of proteins such as transcription factors and kinases (Yang *et al.*, 2006). Hsp104 recognises misfolded aggregates of proteins, effectively separating these aggregates and facilitating the correct folding (Verghese *et al.*, 2012). All of these protein chaperone functions are essential to maintain thermotolerance (and general stress response) in yeast, for example it has been shown that *hsp104* gene mutants are unable to acquire adequate tolerance to heat stress (Sanchez and Lindquist, 1990; Lindquist and Kim, 1996).

HSPs are not only useful against heat stress but also provide general protection against other challenges encountered in brewing fermentations, such as ethanol and osmotic stress (Gibson *et al.*, 2007; Bleoanca and Bahrim, 2013b) via similar mechanisms. Interestingly the expression of genes in response to 'specific stresses', often leads to 'cross protection', where resistance to other stresses is also acquired. A primary example is the yeast General Stress Response (GSR) which is elicited in part due to Stress Response Elements (STRE) in promoters of a range of genes necessary for initiating specific stress responses (Estruch, 2000). The genes *m*sn2 and *msn4* encode homologous zinc finger proteins (Lee *et al.*, 2008) which are an essential component of the general stress response, responsible for binding STRE elements and activating expression of the relevant gene. For example *HSP12* and *hsp104* are among the many genes which have promoters containing STRE elements and where expression relies in the activity of Msn proteins (Martínez-Pastor *et al.*, 1996; Estruch, 2000; Watanabe *et al.*, 2009). Upon exposure to stress, the Msn2 protein is translocated from the cell cytoplasm to the nucleus to aid in stress response gene expression (Hao and O'Shea, 2011).

Another similar element within the GSR is triggered via the cAMP-protein kinase A (PKA) pathway and the *ras* genes. *Ras* genes encode GTP binding proteins which can transfer signals from the external environment and convert them into intracellular response, often utilising the cAMP-PKA cascade (Thevelein and De Winde, 1999; Estruch, 2000; Tamaki, 2007). In yeast these signalling cascades are constitutively active and negatively regulate the expression of genes involved in stress response such as *msn2/4* and trehalose accumulation (Stanhill *et al.*, 1999; Estruch, 2000). Upon

exposure to stress, the signalling cascade is repressed and therefore releases control over the stress response (Estruch, 2000). Similarly, the high osmolarity glycerol response (HOG) pathway also utilises a kinase cascade signalling type system, one of which is the Hog1p protein kinase found to accumulate and interact with Msn2/4 (Estruch, 2000; Alepuz *et al.*, 2001; Dihazi *et al*., 2004). The action of Hog1p and, indeed, the entire HOG pathway are important in the expression of the enzymes GPD1 and GPP2, essential for catalysing the production of glycerol in response to osmotic stress (Tamás *et al.*, 1999). This allows the cell to produce and use glycerol as a compatible solute; when low cellular water content occurs as a result of osmotic stress and turgor pressures, this allows intracellular function to continue (Nevoigt and Stahl, 1997).

Finally, the Environmental Stress Response (ESR) in yeast represents a network regulated by the expression and repression of over 900 genes (Gasch, 2003). This complex network is controlled by components such as the transcription factor Msn2, genes encoding ribosomal proteins (RP) and ribosome biogenesis (RIBI) genes, and is essential for yeast cells to respond to a wide variety of stresses such as temperature shock, oxidative stress, nutrient depletion and salt stress (Gasch *et al.*, 2000, 2017). As expected, genes that induce the environmetal stress response increase in expression when stress is imposed, coupled with reduced RP and RIBI gene expression. When stress is removed, the opposite profile of gene expression is observed.

The fluctuating and intense nature of the stresses observed in brewing fermentations means that yeast cells need to have a degree of flexibility in their stress response. To survive, it is essential to have the ability to tailor the response to the stress, while also prioristising growth and cell division. As a result, microorganisms such as yeast may utilise survival strategies that lead to the rise of distinct sub-populations of cells with unique phenotypes. This functions to allow fractions within a population to work distinctlty, but synergistically, increasing the chances of survival of the culture. The variation in ability within these sub-populations is reffered to as ‘heterogeniety’ and will be discussed further in the following sections (Sumner and Avery, 2002; Gasch *et al.*, 2017).

1.9.2 Cell protectants

Yeast have adapted specific responses to the different stresses they are exposed to, including producing compounds for their protective properties. The stress protectant trehalose, for example, is accumulated in response to ethanol, osmotic, heat and oxidative stress (Hottiger *et al*., 1987; Majara *et al*., 1996; Divate *et al.*, 2016). Trehalose acts by increasing the stability of the plasma membrane; in the case of ethanol stress this prevents ethanol induced membrane leakage (Mansure *et al.*, 1994). Further to this it can also be used as a reserve carbohydrate when presented with nutrient depletion, or due to inability to take up fermentable sugars as a result of osmotic pressures. This forms part of the survival strategy, where the reserve carbohydrate can be utilised (Panek, 1963). In a similar way, glycogen is known to act as a reserve energy supply, essential for maintaining the pool of ATP within in the cell and provide substrates for protein production (François and Parrou, 2001) and rapid energy production in response to a variety of stresses including osmotic, heat and oxidative (Smith *et al*., 1998; Possik *et al.*, 2015). Furthermore, the osmolyte glycerol is also essential in the maintenance of a healthy turgor pressure in response to osmotic stress and for its use as a compatible solute, which can be stored and used as a reserve carbon source (Hohmann, 2002).

1.10 Heterogeneous strategies for survival

1.10.1 Bet Hedging

A widely accepted theory explaining the benefits of innate variation within a population is that of 'bet hedging'. Bet hedging acts as a mechanism for survival by trading population fitness for an increased capacity for adaptation. The strategy results in reduced mean fitness in a population, with the appearance of low occurrence phenotypes. Under 'normal' conditions these would be at a disadvantage compared to the main population (Grimbergen *et al.*, 2015; Martins and Locke, 2015). Such disadvantages include artefacts such as reduced growth or reproduction rates, resulting from different metabolic or gene expression profiles (Veening *et al.*, 2008). However, this mode of activity can become preferential when the environment changes, such that the main population no longer has a fitness advantage (Philippi and Seger, 1989). For example, it has been shown that an increase in environmental stress can lead to the survival of low-occurrence sub-populations, which have a fitness advantage due to their ability to proliferate under these new conditions thus allowing the population to thrive (Philippi and Seger, 1989; Levy *et al*., 2012). Philippi and Seger (1989), have described this bet hedging strategy with the adage "don't put all your eggs in one basket". This form of diversity is recognised in yeast populations, for example natural variation between cells forms the basis of many traditional 'breeding' techniques, such as those which have been employed to increase sugar utilisation efficiency in yeast (Attfield and Bell, 2006). *S. cerevisiae* cells typically cannot grow with xylose as as sole carbon source, however through utilising the bet-hedging nature of *S. cerevisiae* and harnessing natural variation, populations of cells can be naturally selected over an extended breeding period that have the ability to utilise xylose. This approach has been desmonstrated to be an effecitive strategy for developing yeast strains that are valuable in bioenergy processes (Attfield and Bell, 2006). Utilising natural and innate varaition in a bet hedging survival strategy may subsequently result in the survival of a group of cells with improved adaptations to adverse conditions, in a Darwinian 'survival of the fitest' mode of population evolution. This can only be achieved with initial population variation. While bet hedging specifically is difficult to

measure and quantify this may be the justification for innate heterogeneity seen in cell populations.

1.10.2 Division of Labour

Another survival strategy leading to heterogeneity within a population is 'division of labour'. Similar to bet hedging, this strategy can be used to resist changes in the environment. An example of this has been shown through studies regarding the formation of biofilms. Diversity in gene expression profiles between cells allows some individuals to specialize in biofilm production, while other cell types may specialise in nutrient acquisition, growth and/or environmental stress resistance (Kearns, 2008). In this way, cells act to divide the 'responsibilities' (labour) of the culture, increasing the survival chances of the overall population. This type of division of labour is relatively common in yeast populations, where *S. cerevisiae* cell populations can display a range of phenotypes with differing abilities. This is not only related to biofilm formation, but also reflected in differences with respect of quiescence, apoptosis and flocculation (Wloch-Salamon *et al*., 2017; Opalek and Wloch-Salamon, 2020). Division of labour allows individual cells to carry out specific tasks to the benefit of the whole population, rather than switching between tasks which could ultimately act as an energetic and metabolic hindrance (van Gestel *et al*., 2015).

1.10.3 Specialist vs Generalist

Both bet hedging and division of labour strategies can broadly be considered to fall under the umbrella heading of 'specialist vs generalist'. This overarching definition dictates that 'specialist' cells within a population are adapted for coping in a single, stable and favourable environment with relatively low metabolic and energetic cost (Futuyma and Moreno, 1988). However, when conditions change and a stress is imposed on the population, such cells may lack the necessary adaptations to persist and survive (Van Tienderen, 1991). In contrast, 'generalist' cells are able to exploit multiple energy sources and habitats, since they are able to react to changing and stressful environments due to the presence of cells with a broader range of stress

response adaptations (Jarosz *et al.*, 2014; Rodrigues, 2017). Heterogeneity observed in populations harbouring a high number of generalist cells are those that typically employ strategies such as 'bet hedging', 'division of labour', or a combination of the two. However, often this requires a cost, typically paid with reduced growth rate (Van Tienderen, 1991). In a heterogeneous and fluctuating environment, cell populations evolve towards a compromise between the optimum constitution for a specific environment and being able to cater for all environmental eventualities. This results in a generalist and heterogeneous population, able to react and survive under changing environmental conditions (Futuyma and Moreno, 1988; Van Tienderen, 1991). Examples of each of these strategies and the overall 'specialist vs generalist' strategies of survival can be seen in Figure 1.2. A prime example of such behaviour is that seen in wild yeast populations inhabiting highly polluted sites. In these environments, various pollutants such as lead and copper are present at toxic concentrations. It has been shown that this results in the occurrence of highly heterogeneous populations of *Candida sake*, with varying abilities to withstand the pollutant stress from cell to cell (Holland *et al.*, 2014). In the study conducted by Holland et al., (2014), when comparing *C. sake* populations taken from polluted sites to populations taken from unpolluted sites, it became apparent that latter conferred a much *lower* degree of cell-to-cell heterogeneity in response to pollutant stress when compared to those taken from polluted sites, which showed *greater* diversity (heterogeneity).

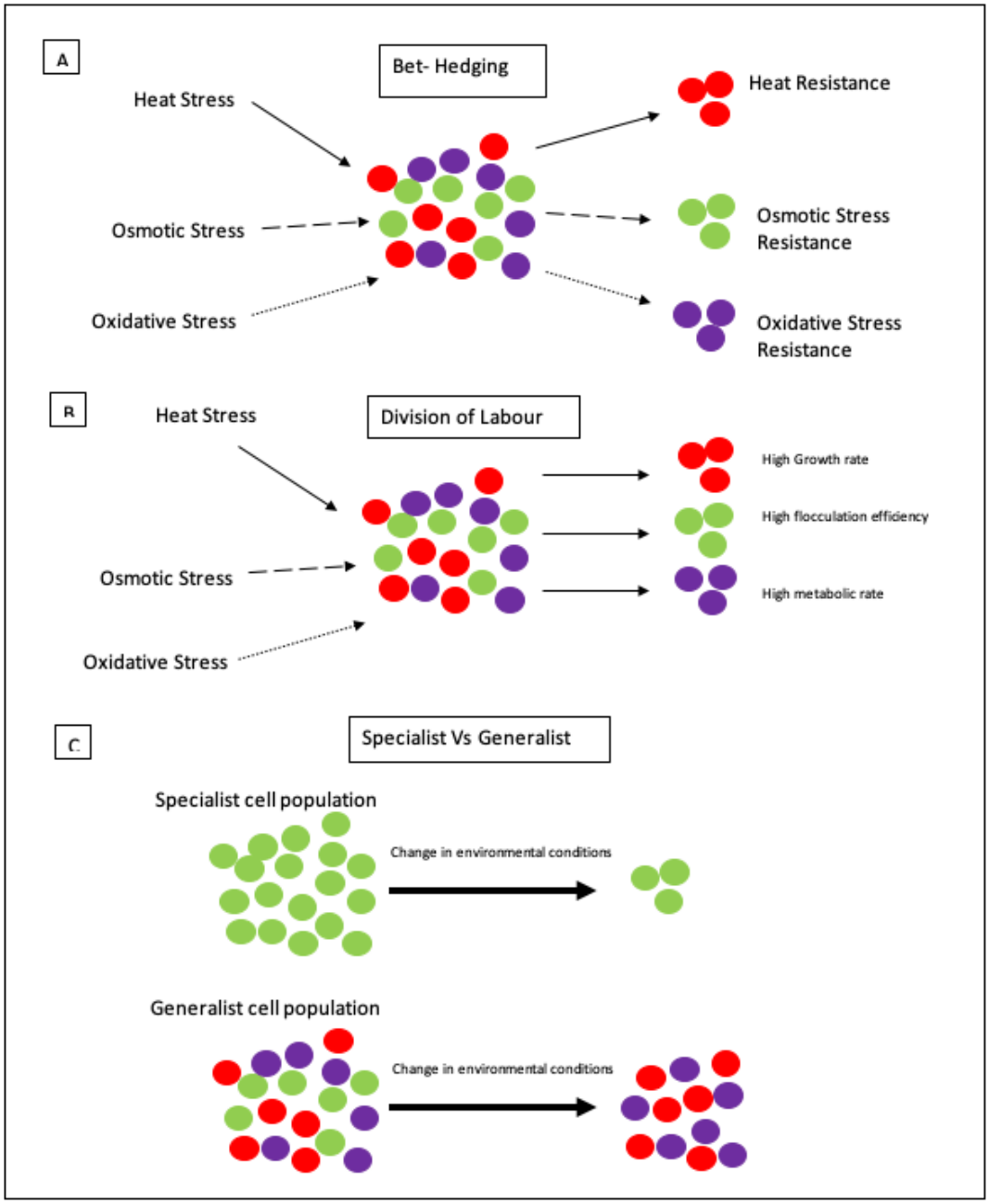

Figure 1.2. Heterogeneous population survival strategies. Displayed here are three strategies of survival that cause heterogeneity in microbial populations. Each coloured dot resembles a cell in a population. The first strategy A: Bet Hedging shows a highly heterogeneous cell population where cells have varying abilities to withstand different environmental stresses (in this example heat, osmotic and oxidative stress). Hedging their bets in normal conditions and in the event stress is imposed, the sub-population of cells that are more adapted to resisting the particular stress are able to survive. The second strategy B: Division of labour shows cells can have a pre-determined role within the population, for example some may have a high growth rate, others a high flocculation efficiency and some with increased metabolism. Effectively dividing the labour of the overall population to smaller sub-populations which allows cells to carry out their function especially when stress is

imposed, without having to expend energy switching physiological states. Finally, C: Specialist vs Generalist demonstrates two mechanisms of functioning, specialist populations contain cells that are ideally adapted for one environment, so that they grow and thrive in this environment in the most efficient and least costly way. However, if the environment changes, cells within the population will not be able to respond and may lead to cell death. The generalist approach sees small groups of cells all with ranging abilities to survive at varying levels of stress, similar to bet hedging, this therefore means when the environment does change, sub-populations with the fitness advantage to resist the new conditions will allow the population to continue to survive and grow, with only those non-adapted cells dying.

1.11 Genotypic vs Phenotypic variation

1.11.1 Genetic variation present in brewing yeast populations

Due to the asexual nature of brewing yeast, with budding as the predominant form of growth, it is expected that a yeast population should have a large degree of genetic uniformity. However, it has been known for many years that variants are relatively common in industrial cultures. Furthermore, developments in molecular techniques such as DNA fingerprinting and genome sequencing have allowed these to be characterised in order to reveal the extent and nature of genetic variation in brewing cultures. The genetic variation observed in brewing yeasts is often a result of chromosome rearrangements which can be caused by deletions, duplications and migration of transposable elements (Adams *et al.*, 1992; Longo and Vezinhet, 1993; Casey, 1996). One example stems from research which used chromosome fingerprinting to analyse a brewing yeast stock maintained over a period from 1958-1985, and which was subsequently found to exhibit at least 7 different chromosome fingerprints spanning these 30 years (Casey, 1996). Further to this, extended cultivations of yeast, using cultures of very high generational age (100+) have also shown large chromosome variation in length, arrangements and insertions (Adams *et al.*, 1992; Longo and Vezinhet, 1993). Another example of yeast genetic variation is that seen in respiratory deficient cells (or petite mutants) (Šilhánková *et al.*, 1970) these cells have either damaged mitochondrial DNA, or no mitochondria at all (Nagley and Linnane, 1970; Castrejón *et al.*, 2002; Doudican *et al.*, 2005; Gibson *et al.*, 2007;

Gibson *et al*., 2008; Jenkins *et al.*, 2009). These mutations are caused by prolonged exposure to stresses such as ethanol and oxidative stress which result in mtDNA damage and genetic variation (Doudican *et al.*, 2005; Gibson *et al.*, 2007).

Exposing yeast to stresses continually, as seen during serial re-pitching, can increase the likelihood of mutation, resulting in chromosome polymorphisms developing over time. This phenomenon occurs gradually and is often referred to as genetic drift (Powell and Diacetis, 2007; Meier-Dörnberg *et al.*, 2017). Such mutations can be random in nature, caused by transposable element integration and mitotic segregation events (Lewis *et al*., 1976), however they are also likely to be related to fermentation-specific phenotypes such as flocculation. Repeat sequences within flocculation genes have been suggested to act as primary sites for genetic change, which is reflected in the many reports of increased flocculation during serial re-pitching (Sato *et al*., 2001; Large *et al.*, 2020). Irrespective, it has been shown that under ideal conditions, yeast populations will experience genetic change after around 275 generations (Wilke and Adams, 1992), although under stressful conditions, they occur at the more advanced rate of 30-40 generations.

The occurrence of mutations and genetic drift are also dependent on the yeast strain used. Examples of this can be seen in the respiratory deficient petite mutations where both frequency and natural propensity of mutations can vary largely between strains (Jenkins *et al.*, 2009). In addition, in a study by Thorne (1968), the bottom fermenting yeast *Saccharomyces carlsbergenesis* (*pastorianus)* was used in a continuous fermentation lasting 9 months. Following this time period, 48 single cells were obtained by micromanipulation and grown into cell cultures. These isolates showed mutant populations with differing flocculation abilities, metabolism, morphology, flavour production and fermentation performance as a direct result of genetic mutation and genetic drift (Thorne, 1968). Contrary to this, Powell and Diacetis (2007) conducted a study whereby cultures of 98 and 135 generations (serial re-pitchings) were analysed for genetic differences in δ regions using RAPD-PCR fingerprinting, and in frequency and location of transposons in an attempt to detect genetic variation compared to initial stocks. However, no mutations or genetic drift could be detected

(Powell and Diacetis, 2007), indicating that intrinsic strain stability and industrial conditions are important in determining the potential for genetic drift.

1.11.2 Detecting genetic variation

As mentioned previously, there are many methods used to detect variants within a yeast population. At the basic level, colony morphology variants can be visualised using Wallerstein Laboratory Media (Pallmann *et al.*, 2001; Powell and Diacetis, 2007). Similarly, variants in yeast populations caused by the occurrence of petite mutations can be detected by plating cells on YEPG media (Lee *et al.*, 2001). This allows detection of petites as they are unable to metabolise glycerol, so are unable to grow on these plates (or form extremely small colonies after an initial period of growth) (Lee *et al.*, 2001). A more precise method of detecting petites can also be performed using the triphenyl tetrazolium chloride (TTC) overlay technique, where colonies grown on YDP agar are overlaid using TTC. Actively respiring cells metabolise the TTC, producing formazan and red pigmentation, while respiratory deficient petite mutants cannot and remain a normal white colour (Boeker-Schmitt *et al*., 1982; Barclay *et al.*, 2001; Lee *et al.*, 2001).

Chromosome variation and genetic drift between cells in a population can also be detected with relative ease. Using pulsed-field gel electrophoresis, variation in chromosome length can be detected between isolates (Hage and Houseley, 2013). Genome-wide changes can also be identified using amplified fragment length polymorphisms, using restriction enzymes such as EcoRI, PstI and SalI which cut purified DNA at specific restriction sites, resulting in fragments of DNA that can be visualised by gel electrophoresis. These bands form a restriction profile that can be compared between isolates highlighting genetic drift and can be combined with analysis of specific targets such as transposons (Wightman *et al*., 1996).

Improvements in sequencing technology also allow for a more targeted approach where specific regions of the genome can be analysed for sequence divergence, in

addition to whole genome sequencing which is now possible in relatively high throughput (Nakao *et al.*, 2009).

A similar technique aimed at examining the frequency and location of repeat sequences within the genome is by PCR amplification of interdelta sequences within the yeast DNA. These sequences of repetitive DNA flank transposons and the number, location and length is unique to each yeast strain (Xufre *et al.*, 2011). For example, in the yeast strain S288C, around 300 interdelta regions are present, displaying varying lengths from 70bp-1kb. Analysis of these produces a unique strain-specific fingerprint when amplicons are separated by gel electrophoresis (Legras and Karst, 2003). This technique also provides a useful tool for analysis of genetic change, since both gross- and small-scale changes to the genome are reflected in the location of transposons and can be rapidly detected by fingerprint changes using gel electrophoresis.

1.12 Sources of phenotypic heterogeneity in industrial yeast

While genetic variants can be found within brewing yeast populations, the frequency is not as high as might be expected, primarily as a result of genetic stability and favourable cell attributes in relation to stress factors encountered (Powell and Diacetis, 2007; Ekberg *et al.*, 2013). As mentioned previously, genetic uniformity is expected throughout brewing yeast populations, due to the asexual nature of division in production strains. However, it is perhaps underappreciated that a truly homogenous culture will rarely be seen, with physiological differences arising due to cell-to-cell variation in factors such as size, age and viability. These can in turn cause variation between isogenic cells, a phenomenon referred to as phenotypic heterogeneity. For example, variation in size and age also influences the flocculation ability of yeast cells resulting in a range of flocculation efficiencies. Furthermore, the ability to utilise various wort sugars is closely linked to cell age and size (Powell et al., 2003), demonstrating that while genetic homogeneity can be achieved, true population physiological heterogeneity is seldom observed.

1.12.1 Viability and vitality

At the very basic level, perhaps the most obvious marker of heterogeneity in industrial yeast cultures is related to population viability. The fact that a population comprises live and dead cells indicates that there is variation between cells, a phenomenon that is likely to be exacerbated in processes where high levels of stress are encountered, such as high gravity brewing (Casey *et al*., 1984; Saldi *et al.*, 2014). While heterogeneity in viability can be determined easily, vitality is much harder. As such the presence of heterogeneity in brewing yeast cell 'activity' is a relatively unknown attribute beyond indirect analyses. However, an example of how this can impact fermentation performance has been demonstrated in *Lactobacilli* yoghurt fermentations used in the production of lactic acid, a useful industry product (Komesu *et al.*, 2017). When *Lactobacilli* are exposed to a variety of environmental stresses such as pH stress and oxygen limitation (relevant to conditions experienced in beer fermentations), Alonso et al., (2014) provided evidence that 3 distinct populations of cells could be obtained with varying degrees of health: metabollically active cells, damaged cells and finally dead cells. Interestingly, the quantity of 'damaged' and 'dead' cells in these yoghurt fermentations made up a sizeable proportion of the entire population; even at the beginning of fermentation they comprise up to 46% of the biomass (Alonso *et al.*, 2014). While such extremes are unlikely to be encountered in brewing yeast, it is evident that such a loss of population activity could hugely hamper process efficiency and performance.

1.12.2 Cell Age

Another primary form of phenotypic heterogeneity within any population is related to an individual's age. This is true for most microorganisms, including yeast used for brewing, where previous studies have shown a number of age-related changes over time (Powell et al., 2003). Historically, analysing the impact of age at the cellular level was problematic, however single cell technologies such as flow cytometry and fluorescence microscopy are able to facilitate this form of analysis. Fortuitously, yeast cell age is determined by replicative rather than chronological lifespan and can be

determined easily by monitoring the number of replications a cell has undertaken. This can be achieved in several ways, although a simple mechanism for this is to analyse for the presence of bud scars (Beran, 1968). Bud scars remain on the surface of mother cells following division (Section 1.6.2) and can be visualised through staining and enumeration to reveal the age of an individual cell (Horisberger and Rosset, 1976). Utilising similar techniques, variation in brewing yeast cell age has been shown to occur (Barker and Smart, 1996). Furthermore, it has been demonstrated that age determines key physiological properties in yeast. Interestingly, young does not always mean 'good' and it has been shown the newly produced daughter cells are less efficient at utilising wort sugars compared to mixed age populations (Powell et al., 2003). While this example is likely to be due to cell cycle development rather an indication of any defects *per se*, it is interesting to note that cell size, metabolism, mitochondrial DNA stability and stress response can all vary from cell-to-cell due to differences in age (Costa & Moradas-Ferreira, 2001; Laun et al., 2001; Powell et al., 2003).

From a practical brewing perspective, cell age has also been shown to have an impact on flocculation efficiency, with older cells appearing to be more flocculant. As above, this may be attributed to the relative morphologies of daughter and aged cells; newly produced individuals exhibit a smoother and less hydrophobic cell surface, whereas older cells are bigger (Rockenfeller and Madeo, 2008), more wrinkled, more hydrophobic (Jin *et al.*, 2001) and consequently more flocculant (Powell et al., 2003). Variation in replicative age has also been shown to correlate with internal pH, where cells that have produced the most daughter cells have a higher intracellular pH (Imai and Ohno, 1995). Internal pH has crucial links to membrane transport systems, yeast growth and metabolic abilities (Rowe *et al*., 1994; Imai and Ohno, 1995). Therefore, variation in proliferative age and internal pH between individual cells could potentially result in cells with a spectrum of physiological properties.

1.12.3 Brewing yeast collection and cropping

During fermentation, yeast cells collect in the cone of a fermenter due to the processes of flocculation and sedimentation. In this environment, cells are exposed to a variety of stresses including temperature fluctuations, ethanol and nutrient deprevation. In a previous study, Powell and Smart (2004), found that yeast cells in the cone resided in a highly variable environment. For example, alcohol was observed to be present in the range of 6.5-8.5%, and specific gravity varied between 1.006-1.009 at different locations. In addition, parameters such as pH and temperature were also heterogeneous, creating a series of micro-environments, poetentially imposing varying levels of stress on a population. The authors argued that these intensified micro-environments had the potential to lead to the accumulation of distinct sub-populations of cells. The same authors also demonstrated that sub-populations with different attributes and abilities did indeed exist, including differences to flocculation, fermentation performance and flavour production (Powell *et al*., 2002). This could therefore impact batch-to-batch consistency if continual selection of sub-populations over time were cropped and subsequently re-pitched (Powell & Smart, 2004).

Interestingly, variation in cell age has also been reported within the yeast cone associated with brewing fermentations (Section 1.12.2). Yeast cells taken from the cone of a conical fermenter showed a range from 0-6 bud scars (Powell and Smart, 2004; Kuřec *et al.*, 2009). This trend was also linked to cell size, which varied considerably within the population (Powell & Smart, 2004). Furthermore, yeast cells throughout the cone displayed heterogeneity in flocculation efficiency, with yeast at the bottom of the cone being ~77% flocculent, those in the middle ~87% and those at the top ~79%. Interestingly the distribution of flocculation efficiency throughout the cone may correlate with the distribution of cell age, with larger and older cells being located in the middle. This not only provides further evidence that cell age may be linked to fermentation properties (Bony et al., 1998; Powell & Smart, 2004), but is also indicative of the presence of variation within industrial brewing yeast cultures in general.

1.12.4 Membrane structure

The membrane composition of a yeast cell is integral to many environmental stress factors, as described in Section 1.9 parameters such as lipid composition (Swan and Watson, 1999), membrane fluidity (Swan and Watson, 1999) and membrane potential (Moskvina *et al*., 1999) all impact a cells resistance to stress. Interestingly, variation in membrane composition has been reported between yeast cells (Guyot *et al.*, 2015). Due to the relationship between membrane composition and stress resistance, this variation may have a major impact on a cells capacity to survive during industrial fermentations, which may affect performance during industrial fermentations.

1.12.5 Gene expression

Phenotypic heterogeneity is inevitably a consequence of a variety of complex effectors at the molecular level, as well as being impacted by environmental conditions. Variation in cell-to-cell gene expression, termed 'stochastic gene expression', can result from differences in transcription, translation and feedback mechanisms (Guimaraes *et al*., 2014). For example, it is known that gene expression stochasticity can cause variation in flocculation between yeast cells in an isogenic population. This is because the ability of a cell to 'floc' is tightly regulated by the expression of *FLO* genes, including *FLO*1, 5, 9, 10 and 11 (Bony *et al*., 1998). Expression of these genes is dictated by a complex network of transcription factors, bistable switches and feedback loops therefore resulting in a system prone to noise in expression. This expression 'noise' in the *FLO* genes is key to eliciting variation in flocculation ability between cells (Bony *et al*., 1998; Halme *et al.*, 2004; Verstrepen *et al*., 2004).

It should also be noted that key yeast stress response pathways, including GSR and ESR (Section 1.9.1) are also subject to gene expression stochasticity. In a study coupling the expression of the yeast stress response gene *HSP104* to a GFP reporter, Attfield *et al.*, (2001) utilised single cell flow cytometry to visualise differences in GFP intensity. Based on this, *HSP104* gene expression from cell-to-cell could be monitored in response to exposure to both heat and ethanol stresses. When used in conjunction

with propidium iodide (PI) as a viability stain, it was found that increases in *HSP104* gene expresssion positively correlated with PI exclusion from the cell (i.e. improved cell health), but that the level of GFP intensity varied from cell-to-cell. This indicated that sub-populations of cells existed with greater levels of *HSP104* expression, greater resistance to heat and ethanol stress and thus were better adapted to survive these conditions (Attfield *et al.*, 2001). Similarly, using single cell RNA sequencing, it has been shown that differences in mRNA content exist between yeast cells. Gasch *et al.*, (2017) demonstrated a degree of heterogeneity between genetically identical cells related to genes involved in the ESR, ribosome biogenesis, and ribosomal protein-encoding genes causing variation in the stress response between cells (Gasch *et al.*, 2000, 2017).

While heterogenety in yeast populations is undeniably present, the full extent of heterogenity in response to brewing related stresses remains unclear. Although cell-to-cell heterogeneity does exist within brewing yeast strains, the extent to which this occurs in different strains and the impact of this on fermentation remains unexplored. Furthermore, the causitive agents and sources of heterogeneity are likely influenced by epigenetic factors such as gene expression variation. The natural presence of heterogeneity within wild microbial populations leads to the question as to whether this is a useful attribute in controlled industrial fermentions, or whether it may prove to be a hinderance.

1.13 Is heterogeneity a desirable attribute?

Despite the advantages to a population in using heterogeneous survival strategies (such as bet hedging under stressful and fluctuating environments), heterogeneity is likely to be predominantly viewed as an undesirable trait. In bioprocesses, cell-to-cell heterogeneity can hamper the overall consistency of the production process (Wang *et al.*, 1995; Alonso *et al*., 2012; Alonso *et al.*, 2014). Using a previous example (Section 1.12.1), where *Lactobacillus casei* are used in yoghurt production, cell isolates display a remarkable heterogeneity in survival rate, such that a sizeable proportion (46%) of the population do not survive and therefore demonstrate reduced lactic acid production (Alonso *et al.*, 2014). Another example can be seen in the production of IFN-α recombinant protein production in yeast. The protein product, intended for human therapeutic use, must have a very specific and uniform configuration with humanised post-translational modifications to ensure acceptance by the human body (Tuite *et al.*, 1982; Hitzeman *et al.*, 1983; Katla and Sivaprakasam, 2019). However, the production of human interferons such as IFN-α in yeast can be incredibly heterogeneous, with protein product varying in sequence, polymerisation, extent of degredation and degree of processing (Hitzeman *et al.*, 1983; Wang *et al.*, 1995). This can result in the need for further downstream processing and rejection of some of the product, making the process both inefficient and costly.

Conversely, heterogeneity in brewing yeast could be considered to be beneficial. For example, flocculation variation could allow sedimentation at different points throughout fermentation, allowing the brewer to crop a large amount of yeast after attenuation (in a process called 'warm cropping'), while also leaving non-flocculated healthy yeast in suspension to aid in the reduction of VDK, a group of undesired off flavours in beer (Powell *et al*., 2003b; Kalayu, 2019). This initial cropped yeast would be of higher quality due to eliminating the lengthy period of 'down-time' associated with the cone, and could be re-pitched quicker into a subsequent fermentation. In addition, to aid in yeast handling, current brewing yeast strains are often expected to be able to produce a range of products and ferment effectively under a variety of different conditions. It is possible that phenotypic flexibility here would allow yeast to

adapt to these processes more efficiently. A related example is the trend towards high gravity brewing, where key stresses are elevated but expectations of the yeast remain the same. In such instances, heterogeneity in response to stress may be an essential bet-hedging mechanism which could allow adaptation of the culture and ensure performance.

Convention would, however, suggest that homogeneity is beneficial for precisely the opposite reasons described and debate above: if a population is consistent in nature then process efficiency should be maximised. However, it is likely that if a cell population is strictly homogeneous in phenotype it will be less able to respond to a rapidly changing environment. In complex processes such as brewing fermentations, with multiple and varying environmental stresses, a degree of heterogeneity may function to allow populations to adapt and thrive. Therefore, it is likely that bet hedging, division of labour and specialist/generalist strategies are employed by existing brewing yeast strains to varying degrees. However, the extent to which this occurs and its impact on performance and overall fermentation characteristics remains unknown. This is largely because industrial evaluations of yeast cultures depends on analysis of a representative population (i.e an average assessment) of cells. Similarly, investigations into the stress response in yeast have focused on population analysis and measuring cell-to-cell heterogeneity is a relatively new concept that relies on the development and implementation of single cell analytical techniques.

1.14 Importance of single cell technology in measuring heterogeneity

Developments in single cell technologies such as flow cytometry, fluorescence activated cell sorting (FACS), single cell RNA-sequencing and microscopy have led to a number of cellular heterogeneity studies (Sumner and Avery, 2002; Kimura *et al.*, 2022). As described above, measuring differences in performance (or functional attributes) between cultures has historically relied on data reflecting an entire population. Mean (average) data generated in this way can be useful, for example comparing brewing yeast strains for ethanol tolerance can give useful information to predict fermentation performance. However, using population data can also be misleading; two yeast populations may reveal an almost identical maximum ethanol tolerance, when in reality it may be that one population contains only a proportion of cells that are tolerant to ethanol, while in the other all cells may be tolerant. To understand and distinguish these two different strategies, single cell technology can be a powerful tool. There have been several studies using bioprocess-relevant organisms that utilise flow cytometry to indicate variation in a population. Many of these use fluorescent reporters like GFP coupled to a gene of interest, for example in yeast the expression of *HSP104* has been coupled with a GFP reporter and single cells measured using flow cytometry revealing differences in GFP intensity and therefore *HSP104* gene expression from cell-to-cell (Attfield *et al.*, 2001)(Section 1.12.5). Developments in mRNA sequencing have also led to the emergence of single cell RNA-seq, which can reveal detailed information about individual cells within a population. For example, this approach has been used to provide evidence that the environmental stress response (ESR) is heterogeneous within yeast populations. This was achieved by analysing the mRNA content of target genes and measuring variation between individual cells (Gasch *et al.*, 2017).

1.15 Aims

Population heterogeneity is known to exist in wild yeast populations, and mixed communities such as biofilms (Levy *et al.*, 2012; Holland *et al.*, 2014; Wloch-Salamon *et al.,* 2017). However, it remains largely unexplored in industrial fermentation systems, primarily due to the belief that the asexual mechanism of yeast division yields genetically identical cells of equivalent physiological character. However, there is evidence to suggest that, like most organisms, yeast populations utilise non-genetic (phenotypic) heterogeneous strategies in order to survive stressful environments. As a result it is currently unknown to what degree phenotypic heterogeneity is present in yeast strains used in stressful industrial fermentations (such as brewing) and how any heterogeneity may affect fermentation performance. As such, this study aims to determine the degree of phenotypic heterogeneity in a variety of brewing yeast strains used for commercial beer production. Specifically, we aim to study the presence and extent of cell-to-cell variation relating to the innate stress response of isogenic cell populations. Furthermore, we aim to investigate factors contributing towards this heterogeneity, including cell age and stress related cell components, in the hope of determining the source of non-genetic heterogeneity. To achieve this, we aim to develop an accurate and high throughput method of acquiring cells of different ages that can subsequently be used to investigate the innate properties of cells and determine the presence of heterogeneity, whilst revealing if this is an inherited or acquired characteristic in isogenic populations. Finally, we aim to study the genotypic and phenotypic contribution to heterogeneity using a combination of quantitative metabolomics and single cell RNA sequencing. The goal here is to ascertain both the degree of physiological variation and commonalities in source between strains and to determine the impact of epigenetic factors on the observed population heterogeneity. This work therefore aims to provide a greater understanding of the intricate dynamics of the yeast stress response in industrial brewing yeast cultures, allowing the relationship between a yeast strain and both its versatility and its suitability for a variety of stressful and challenging fermentation types to be assessed.

Chapter 2. Materials and methods

2.1 Yeast strains and growth medium

In this study two industry relevant *Saccharomyces cerevisiae* ale yeasts, three *Saccharomyces pastorianus* lager yeast strains (two Frohberg and one Saaz type), a wild yeast strain belonging to the species *Brettanomyces anomalus* and a Norwegian Voss Kveik strain were investigated (Table 2.1). These strains were chosen to represent a broad spectrum of yeast isolates, reflecting the diversity of yeast strains used across the brewing industry. Each strain was maintained and grown in YPD broth media (1% (w/v) yeast extract (Oxoid, USA), 2% (w/v) peptone (Oxoid, USA) and 2% (w/v) dextrose (Fisher Scientific, UK) with shaking at 25°C. Solid YPD media for both growth and storage were prepared by supplementing liquid YPD broth with 1.5% (w/v) agar no. 3 (Oxoid, USA). All media types were autoclaved at 121°C, 15psi for 15 mins to achieve complete sterilisation.

Table 2.1 Studied yeast strains

Strain	**Type**
NCYC 1332	Ale- *Saccharomyces cerevisiae*
M2	Ale- *Saccharomyces cerevisiae*
CBS 1260	Lager (*S. pastorianus*)- *Frohberg*
CBS 1174	Lager (*S. pastorianus*)- *Saaz*
W34/70	Lager (*S. pastorianus*)- *Frohberg*
Kveik	Voss Kveik- *Saccharomyces cerevisiae*
BA	*Brettanomyces anomalus*- Wild Yeast

2.2 Yeast Storage

2.2.1 Long term

A culture library of all seven yeast strains was maintained cryogenically. Liquid yeast cultures in YPD broth were grown at 25°C to stationary phase. Subsequently 500µl of yeast culture and 500µl of 50% (v/v) sterile glycerol (Fisher Scientific, UK) were mixed, added to 1.5ml cryovials (Nalgene Nunc International, UK) and stored at -81°C for long term storage.

2.2.2 Short term

Short term yeast cultures were maintained on YDP agar slopes. 15ml of autoclaved liquid YPD agar was added to 30ml glass universals and laid at an angle inside a class II sterile cabinet to cool and set. Following this, sterile loops were used to streak liquid yeast culture onto the slope aseptically and slopes incubated at 25°C in a static incubator (Sanyo, Japan) for 5-7 days allowing growth to cover the entire slope. Once full coverage was achieved, the slopes were stored at 4°C.

2.3 Total and viable cell counts

2.3.1 Cell count protocol

Cell counts were performed using an Olympus BH-2 light microscope at 400x magnification in conjunction with a haemocytometer (Weber Scientific International Ltd, UK). Yeast cells in liquid media were diluted in sterile RO water to approximately $1x10^6$ cells/ml (OD_{600} = 1) and 10µl of the suspension placed onto a haemocytometer chamber with a glass cover. For statistically reliable and reproducible calculations, ≥ 200 cells were counted and buds were enumerated only when greater than half of the size of the mother cell. To ascertain the cell concentration in the original culture the following calculation was used:

Cell/ml (original culture) = Number of cells in counted grid x 10^4 (volume of counting area) x dilution factor

2.3.2 Viability

Yeast cell viability was assessed using the stain methylene blue (Pierce, 1970). Methylene blue powder (Sigma-Aldrich, UK) was dissolved in 2% sodium citrate (w/v) (Fisher Scientific, UK) to achieve a final concentration of 0.01%. As in Section 2.3.1, yeast cells were prepared in solution at 1x10^6 cells/ml (OD_{600} = 1) and mixed with methylene blue at a 1:1 ratio. The solution was then mixed by pipetting and incubated at room temperature for 5 mins. Cells were counted and viability determined using a haemocytometer. Subsequently the ratio of blue coloured cells (non-viable) to normal coloured cells (viable) was calculated:

Yeast cell viability (%) = total cells – dead cells x 100

2.4 Growth curve analysis

Starter cultures were first produced by inoculating yeast from a YPD slope into 10ml of liquid YPD media. Cultures were incubated in an orbital shaker (Certomat BS-1, Sartorius, USA) at 120rpm at 25 ^oC for 48 hrs. For high-throughput growth curve analysis, yeast cultures were diluted to 10^8 cells/ml using sterile RO water and 2µl was mixed with YPD to a total volume of 200µl in wells within a 96 well plate (Corning, USA), such that the final cell concentration was 10^6 cells/ml. Each strain and associated conditions were tested in triplicate to ensure statistical accuracy and reproducibility. 96-well plates were sealed with an aerobic film (Fisher Scientific, UK) and incubated in an automated plate reader (Infinite 200 PRO, TECAN, UK) at 25^oC for 96 hrs, with OD_{600nm} measurements taken at 15 min intervals. Data was collected using the synchronised Magellan data analysis software (Tecan, UK), converted to excel data and analysed on GraphPad PRISM 9 (Graphpad Software Inc., USA).

2.5 Strain identification - Permissive growth temperature

Yeast cultures were cultivated at a range of temperatures in order to aid the differentiation of brewing yeast type. Specifically, it is known that the temperature at which a yeast strain can grow differs between ale (can grow at 37 °C) and lager strains (cannot grow at 37 °C), allowing broad species identification. To achieve this 10ml YPD media was inoculated with a loop-full of yeast taken from agar slopes. These were subsequently incubated at 25°C in an orbital shaker (Certomat BS-1, Sartorius, USA) (120rpm) for 48hrs. Yeast cultures were then centrifuged at 4000rpm for 3mins, with the YPD media supernatant being discarded and the yeast pellet washed twice in sterile RO water. Yeast pellets were then re-suspended in 2ml of sterile RO water an total/viable cell counts were performed (Section 2.3.2). Viable yeast cells were serially diluted to a final concentration of 10^6, 10^5, 10^4, 10^3 and 10^2 cells/ml. Subsequently, a volume of 5µl of each dilution (for each strain) was pipetted onto YPD agar plates, as shown in Figure. 2.1. This was performed in triplicate with the intention for one plate to be statically incubated aerobically at 4 °C, another at 25 °C and a final plate at 37 °C. Growth was assessed visually and images procured using a GelDoc-It®2 Imager (UVP LLC, UK) under ultra violet light allowing the visualisation of small CFUs that were difficult to distinguish with standard photography.

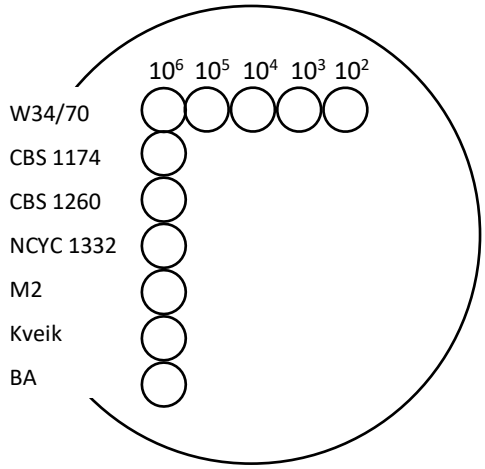

Figure 2.1. Schematic diagram of spot plate layout

2.6 Yeast genus and species characterisation

2.6.1 The extraction of genomic DNA

For molecular analysis of genomic yeast regions, including ITS region analysis and Inter Delta PCR, genomic DNA was extracted as described by Legras and Karst (2003). Yeast strains were initially propagated in 10ml YPD at 25°C under shaking conditions (Certomat BS-1, Sartorius, USA) at 120rpm for 48hrs. 1.5ml of yeast culture was transferred to a micro tube (Starstedt, UK) and centrifuged at 5000rpm for 5 mins. The supernatant was taken off the yeast pellet and the pellet re-suspended in 400µl cell lysis buffer (Tris 10mM, pH7.6, EDTA 1mM, NaCl 100mM, Triton X-100 2% (w/v), sodium dodecyl sulphate 1% (w/v))(Fisher Scientific, UK), subsequently 400µl of phenol/chloroform/isoamyl alcohol (25/24/1 v/v) (Sigma- Aldrich, UK) and 0.4g of glass beads (0.45-0.55mm acid washed, Sigma- Aldrich, UK) were added. This mixture was vortexed for 4 mins. Subsequently 200µl of Tris EDTA (pH 7.6) buffer (Sigma-Aldrich, UK) was added and the mixture centrifuged at 6000 rpm for 5 mins. Following centrifugation, the clearly separated upper phase was pipetted into an Eppendorf tube and 500µl of chloroform/ isoamyl alcohol (98/2 v/v) (Sigma-Aldrich, UK) was added and gently agitated by inverted mixing and centrifuged at 14000 rpms for 2 mins. The resultant upper aqueous phase was pipetted into a new Eppendorf tube and 2 volumes of ice-cold ethanol added to each tube. This mixture was then centrifuged at 14000 rpm for 5 mins and the ethanol-based supernatant discarded. The nucleic acid pellet was then left to dry for at least 15 mins to rid any residual ethanol and finally dissolved in 50µl 10mM TE buffer pH 8.0 (Fisher Scientific, UK). DNA was either used immediately for PCR, or stored at -20 °C for use at a later time.

2.6.2 Species differentiation using ITS PCR

ITS PCR was performed based on the method described by White *et al* (1990). ITS PCR reaction used the primers ITS1 and ITS4 with sequences 5' TCCGTAGGTGAACCTGCGG 3' and 5' TCCTCCGCTTATTGATATGC 3' respectively (Sigma- Aldrich, UK). The PCR master mixture consisted of 36µl sterile RNAse free molecular grade water (Sigma-Aldrich, UK), 5µl 10x Mg-free buffer (New England Biolabs), 5µl 2.5mM $MgCl_2$ (New England Biolabs), 5µl 0.2mM deoxynucleotide triphosphates (dNTPs) (New England Biolabs), 0.5µl of each of ITS1 (100µM) and ITS4 (100µM) primers and 0.5µl (2.5 units) Taq DNA polymerase (New England Biolabs). Each component was multiplied by the number of samples to make an initial 'master mix'. Subsequently, 48µl of master mix was added to 2µl extracted DNA (section 2.6.1) in PCR tubes and placed in a thermal cycler (TC-512 Techne, UK), with a pre-heated lid. The following programme was run: 95 °C for 5 mins followed by 35 cycles of 95 °C for 1 min, 55 °C for 2 mins, 72 °C for 2 mins and a final extension of 72 °C for 10 mins. Following completion, the samples were held at 4 °C until required.

2.6.3 RFLP analysis of ITS PCR product

The products from the ITS PCR amplification (Section 2.6.2) were analysed for restriction fragment length polymorphisms (RFLP's) using the restriction enzymes *HaeIII, HinfI* and *CfoI*. These were added to DNA in independent reactions in order to digest the PCR product (New England Biolabs, UK). The reaction mixture consisted of 8µl PCR product, 1µl restriction enzyme (*HaeIII, HinfI, CfoI*), 2µl of the respective 10x buffer (New Engalnd Biolabs, UK) and 9µl sterile RNAse free water (Sigma- Aldrich, UK). Samples were incubated in a 1.5ml Eppendorf tube for 1 hr at 37 °C in a water bath.

2.6.4 Visualisation of ITS PCR amplicons and RFLP DNA fragments

Electrophoresis was used to resolve DNA products via a 1.5% (w/v) agarose gel (ITS PCR products) and 2% (w/v) agarose gel (RFLP products) (Sigma-Aldrich, UK), containing 0.2µg/ml ethidium bromide (Sigma- Aldrich, UK). Gels were submerged in TAE buffer (4.85 g/l TRIS, 1.14 ml/l glacial acetic acid and 0.37 g/ EDTA) (Fisher Scientific, UK) within a gel electrophoresis tank. Aliquots of 5-10µl (depending on purity) of ITS PCR or RFLP products were combined with 5x DNA loading dye (New England Biolabs, UK) to a final 5% (v/v) concentration, and DNA fragments were separated based on their size at 80mV for 120+ mins, until sufficient resolution was achieved. After completion, agarose gels were visualised using ultraviolet light and a GelDoc-It®2 Imager (UVP LLC, UK). DNA fragment length was determined by comparison to a reference 100bp ladder, in which 5µl of the ladder (New England Biolabs, UK) was pipetted into a spare well located within the same gel.

2.7 Yeast strain identification by DNA fingerprinting using amplification of interdelta regions

A more detailed analysis of the genetic constitution of each yeast was performed using a recognised fingerprinting method based on interdelta regions of the genome. Delta sequences are linked to yeast transposons and amplification of regions between the delta sequences allows for the accurate identification of yeast strains based on the production of multiple amplicons (Legras and Karst, 2003).

2.7.1 Interdelta PCR reaction

Initially, yeast genomic DNA was obtained through extraction as described in Section 2.6.1. Subsequently, interdelta sequences were amplified by PCR using the primer pair delta 12 (10µM) and delta 21 (10µM), with the sequences 5'-TCAACAATGGAATCCCAAC-3' and 5'-CATCTTAACACCGTATATGA-3' respectively (Sigma- Aldrich, UK). A master mixture of 33µl sterile RNAse free water (Sigma-

Aldrich, UK), 10µl 5X Phusion HF buffer (New England Biolabs, UK), 2µl 2.5mM MgCl2 (New England Biolabs, UK), 1µl dNTPs (New England Biolabs), 1µl of the forward (delta 12) and reverse (delta 21) primers and 0.1µl Phusion HF DNA polymerase (New England Biolabs, UK) was prepared immediately prior to analysis. To create this, each component of the master mix was multiplied by the number of reaction samples needed and mixed in an Eppendorf tube and kept on ice. For each reaction, 48µl of master mix and 2µl DNA template were mixed by pipetting in a PCR reaction tube. DNA fragments were amplified using the following PCR protocol: 30secs at 98°C preceding 38 cycles of: 10 secs at 98 °C, 30 secs at 50 °C, 90 secs at 72 °C and a final elongation of 10 mins at 72 °C. Following this, samples were held at 4 °C until required. For visualisation of the interdelta PCR products, the electrophoresis method in Section 2.6.4 was applied and DNA was resolved using a large 2% (w/v) agarose gel at 80mV for 3+ hours until sufficient resolution was achieved. The agarose gel was visualised under ultraviolet light using a GelDoc-It®2 Imager (UVP LLC, UK). The size of DNA amplicons resolved on the gel were determined visually by reference to a 1kb ladder (New England Biolabs, UK) loaded into a spare well within the same gel.

2.8 Brewing stress tolerance determination via spot plate analysis

Spot plate analysis was used to determine the susceptibility and tolerance of yeast strains to ethanol, oxidative, osmotic, copper and zinc stress, based on the growth of cells from each yeast culture. This was performed primarily to establish the concentrations of each stress appropriate for subsequent heterogeneity analysis (Section 2.9). To achieve this, YPD agar was supplemented with various substances to create each of stress factors above. YPD-agar (at 50 °C) was supplemented with ethanol (Fisher Scientific, UK), sorbitol (osmotic stress) (Sigma- Aldrich, UK), hydrogen peroxide (oxidative stress) (Fisher Scientific, UK), zinc sulphate heptahydrate (Sigma-Aldrich, UK) and copper sulphate pentahydrate (Sigma- Aldrich, UK) in order to produce stress plates. These plates were produced to a final volume of 20ml, with final concentrations of 0-30% (v/v) ethanol, 0-60% sorbitol (w/v), 0-8mM hydrogen

peroxide, 0-8mM zinc sulphate heptahydrate and 0-6mM copper sulphate pentahydrate.

Plates were inoculated with yeast that had been cultivated from agar slopes into 10ml YPD and incubated for 48 hrs at 25 ^{o}C in an orbital shaker at 120rpm. Yeast cells were centrifuged at 4000rpm for 3 minutes and washed twice with sterile RO water. Viable cell counts were performed as described previously (Section 2.3.2) and suspensions of yeast were created at 10^6, 10^5, 10^4, 10^3, 10^2 viable cells/ml in sterile RO water. Subsequently, 5µl of each diluted cell aliquot was then 'spotted' in a grid format onto the supplemented YPD agar plates as described previously (Section 2.5; Figure 2.1). Plates were incubated for 7 days in a static incubator (Sanyo, Japan) at 25 ^{o}C.

2.9 Analysis of phenotypic heterogeneity using stress dose response analysis

2.9.1 Induction of stress

In order to evaluate and quantify heterogeneity in the yeast stress response, the growth kinetics of cell populations derived from each yeast strain were investigated. To achieve this, cell populations were prepared from YPD agar slopes, by cultivating yeast in 10 mL of liquid YPD for 48hrs at 25°C and 120rpm in an orbital shaker (Certomat BS-1, Sartorius, USA) until exponential growth phase was achieved. Subsequently, cell populations were enumerated and diluted such that 5000 viable cells were seeded into individual wells within a 96 well plate. Each well contained a combination of YPD and increasing concentrations of a defined stressor (ethanol, sorbitol, hydrogen peroxide, copper sulphate and zinc sulphate), made to a total volume of 200 µL. An example of this procedure is detailed in Table 2.2 using ethanol stress as an example. The final concentration range for each stressor was: 0-25% (v/v) ethanol, 0-8mM hydrogen peroxide, 0-60% (w/v) sorbitol, 0-8 mM copper sulphate pentahydrate and 0-10mM zinc sulphate heptahydrate. Yeast cell seeds were exposed to these stresses in a grid format, with gradually increasing concentrations of stressor

as shown by the example illustrated in Figure 2.2. Following inoculation, samples were incubated aerobically at 25°C for 72hrs in a static incubator (Sanyo, Japan) and the activity of each yeast sample was determined using MTT staining, as described below.

Table 2.2. Combinations of YPD media and ethanol applied to achieve the desired concentrations of ethanol stress for exposure to 5000 yeast cells.

YPD/µL	100% (v/v) Ethanol/µL	Yeast cells (totalling 5000 cells)	% (v/v) Ethanol (Stressor)
190	0	10 µL	0
180	10	10 µL	5
175	15	10 µL	7.5
170	20	10 µL	10
165	25	10 µL	12.5
160	30	10 µL	15
150	40	10 µL	20
140	50	10 µL	25

2.9.2 Determination of population stress tolerance and heterogeneity (MTT assay)

To measure the degree of heterogeneity in stress response of each yeast strain, yeast activity was measured using a stain known as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (van Meerloo *et al*., 2011). This approach relies on the capacity of yeast to metabolise and reduce the MTT tetrazolium salt to an insoluble formazan crystal form which can be quantified (Sánchez and Königsberg, 2006). To determine yeast activity through MTT staining, the entire 96 well plate (prepared as in Section 2.9.1) was first centrifuged at 2500rpm for 5 mins using a plate centrifuge (MSE Centaur 2, Sanyo, Japan) in order to pellet the yeast cells and the media was subsequently pipetted from each well. Subsequently, 100 µL of fresh media (YPD) and

10 µL of >98% MTT (VWR, UK) were added to each well, and cells were incubated for 4hrs at 25°C. The plate was then centrifuged again and 85 µL of media was removed, leaving dark grey formazan crystals. For accurate quantification, these crystals were dissolved in 50 µL DMSO (anhydrous ≥99.9%) (Sigma-Aldrich, UK) to release a purple colouration. The intensity of this purple colour was determined by measuring the absorbance at OD_{570nm} directly from 96 well plates using a plate reader (Infinite 200 PRO, TECAN, UK). This process is depicted in the schematic shown below (Figure 2.2). The relative intensity of the purple colour represents the number of healthy viable cells present in each well. It should be noted that although the MTT assay determines activity of cells at the point of analysis, this is reflective of the capacity of the initial 5000 cell population to survive and produce new cells under the stress conditions applied.

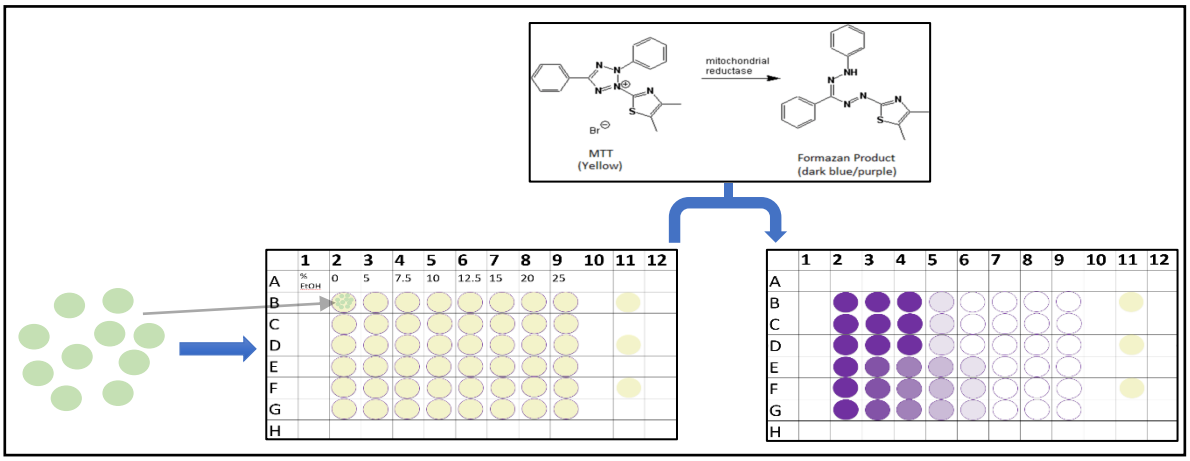

Figure 2.2. Protocol for determining yeast stress response heterogeneity. 5000 cells were initially seeded in triplicate into wells containing different level of stress (ethanol is show here as an example) up to a volume of 200 µL. Typically, 2 strains were analysed on each plate at a given time. After 3 days of growth at 25°C, fresh media and MTT were added. The latter was converted to formazan crystals by the actively metabolising cells. With the addition of DMSO, these crystals turned purple and the degree of colouration was measured by spectrophotometry.

2.9.3 Assessment for the capacity of MTT to predict viable cell number

To ensure accuracy and determine the working range of the MTT assay for this study, increasing concentrations of brewing yeast cells (0-10^8 cells/ml) were seeded into YPD to a total volume of 200µl and the MTT cell cytotoxicity assay performed on these cell seeds (as per section 2.9.2). The standard curve in Figure 2.3 displays the spectrophotometry data obtained at 570nm (Infinite 200 PRO, TECAN, UK). An OD570nm reading of 3.42 corresponded to 10^8 cells/ml and 0.10 for 0 cells/ml. This graph provides evidence suggesting the working detection range of the MTT assay is from ~20000 cells to in excess of 10^7 cells (in 200 µL cultures) and between these two limits there is a linear relationship, meaning small differences in OD can accurately correlate to an increase in viable cell number. It is worth noting here that the viability of the cell cultures used was 99.2% viable.

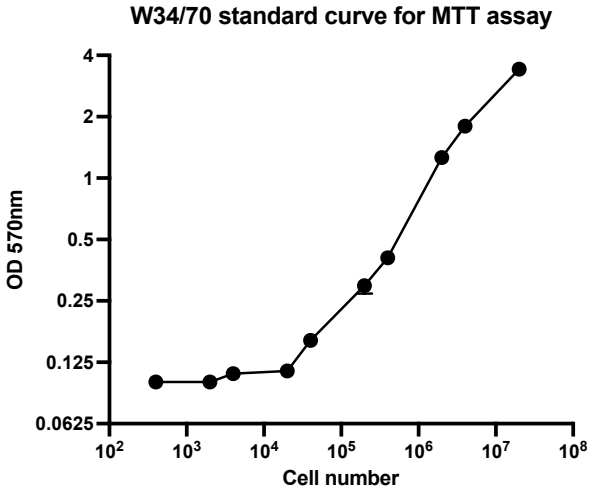

Figure 2.3. MTT standard curve for strain W34/70. Graph depicting the OD 570nm readings taken for a variety of cell concentrations (0-10^8 cells/ml) having undergone the MTT assay.

2.9.4 MTT assay and heterogeneity determination data analysis

2.9.4.1 Dose response curves

The colourimetric data obtained through the use of the MTT assay obtained in section 2.9.2 was formatted into dose response curves in the data analysis software GraphPad PRISM 9 (Graphpad Software Inc., USA). Firstly the percentage viability was evaluated by comparing the colour obtained in each condition to a control (non-stressed) which represented 100% viability. From this, sigmoidal dose response curves were obtained, these curves possessed a point of inflection at which point the hillslope gradient of the curve was measured using a four parameter logistic equation displayed below:

Equation 2.1. Four- parameter logistics equation

$$y = a + \left(\frac{b-a}{1+10^{((Logc-x)\times d))}}\right)$$

y = Percentage viability

x = Stressor concentration

a = Minimum y value (infinite stress)

b = Maximum y value (no stress)

c = Point of inflection

d = Hillslope

The obtained dose response curves were modified, such that the maximum viability for each condition was measured at x=0. Dose response curves that contained curves with a gradual hillslope gradient would represent a gradual decline in viability as the stressor concentration increases, as a result of a greater degree of heterogeneity. Compared to slopes with a steeper gradient and more sudden decline in viability, due to the absence of cell-to-cell heterogeneity. Heterogeneity in each cell population was quantified using this hillslope gradient obtained by differentiating the equation above. IC-50 values were also obtained from these dose response curves, this value was measured at the stressor concentration at which point the viability of the cell population reduced to 50%. This was used in order to compare values of maximum tolerance between strains.

Finally, in order to identify significant differences in hillslope (heterogeneity) between strain, Tukeys multiple comparison test was used with P values below 0.05 representing significant differences.

2.10 Analysis of stress-related cell targets using fluorescent staining

In order to quantify and determine the integrity of specific cell components, the stains Mitotracker Green FM (Fisher Scientific, UK), Nile red (Sigma-Aldrich, UK), $DiOC_6$ (Sigma- Aldrich, UK), Laurdan (Fisher Scientific, UK) and Filipin III (Sigma-Aldrich, UK) were applied to cell suspensions, targeting mitochondrial mass, mitochondria membrane potential (MMP), internal membrane integrity, membrane fluidity and membrane sterol content respectively.

2.10.1 Fluorescent staining of targeted cell structures

Yeast cultures were taken from agar slopes and grown in 10 mL YPD at 25°C for 48hrs in an orbital shaker (Certomat BS-1, Sartorius, USA). These starter cultures were then centrifuged at 6000rpm for 3 mins and washed twice in the respective buffers for each stain: HEPES (10mM, pH7.4, Sigma- Aldrich, UK) (for MitoTracker Green and $DiOC_6$) or PBS (10x, Sigma- Aldrich, UK) (for Nile red, Filipin III and Laurdan). Cell cultures were then enumerated and assessed for viability (Sections 2.3.1 and 2.3.2). Aliquots were then diluted to a total volume of 10^6 cells/ml in the relevant cell stain buffer (as above). Each was stained to a specific final concentration as specified here: 100nM-MitoTracker green FM, 175nM- $DiOC_6$, 0.3 µg/ml Nile red, 250µM Laurdan and 5 µg/ml Filipin. Once prepared, samples stained with MitoTracker Green, $DiOC_6$ and Laurdan were incubated for 20 mins. Those cultures stained using Nile red were incubated for 10 mins and Filipin stained cells were incubated at 5 mins. Irrespective of the stain, each culture was place in a static incubator (Sanyo, Japan) in a dark environment and at 25°C for the specified time. Following this each sample was washed twice in the relevant stain buffer (as above) with separation by centrifugation at 4000rpm for 3

mins, and finally re-suspended in fresh buffer. These samples were then stored on ice in the dark until used for analysis.

Stained cells were subsequently visualised using fluorescence microscopy (Nikon Eclipse Ci with CoolLED pE-300 LED illumination) using 100X (oil) objective. Fluorescence was detected using UV light excitation and GFP emission filter for Mitotracker and $DiOC_6$, blue light excitation with CY3 emission filter for Nile red and UV light excitation with emission detected using the DAPI filter for Laurdan and Filipin. Fluorescence 2D images were procured and confirmation of successful staining assessed using the NIS-Elements analysis software (Nikon, UK).

2.10.2 Heterogeneity assessment of stress related cell targets

In order to determine variation between cells (heterogeneity), flow cytometry was used in conjunction with the stains described previously (Section 2.10.1). In each instance, cells were analysed by flow cytometry (Beckman Coulter Astrios EQ) with data analysed using Kaluza (Beckan Coulter, USA) and FlowJo (FlowJo, USA). The cells in each stained population were initially analysed for the way in which each cell scattered the source light at a wavelength of 488nm. Scattered light was detected using both forward and side scatter detectors to gain greater detail of the cells within the populations. The cells were analysed initially by cell size (forward scatter - area) against granularity (side scatter - area), in order to identify the main population of yeast cells. This population was then analysed by forward scatter height vs forward scatter width in order to identify 'singlets' within the population, discriminating from cell fragments, and from cell doublets and triplets. Finally, a minimum of 500,000 cells from the 'singlet' population were further analysed for flourescence intensity based on the stains Mitotracker Green, Nile red, $DiOC_6$, Laurdan and Filipin. The excitation energy and fluorescence emission detection channel for each of the stains applied can be found in Table 2.3.

Table 2.3. Excitation and emission energies for each cell stain

Cell Stain	Function and cell location	Excitation energy/ nm	Fluorescence emission channel and wavelength/ nm
MitoTracker Green FM	Internal mitochondrial probe. Quantifying mitochondrial mass (Presley *et al.,* 2003)	488	FL17- 513
Nile red	Internal lipid stores. Lipophilic staining targeting neutral lipids (Kimura *et al.,* 2004)	488	Fl18- 576
$DiOC_6$	Internal membranes. Quantifies mitochondrial membrane potential and internal membrane integrity (Johnson *et al.*, 1981)	488	FL17- 513
Filipin	Cell membrane. Specifically staining membrane sterols (Miller, 1984)	355	FL6- 448
Laurdan	Cell membrane. Lipophilic stain used to determine the fluidity and structure of the phospholipid bilayer (Parasassi *et al.*, 1998)	355	FL6- 448

The fluorescence intensity of each cell within the population was detected and a histogram produced using the FloJo analysis software (FloJo, USA). This was performed for each set of experimental conditions, including for each cell population and the relevant fluorescent stain for specific cell targets. Based on the range of detected fluorescence intensities across a cell population, a measure of heterogeneity was obtained. This was achieved by measuring the coefficient of variance across the population from the fluorescence generated by each stain/cell target.

2.11 Isolation and analysis of yeast daughter cells

2.11.1 Staining of chitin structures (bud/birth scars) in yeast

Calcofluor white, wheat germ agglutinin (WGA) and congo red are known to stain the chitin component of yeast bud scars, and this can be used to allow differentiation between daughter and parent yeast cells (Kuřec *et al.*, 2009) (Pringle, 1991). To best isolate purified cultures of daughter yeast cells, each stain was initially assessed for their efficiency in highlighting bud scars. In order to do this, starter cultures of yeast were prepared from agar slopes in 10 mL YPD at 25°C for 48hrs in an orbital shaker (Certomat BS-1, Sartorius, USA). Cell cultures were then centrifuged at 6000rpm for 3 mins and washed twice in sterile phosphate buffer saline (10x, Sigma- Aldrich, UK). Subsequently cell counts and viability analysis were performed (Section 2.3.1 and 2.3.2) and cell suspensions were prepared to a final concentration of 10^6 cells/mL in a final volume of 1ml.

For calcofluor white staining, immediately prior to analysis 100 µL of calcofluor white (1g/L) (Sigma- Aldrich, UK) was added and samples were gently agitated for 20 mins in the dark. Cells were then pelleted at 6000rpm for 3 mins and washed with PBS 3 times to remove excess stain, before being re-suspended in 1ml PBS and kept on ice. This procedure was also followed for Wheat germ agglutinin and congo red. Cell aliquots were prepared in an identical fashion, however, either 200 µL of wheat germ agglutinin FITC fluorescent conjugate (1g/L) (Sigma- Aldrich, UK) or 500 µL congo red (1g/L) (Sigma- Aldrich, UK) was added before gentle agitation in the dark and at room temperature for 15 mins. As above, cells were harvested by pelleting at 6000rpm for 3 mins and washed in PBS 3 times. Finally, each stained pellet was re-suspended in 1ml PBS for analysis and kept on ice.

2.11.2 Bud scar analysis using fluorescence confocal microscopy

Fluorescence microscopy was used to visualise yeast bud scars stained using the procedure described above (Section 2.11.1). This was performed using an LSM 880c fluorescence confocal microscope (Zeiss, UK). Images of stained cells were acquired using a 63x objective, oil (1.4NA), with pinhole set to 1AU. The efficacy of bud scar staining was assessed by taking both brightfield and fluorescence images of single cells in both 2D and 3D, using between 20-50 Z stacks in each instance (depending on the size of the yeast cell). Photomultiplier tube data was converted into 16-bit format and images were analysed (ZEN blue, Zeiss, UK) for accuracy of staining and then the presence/absence of bud scars was assessed. The excitation energies and fluorescence emission detection channels used for the three stains applied can be found in Table 2.4.

Table 2.4 Excitation and emission energies for the studied bud scar stains

Bud scar stain	**Excitation energy/ nm**	**Fluorescence emission channel and wavelength/ nm**
congo red	488	CY3
WGA-FITC	488	GFP
Calcofluor white	405	DAPI

2.11.3 Analysis of population dynamics through bud scar quantification and cell size distribution

Cell populations were stained with calcofluor white, WGA or congo red as previously stated (Section 2.11.1). These samples were then analysed using a Beckman Coulter Atrios EQ flow cytometer (Beckman Coulter, USA) to identify and quantify bud scar structures on individual yeast cells. Initially, stained populations were examined based on cell size and granularity (forward scatter - area against side scatter - area) (Kaluza analysis software, Beckman Coulter, USA), indicated by the fashion in which each cell scattered a 488nm light source. Gating was then performed to select the main

population of yeast cells, and eliminating both large aggregates and smaller cell fragments (Kaluza analysis software, Beckman Coulter, USA). Following this, the desired cell population was investigated based on the size parameters: forward scatter - width and forward scatter - height and then gated in order to select for the main population, consisting of only single cells. This allowed the elimination of cell fragments, as well as doublets and triplets. From this population the coefficient of variance was used to quantify the range of cell sizes encountered (forward scatter - area) in the population of single cells.

For bud scar analysis, the same gated single cells isolated above were inspected for fluorescence intensity, based on each individual stain applied (Section 2.11.1). This was performed firstly to identify, and then to isolate daughter cells from the main population. Calcofluor white staining was visualised using excitation energy of 355nm and emission max at 448nm (Channel FL6), wheat germ agglutinin with FITC fluorescent conjugate at an excitation of 488nm and emission max detected at 513nm (channel FL-17). Congo red was visualised using 488nm excitation and emission detected at an emission max of 620nm (Channel FL-19). Irrespective of the stain applied, cell aliquots were analysed for the fluorescence intensity of scar structures against cell size (forward scatter - area).

2.11.4 Isolation of daughter cells using fluorescence activated cell sorting (FACS)

For daughter cell isolation, fluorescence activated cell sorting (FACS) was employed (Beckman Coulter Astrios EQ). By analysing cells within each population for fluorescence intensity (bud scar structures) and for cell size (forward scatter - area) (Sections 2.11.1 and 2.11.3), the smallest and least fluorescent single cells could be identified and gated. Using FACS this gated sub-population was selected using two independent rounds of sorting, with the smallest and least fluorescent 10-15% cells selected on the first round in ‘enrichment mode’. Liquid droplets containing single yeast cells were then assessed for cell area (forward scatter - area) and fluorescence intensity (bud scar structures) and, if the values obtained for these parameters fell within requirements of the gated area, the cell within the liquid droplet was ejected

into a collection tube. In 'enrichment mode' all cells determined to fall within the gated parameters were sorted, however it is known that this can lead to droplets with multiple cells, or flanking droplets spilling over and being ejected. This initial sort is therefore primarily aimed at sorting large numbers of cells and, as a result, the efficiency is accepted to be relatively poor.

In order to purify the cells selected using the initial sort, a second round of analysis was conducted. At least 500,000 cells from the preliminary sort were selected in each instance. These were then collected by centrifugation at 4000 rpm for 5mins, the supernatant was discarded, and the pellet was re-suspended in fresh PBS (10x Sigma-Aldrich, UK) to a volume of 200µl. This concentrated cell solution was then subjected to a second round of sorting in which 'purification mode' was applied. This mode allows for only positive events (suspected daughter cells) that are both preceded and followed by other positive events to be sorted. This ensures that spill over of cells from outside the desired gate is minimal. This final population of purified cells was then analysed for the presence of bud scars structures via staining (evaluated by confocal microscopy as in Section 2.11.2). In addition, aliquots of 5000 cells were directly sorted into wells of 96 well plates for heterogeneity determination via the MTT assay described in Section 2.9.2. Similarly, individual daughter cells were obtained through the same method with single cells seeded into wells of a 96 well plate for growth analysis section 2.12.

2.11.5 Analysis of population daughter cell purity using imaging flow cytometry

Both standard 'mixed aged' populations and isolated daughter cell populations were stained using calcofluor white bud scar stain, as described in Section 2.11.1. Cells were then analysed for the presence of bud scars using imaging flow cytometry. Stained cell populations were inserted into a flow cytometer (Amnis Imagestream X Mk II (ISX), Luminex, USA) with both brightfield and laser light collected using an objective lens with 40X magnification. Calcofluor white stained cells were excited using the 405nm laser and multi-spectral decomposition was used to collect the brightfield, light scatter and fluorescence emission signals for each cell, therefore producing both brightfield

and fluorescence images, which were subsequently analysed separately or overlaid for further analysis using IDEAS software (Luminex, USA). Using conventional flow cytometry attributes, each cell was also analysed for aspect ratio against area, to identify the singlet population. Subsequent analysis was performed on this singlet population, discounting any fragments or larger groups of cells. From this the 'spot count' feature of the IDEAS software was used to analyse calcofluor white fluorescence images of the studied cell population. The spot count feature enumerates areas of fluorescence over a determined intensity, corresponding to the brightly fluorescent bud scars. Thus, bud scar enumeration was performed on both mixed aged and suspected daughter cell populations in order to assess the purity of cells in the latter (i.e. cells without bud scars) and the efficiency of the FACS protocol overall.

2.11.6 Growth curve analysis of cells stained with calcofluor white

To ensure that calcofluor white did not impact growth characteristics, cell populations were stained with calcofluor white as described in Section 2.11.1. These cells were then enumerated (Section 2.3.1) and 10µl aliquots were seeded into individual wells of a 96 well plate, containing YPD and ethanol from 0-10% (v/v), leading to a total concentration of 10^6 cell/ml in each well, as shown in Table 2.5. The growth of each culture under these conditions was assessed as described previously (Section 2.4), and compared non- stained control samples.

Table 2.5 Culture conditions for growth of calcofluor white stained cells

YPD/µL	100% (v/v) Ethanol/µL	Yeast cells (10^6 cells/ml)	% Ethanol (v/v) (Stressor)
190	0	10 µL	0
180	10	10 µL	5
175	15	10 µL	7.5
170	20	10 µL	10

2.12 Determination of population growth heterogeneity in daughter cells

Yeast cell populations were stained with calcofluor white and daughter cells were isolated using the methods described in Sections 2.11.1 and 2.11.4. However, in this instance, the second round of FACS was performed using 'single cell mode' rather than 'purification mode'. This was performed since it provides the same level of scrutiny of sorting for the desired cells, but also allows individual cells to be sorted directly into individual wells of a 96 well plate. Based on this, single daughter cells were sorted into wells containing 200 µl YPD media. Subsequently an aerobic film (Fisher Scientific, UK) was applied to provide a barrier to the environment, and cells within the 96 well plate were incubated at 25°C. Growth was monitored using an automated plate reader (Infinite 200 PRO, TECAN, UK) as described in Section 2.4, until stationary growth was achieved. Data was collected using the synchronised Magellan data analysis software (Tecan, UK), converted to excel data and growth curves analysed on GraphPad PRISM 9 (Graphpad Software Inc., USA).

In addition to standard growth analysis, single daughter cell growth curves were also produced for cells that had been subjected to cold shock. Once individual daughter cells had been sorted into wells of 96 well plates, the plate was incubated at 4°C for 12 hrs, before being incubated at 25°C for growth curve analysis as above. Irrespective of the experimental growth conditions, the obtained single cell growth curves for both states (normal and cold-shock) were normalised such that each curve was plotted by percentage growth against time (Graphpad Software Inc., USA). From this, the time taken for each growth curve to achieve 50% growth was calculated and the coefficient of variance for this value across each cell population was used as a measure of heterogeneity.

2.13 Assessment of heterogeneity in stress related cell target structures

In order to gain greater insights into heterogeneity within the key stress related cell targets investigated in Section 2.10, the presence of heterogeneity was again assessed within yeast populations of different replicative age. As such daughter cells, cells with 1 bud scar, and cells with 2 bud scars were all investigated. These were assessed by co-staining mixed aged cells with Mitotracker green, nile red and $DiOC_6$ analysing for structures including mitochondrial mass, MMP, internal membrane integrity and neutral lipid content; with the bud scar stain calcofluor white utilised for cell age determination. These co-stained cells were analysed using imaging flow cytometry in order to assess the presence of heterogeneity within each cell population. In addition, the effect of sub-lethal ethanol stress was also tested to observe the impact on heterogeneity in each instance, by culturing cells in 5% ethanol for 48 hrs. This process is outlined in greater detail in the following sections.

2.13.1 Cell compartments stain assessment using imaging flow cytometry

Firstly mixed aged cell population were stained with one of Mitotracker green, Nile red and $DiOC_6$ as in section 2.10.1. Each of the stained cell populations were assessed using imaging flow cytometry (Amnis Imagestream X Mk II (ISX), Luminex, USA) as described in Section 2.11.5. As before, cells were analysed by aspect ratio against area in order to identify the singlet population in each instance. From this, fluorescence images were obtained for each cell using the laser light and the excitation energy relevant to each individual fluorescent stain (Table 2.6). The subsequent emitted fluorescence for each stain was investigated for detection across all emission channels (1-9), in order to determine the extent of fluorescence detected in undesired channels (i.e. stain specificity). If fluorescence was detected in an undesired channel, compensation was performed using the IDEAS analysis software (Luminex, USA) until only fluorescence was detected in the main channel desired for that specific fluorescent stain. This was fulfilled in order to allow the co-staining of Mitotracker green, Nile red and $DiOC_6$ with the bud scar stain calcofluor white, with accurate resolution between the co-stains.

Table 2.6. Excitation and emission energies for stress target stains and bud scar stain

Cell Stain	Excitation energy/ nm	Main fluorescence emission channel
Calcofluor white	405	Channel 7
Mitotracker Green FM (MTG)	488	Channel 2
Nile red	461	Channel 4
$DiOC_6$	488	Channel 2

2.13.2 Co- staining protocol and heterogeneity determination

In order to investigate multiple parameters at the same time, mixed aged cells of the studied strains were first stained with calcofluor white as in Section 2.11.1. Following this, cells were co-stained with one of either Mitotracker green, Nile red or $DiOC_6$ as described in Section 2.10.1. These cell samples were then stored on ice in the dark for a minimum period of time before analysis. The co-stained cell samples were then analysed for cell targets within different age groups of cells in order to identify heterogeneity in nature. Cell samples were injected into the imaging flow cytometer (Amnis Imagestream X Mk II (ISX), Luminex, USA) and the cell aspect ratio against area was used to identify the singlet population as above. As in Section 2.11.5, both brightfield and fluorescence images were taken of each cell, resulting in three images in each instance: a brightfield image, a calcofluor white image indicating bud scars, and a co-stain image, all of which were analysed both separately and overlaid using the IDEAS software (Luminex, USA). The 'spot count' feature of the IDEAS software was again used in order to count the bud scars present on each cell. Single cells of each population were subsequently discarded based on bud scar number, giving a method of separating cells by age and resulting in discrete analyses for cells with either 0, 1 or 2 bud scars. Each group of stained cells was then assessed for the range of fluorescence intensity based on Mitotracker green, Nile red and $DiOC_6$ for each 'age' group. For example, the intensity of emitted fluorescence detected in channel 2 was assessed for newly formed daughter cells (cells with 0 bud scars) stained with

Mitotracker green. From this a histogram depicting the range of fluorescence intensity for cells within the population could be produced. The range of fluorescence intensity was subsequently quantified using coefficient of variance. This analysis was performed for cells with 0, 1 and 2 bud scars for each of the targeted cell structures, giving values of heterogeneity across the respective populations for each. In addition, in order to assess the impact that cell size had on cell-to-cell stain variation, the coefficient of variation was also measured for the cell area of cells within each population.

The analysis of fluorescence heterogeneity using Mitotracker green, Nile red and $DiOC_6$ was also performed using cell populations that had been exposed to sub-lethal ethanol stress. These yeast cultures were pre-cultivated in 10 mL YPD supplemented with 5% (v/v) ethanol for 48hrs at 25°C in an orbital shaker (Certomat BS-1, Sartorius, USA), prior to analysis.

2.14 Metabolomic assessment of stressed vs. unstressed yeast populations

2.14.1 Cultivation of cells for analysis of metabolome

Cultures derived from the yeast strains CBS 1260, M2 and NCYC 1332 were prepared from stocks maintained on agar slopes. In each instance, 10ml of sterile YPD was inoculated with cells and cultures were grown at 25^{o}C for 48hrs in an orbital shaker (Certomat BS-1, Sartorius, USA). Total and viable yeast counts were performed as described in Section 2.3.2 and these were used to prepare duplicate 'master' cell suspensions. Of these, one was used to prepare a control population of 'unstressed' cells, while the second was used to prepare a 'stressed' population. The designated unstressed cells were cultivated in autoclaved YPD, which was also filter sterilised through a 0.2 µm filter (Sigma-Aldrich, UK), at an initial concentration of $1x10^6$ cells/ml. Stressed cell populations were cultivated in 12 ^{o}P hopped wort further supplemented with 5% (v/v) ethanol, and also sterile filtered as above. These

experimental conditions were designed to provide an environment of sub-lethal stress which would be challenging for cells, but not impact viability.

Both control and experimental cell cultures were grown at 25^{o}C for 40 hrs, representing the point at which all cell populations had shifted to stationary phase. During this period of growth, samples were obtained for metabolome analysis at representative and similar points in the respective growth curves. At each point total cell count and viability were again assessed and subsequently 500µl aliquots of each culture were mixed with 500µl of filter sterilized (0.2 µm (Sigma-Aldrich, UK)), ice cold glycerol (50% v/v). These aliquots were frozen at -80^{o}C prior to analysis.

2.14.2 Cell preparation for Liquid Chromatography-Mass Spectrometry

Approximately 1x10^{9} yeast cells were separated from the media, placed into a 2 ml milling tube (Fisher Scientific, UK) and accurately weighed. The cells were subsequently quenched with a 500 µL aliquot of ice cold 80% methanol (Sigma-Aldrich, UK), vortexed for 20 secs then stored at -20^{o}C. In preparation for liquid chromatography mass spectrometry (LCMS) analysis, samples were thawed, ceramic beads added (6 x 2.8 mm) (Fisher Scientific, UK) and cells were lysed in a cold room (8^{o}C) using a Beadmill 24 (Omni International; Kennesaw, GA) at 5.5m/s using 3 x 1-minute cycles with 2-minute dwell time to allow samples to cool. Microscopy was used to confirm that this program lysed all of the cells to the same extent between all yeast strains. Lysates were then centrifuged at 14,000 r.c.f. at 4^{o}C, and the supernatant retained. Each sample was then split into a 100µl portion for reverse phase separation and a 200µL portion for hydrophilic interaction chromatography (HILIC) separation. Reverse phase separation separates less polar compounds with a high retention by using alkyl chains covalently attached to the stationary phase (Lu *et al.*, 2010; Xiu *et al.*, 2014). While HILIC separation is used to identify smaller polar compounds (Xiu *et al.*, 2014). The HILIC aliquot was dried using a Savant SpeedVac centrifugal evaporator (ThermoFisher, UK), and reconstituted in 200µL of acetonitrile (VWR, UK). For each mode of chromatography, a single pooled QC sample was produced as a composite of

all yeast extracts on an equal volume basis. Samples were placed into 0.2ml amber HPLC vials (Sigma- Aldrich, UK) and capped prior to analysis.

2.14.3 Yeast metabolite detection using LCMS

LCMS analysis was performed using an Agilent 1260 Infinity II UHPLC, coupled to an Agilent 6546 tandem Quadrupole – Time of Flight mass spectrometer (Agilent Technologies, UK).

Reverse phase separation was performed using a Kinetex C18 column (2.6u, 150 x 2.1mm; Phenomenex, Macclesfield, UK) held at 40^{o}C. Solvents A and B comprised 5% and 95% acetonitrile in water (v/v) plus 0.1% formic acid. The gradient, flowing at 0.3ml/min, ran from 5% solvent B to 15% at 2mins, rose to 25% at 4 mins, 35% at 8 mins, 45% at 10 mins and 65% at 12 mins. The gradients then moved to 100% at 13 mins, were held until 18 mins, then returned to starting conditions by 18.2 mins, with a further 3.8 mins of re-conditioning. The electrospray ionization (ESI) source used gas and sheath gas temperatures of 320^{o}C, and 350^{o}C respectively, drying gas and sheath gas flows of 8 and 11 L/min respectively, with the nebuliser set to 35 psi. VCAP and nozzle voltages were set to 3500 V and 1000 V respectively, with Fragmenter, Skimmer and Octopole RF voltages of 110, 65 and 750 V respectively. MS1 data (MS1 data represents the initial m/z ratio of all detected molecular ions) was acquired in both (+) and (-) ESI, between 50 m/z-1700 m/z.

HILIC separation was based on the protocol of Dai and Hsiao (2019), and performed using a Poroshell120 HILIC-Z column (2.7u, 150 x 2.1mm; Agilent Technologies) held at 50^{o}C. Solvents A and B comprised water and 85% acetonitrile in water (v/v) respectively, including 10mM ammonium formate (Sigma- Aldrich, UK) at pH 9, plus Infinity lab deactivator additive (Agilent Technologies), at a final concentration of 2.5µM. The gradient flowing at 0.25ml/min, was held at 96% solvent B for 2 mins, then decreased to 88% at 5.5 mins. This was held until 8.5 mins, and decreased to 86% at 9 mins. This was held until 14 mins, then decreased to 82% at 17 mins. The gradient moved to 65%B at 23 mins, held for 1 min, then returned to 96%B at 24.5 mins, with

a further 1.5 mins for re-equilibration. The ESI source used gas and sheath gas temperatures of 225°C, and 350°C respectively, drying gas and sheath gas flows of 13 and 12 L/min respectively, with the nebuliser set to 35 psi. VCAP and nozzle voltages were set to 3500 V and 0 V respectively, with Fragmenter, Skimmer and Octopole RF voltages of 125, 45 and 750 V respectively. MS1 data was acquired in (-) ESI, between 50 m/z-1700 m/z. The UHPLC system (only) was subjected to an overnight 0.5% phosphate wash ahead of HILIC analysis as per technical note 5991-9516EN (Agilent Technologies, 2018).

For MS1 acquisition, the column was conditioned with 8 x 5 µL injections of QC samples for reverse phase separation, and 15 x 3ul injections for HILIC separation. Thereafter, samples were injected in a randomised order, with a QC sample injected after every 5 analytical runs. MS2 data was acquired in Auto MS-MS mode with collision energies fixed at 10, 20 and 40 volts, using separate preferred ion lists to avoid coelution of targets within 0.2 mins. MS2 data is produced using the ions obtained from the MS1 spectra, these molecular ions are selectively fragmented and analysed through another stage of mass spectrometry profiling. The m/z ratio pattern of the resulting fragments is then used in order to generate a fragment map of each MS1 ion, this is used to identify the analyte with greater accuracy.

2.14.4 Mass spectrometry data analysis and analyte identification

For data analysis, the total ion chromatograms (TIC) of repeated QC sample injections were visually assessed to check comparability of runs throughout the data set, in order to maintain column accuracy and validity. Files for each mode of analysis were time aligned to the central QC sample in Profinder software (v10, Agilent Technologies), then batch recursive feature extraction was used to extract all MS1 features (peak height >4000). This resulted in feature pools of 3,440 for (+) ESI reverse phase, 2966 for (-) ESI reverse phase and 2986 (-) ESI HILIC. Extracted files were then exported to Mass Profiler Professional (MPP; v15, Agilent Technologies) for statistical analysis, and initial PCA plots generated to assess data quality. To isolate features of interest between stressed versus unstressed conditions, features were initially filtered on the

basis of significant difference from control ($p<0.01$; one-way moderated t-test) and then either fold change (>15, further limited to peak heights of >10,000) or total abundance (>100k for reverse phase, >500k for HILIC). The shortlists of up/downregulated features were exported as preferred inclusion lists for MS2 analysis. Following this second round of data acquisition, compounds were extracted using Mass Hunter Qualitative Analysis software (v10, Agilent Technologies), and the resulting spectra exported to Sirius GUI software (v4.9.5; Dührkop et al., 2019) for putative identification. The Sirius, CSI:Finger ID (Dührkop et al., 2015) and Canopus (Feunang et al., 2016; Dührkop et al., 2020) modules were employed to provide *de novo* chemical formulae prediction, identity match scores and to predict chemical class, respectively. The Yeast Metabolome Database and associated mine (as of November 2021) were employed for CSI:Finger ID analysis. Successfully annotated compounds were manually assembled into entity lists within MPP for heatmap generation.

2.15 Single cell RNA sequencing

2.15.1 Cell preparation

Cultures derived from the yeast strains CBS 1260, M2 and NCYC 1332 were prepared from stocks maintained on agar slopes. In each instance, 10ml of sterile YPD was inoculated with cells and cultures were grown at 25^{o}C for 48hrs in an orbital shaker (Certomat BS-1, Sartorius, USA). Total and viable yeast counts were performed as described in Section 2.3.2 and these were used to prepare duplicate 'master' cell suspensions. Of these, one was used to prepare a control population of 'unstressed' cells, while the second was used to prepare a 'stressed' population. The designated unstressed cells were cultivated in autoclaved YPD, which was also filter sterilised through a 0.2 µm filter (Sigma-Aldrich, UK), at an initial concentration of 1x10^{6} cells/ml. Stressed cell populations were cultivated in 12 ^{o}P hopped wort further supplemented with 5% (v/v) ethanol, and also sterile filtered as above. These experimental conditions were designed to provide an environment of sub-lethal stress which would be challenging for cells, but not impact viability.

Both control and experimental cell cultures were grown at 25^{o}C for 40 hrs, representing the point at which all cell populations had shifted to stationary phase. During this period of growth, samples were obtained for gene expression analysis at representative and similar points in the respective growth curves. At each point total cell count and viability were again assessed and subsequently 500µl aliquots of each cell culture were mixed with 500µl of filter sterilized (0.2 µm (Sigma-Aldrich, UK)), ice cold glycerol (50% v/v). These aliquots were frozen at -80 ^{o}C prior to sequencing.

2.15.2 Single cell library preparation

Cell aliquots were thawed on ice and cells pelleted at 3000rpm for 3 mins at 4^{o}C. Subsequently the cell pellet was resuspended in ice-cold filter sterilised PBS pH 7.2 (1L 10x PBS was prepared by adding into 800 mL MilliQ water (Sigma- Aldrich, UK): 81 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 (Sigma- Aldrich, UK), pH adjusted to pH 7.2 (with HCl solution) and volume up to 1L with MilliQ water). Cell aliquots were prepared to a final concentration of 10^6 viable cells/ml, with the target of achieving greater than 500 single cells for sequencing. For library preparation and barcoding, the Chromium Single Cell kit (v2) protocol was used (10x Genomics, USA). However, in this instance, 100x zymolyase (100mg/ml zymolyase (AMSBIO, UK) was dissolved into 1x reverse transcriptase buffer (Quantiscript RT Buffer), replacing a 1µl water allocation in the reverse transcription master mix. Irrespective, the total reaction volume was made up to 100 µl. The microfluidic Chromium Single Cell Chip (10x Genomics, US) was prepared as described by the manufacturer; a cell suspension was added to the single-cell master mix and immediately transfer to the microfluidics chip. Hydrogel beads (10x Genomics, US) were added and partitioning oil according to the protocol. Cells were encapsulated with hydrogel beads using the 10x Genomics Chromium device. After emulsification, reverse transcription, clean-up, cDNA amplification and library construction were all performed as detailed in the Chromium Single Cell kit (v2) protocol (10x Genomics, USA).

2.15.3 Single cell sequencing and data analysis

Single cell RNA sequencing was performed in accordance with the Chromium Single Cell kit (v2) protocol (10x Genomics, USA). Once cell libraries were prepared (Section 2.15.2), sequencing was performed using the Illumina NextSeq 500 platform (Illumina, USA), generating >50,000 reads per cell. This required 85-100 million reads per sample using the Illumina NextSeq500 high Output 150 cycle kit (Illumina, USA). Sequencing data was processed using the Cell Ranger software (10x Genomics, USA), producing .cloupe files for single cell gene expression of the cell populations. A summary of the parameters investigated and the output files obtained can be found in Table 2.7.

Table 2.7 Obtained list of .cloupe files for single cell gene expression

.cloupe File
Unstressed CBS 1260
Unstressed M2
Stressed CBS 1260
Stressed M2
Unstressed CBS 1260 vs stressed CBS 1260
Unstressed M2 vs stressed M2
Unstressed CBS 1260 vs M2
Stressed CBS 1260 vs M2

In order to produce the data files, the sequencing data was aligned using the reference genome belonging to the *Saccharomyces* strain S288c. The procured .cloupe files were then analysed using Loupe Browser 6 (10x Genomics, USA). From this, comparisons in single cell gene expression between experimental conditions could be performed. Gene names and functions were obtained through the *Saccharomyces* Genome Database (https://www.yeastgenome.org).

Chapter 3. Genotypic and phenotypic characterisation of brewing yeasts and the physiological response to brewing stress

3.1 Introduction

Species belonging to the genus *Saccharomyces* (literally meaning 'sugar fungus') are widely used in brewing fermentations. This is because they exhibit a range of desirable attributes, including good sugar consumption, ethanol production and the ability to create desirable flavour compounds (Lodolo *et al.*, 2008). Furthermore, such yeasts are robust and relatively tolerant to stress factors associated with industrial fermentations and handling of cultures. Within the *Saccharomyces* genus there are two key species that are utilized in brewing: *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*. Historically the most common type of yeast was *S. cerevisiae,* used to produce ale-type products. Indeed, the term *cerevisiae* originates from the Latin for 'beer' (Mortimer, 2000). More recently, use of the yeast species *S. pastorianus* has surpassed *S. cerevisiae,* at least in terms of beer volume, since the latter is widely used in the production of lager products, which are popular for their 'clean' flavour profile (White and Zainasheff, 2010).

The genetic diversity of yeasts used in the brewing industry reflects the current range of styles and flavours of beers being produced by breweries. It is known that strains belonging to the species *S. cerevisiae* and *S. pastorianus* have evolved separately, with *S. cerevisiae* yeasts being of distinctly older lineage (Nguyen *et al.*, 2011; Gallone *et al.*, 2016). There is strong evidence to suggest that strains belonging to the species *S. pastorianus* have arisen more recently, as a result of the natural hybridization between two parental strains, currently believed to be *S. cerevisiae* and *S. eubayanus* (Dunn and Sherlock, 2008; Nguyen *et al.*, 2011; Gallone *et al.*, 2016). There are at least two main groups of lager yeast, designated as Saaz and Frohberg types (Dunn and Sherlock, 2008; Monerawela *et al.*, 2015; Gallone *et al.*, 2018). The Frohberg group possess most of the DNA from each parent and comprises the majority of current lager

production strains, including the commonly used strain W34/70 (Monerawela *et al.*, 2015). In contrast, the Saaz group has lost a significant proportion of the *S. cerevisiae* genome and includes strains previously designated as *S. carlsbergensis* (Walther *et al.,* 2014). As a consequence of these genetic differences, each group possesses significantly different attributes, such as optimum growth temperature, attenuation capabilities and maltotriose utilisation (Gibson *et al.*, 2013).

Despite variation between lager yeast strains, there is considerably greater diversity within *S. cerevisiae* ale yeasts. This is because these organisms have undergone domestication over several centuries, which means that a broad spectrum of yeasts have evolved with their own unique qualities. Irrespective of this diversity, all ale yeasts are often referred to as 'top fermenting', due to their flocculation characteristics, and perform best at temperatures of between 20-30°C (Walker and Stewart, 2016). While much is known about ale yeast evolution (Gallone *et al.*, 2016, 2018), there has been recent interest in farmhouse brewing yeasts, such as those historically and currently still found in Norway (Preiss *et al.*, 2018). These organisms appear to be unique in that they ferment quickly and produce an acceptable portfolio of flavour compounds at high temperatures. It is believed that these strains, referred to as Kveik yeast, may form an independent group of closely related yeasts strains, being distinct in both genome and fermentation properties (Garshol, 2020). In addition to traditional 'ale' and 'lager' strains, alternative types of yeast are sometimes also used for beer production, albeit almost exclusively for niche products. One such yeast is *Brettanomyces*, which is often associated with beer spoilage, but in some instances can lend unique qualities to a beverage. *Brettanomyces* yeast produce a range of acids (Moktaduzzaman *et al.*, 2016; Serra Colomer *et al*., 2019) and phenolic compounds (Harris *et al.*, 2009; Serra Colomer *et al*., 2019) which are desirable in certain sour and lambic beers (Spitaels *et al.*, 2014). Consequently, it can be seen that when considering the total yeast flora within the industry, there is a significant amount of diversity. This genetic diversity is important in generating products with unique characteristics, often as a result of different characteristics in relation to nutritional requirements, growth rates, flavour production, sugar utilization, flocculation characteristics and stress tolerances (Gallone *et al.*, 2016; Walker and

Stewart, 2016; Capece *et al.*, 2018). However phenotypic variation is not only attributed to gross genetic divergence. Within a species, variation may occur as a result of differences in gene expression, genetic feedback loops and metabolic networks (Attfield *et al.*, 2001; Gasch *et al.*, 2017). In this section, a diverse group of brewing yeast strains were selected to investigate the variation in response to key fermentation parameters.

This study aims to identify, differentiate and characterise brewing yeast strains based on growth kinetics, permissive growth temperatures, their tolerance to various brewing related stresses and via genetic fingerprinting using PCR based techniques. Specifically, the impacts of ethanol, osmotic and oxidative stresses were examined to determine how phenotypic characteristics vary between strains, and to identify the absolute tolerance limits of each yeast.

3.2 Analysis of brewing classification by permissive growth temperature

One of the classical mechanisms for classification of lager and ale brewing strains is through analysis of permissive growth temperature (Kishimoto and Goto, 1995; Libkind *et al.*, 2011). Ale strains are able to grow at 37°C, while lager yeasts can only be cultivated at colder temperatures (Baker *et al.*, 2019). Although disparity in growth tolerance offers a potential mechanism for classification of traditional brewing species, the response of non-*Saccharomyces* brewing strains (including Kveik yeasts) has not been as widely explored. To investigate this, each yeast strain was serially diluted from $1x10^6$ cells/ml to $1x10^2$ cells/ml and 'spotted' onto YPD agar plates as described in Section 2.5. Subsequently these agar plates were incubated at 4°C, 25°C and 37°C for 7 days before visual analysis for the production of biomass.

It can be seen that there were differences in the growth performance between each of the brewing yeast strains (Figure 3.1). When cells were cultivated at 37°C only the ale strains (NCYC 1332, M2 and Kveik) and the *Brettanomyces anomalus* yeast (BA)

survived, with no signs of growth inhibition at any cell concentration. Although BA did display growth, there was observable inhibition at lower cell concentrations, manifested by smaller spot sizes indicating a reduced number of colonies. On the other hand, no growth was apparent in the lager strains at all. However, as anticipated, all strains thrived at 25°C, with no signs of growth inhibition signifying a likely temperature optimum. When the temperature was reduced to 4°C, growth was restricted and was typically only seen when high concentrations of starting culture were provided ($1x10^6$ cells/ml). This was true for all strains except for the *Brettanomyces anomalus* yeast (BA), which was unable to grow at this temperature. In contrast, the lager strain CBS 1174 appeared to be the most tolerant at this temperature, displaying further growth at 10^5 cells/ml. The results shown here lend themselves with what is previously known of lager and ale strains; at higher temperatures *S. cerevisiae* strains are able to thrive, while the more cold tolerant lager strains are unable to grow (Baker *et al.*, 2019). Although small amounts of growth were seen in most strains at 4°C, the Saaz yeast CBS 1174 performed the best, perhaps indicative of the higher portion of the cold tolerant *S. eubayanus* within the genome as suggested previously (Gibson *et al.*, 2013; Baker *et al.*, 2019; Langdon *et al.*, 2019).

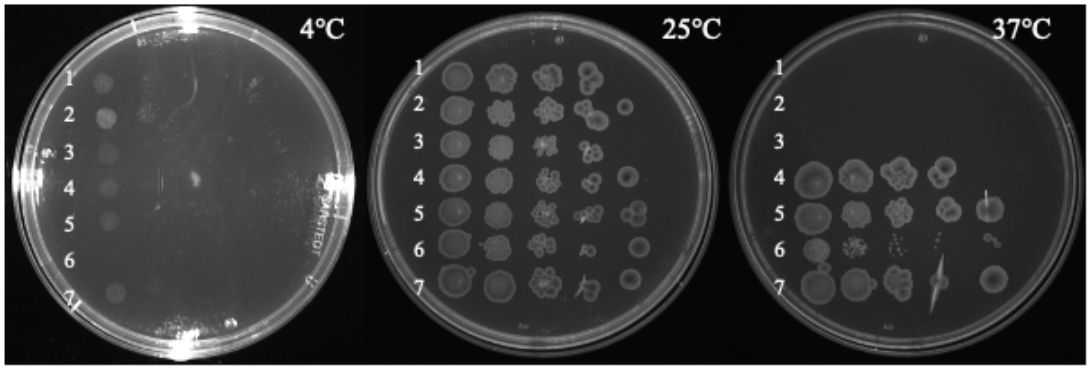

Figure 3.1. Yeast strain determination based on growth temperature tolerance and preference. Cell seeds of 5µl containing decreasing cell concentrations of 10^6 to 10^2 (left to right) were spotted on YPD-agar plates and incubated at three test temperatures of 4°C, 25°C and 37°C for 7 days aerobically. 1- W34/70, 2- CBS 1174, 3- CBS 1260, 4- NCYC 1332, 5- M2, 6- BA, 7- Kveik.

3.3 Genus and species identification through RFLP analysis of ITS region

Although analysis of temperature tolerance provides data that is useful in the broad characterization of yeasts, advances in molecular biology have allowed alternative methods to be developed for more precise identification. At the current time, the internal transcribed spacer (ITS) region of the yeast genome acts as the primary fungal barcode marker (Fujita *et al.*, 2001; Pham *et al.*, 2011). Consequently, each yeast strain was characterized to the genus and strain level based on the amplification of this specific region of the genome. This was performed using primers designed to amplify the internal transcribed spacer region, including the 5.8S rRNA gene. To enhance specificity, each PCR product was also digested using the restriction endonucleases *Hae*III, *Hinf*I and *Cfo*I according to Section 2.6.3. Each yeast was identified by comparing the size of the ITS region and their derived restriction fragments to previously published reference data (Esteve-Zarzoso *et al.*, 1999; Pham *et al.*, 2011; Bokulich and Mills, 2013).

It can be seen from Figure 3.2(a) that all of the strains except for the *Brettanomyces* yeast (BA) yielded an ITS region of approximately 880bp in length, correlating with previous analyses of *Saccharomyces* yeasts. The wild yeast strain BA displayed an ITS region length ~500bp. Further analysis was required to differentiate the *Saccharomyces* species. To achieve this, RFLP analysis of the ITS DNA products was conducted using the restriction endonucleases *Hinf*I, *Hae*III and *Cfo*I. The *Hinf*I digestion (Figure 3.2b), was not sufficient to discriminate between species, with all *Saccharomyces* strains exhibiting fragment lengths of ~365bp and ~130bp. However, BA was again disparate with band lengths of ~225bp and 80bp. *Cfo*I digestion (Figure 2c) also revealed identical fragment patterns for all strains expect for BA. Bands were observed at ~340bp, 320bp and potential faint bands representing fragments around 120bp among the conventional brewing strains. While BA differed in fragment profile possessing a DNA band at ~270bp.

Digestion using the enzyme *Hae*III was the most useful in differentiating each yeast species. Analysis of the restricted fragments (Figure 3.2d) indicated that strains W34/70, CBS 1260, NCYC 1332, M2 and Kveik all displayed 4 bands of ~320bp, 220bp, 180bp and 140bp, typical of *S. cerevisiae* yeast and Frohberg type *S. pastorianus* yeast (Pham *et al.*, 2011). However, CBS 1174 and BA both had different and distinct profiles. CBS 1174 showed 3 fragments of length of ~500bp, 220bp and140bp (indicative of a Saaz type lager yeast) and BA yielded 2 fragments of ~400bp and 110bp. Although the *Hae*III RFLP only highlighted contrasting fragment profiles between 3 groups of yeasts, it did allow differentiation between BA, *S. cerevisiae* and Saaz lager yeast, and Frohberg type lager strains. Interestingly, the results from the *Hae*III digest of ITS regions show that Frohberg type lager strains each produced 4 bands of the same size as the *S. cerevisiae* ale strains, meaning that this method was not suitable for differentiating this type of lager (*S. pastorianus*) yeast from *S. cerevisiae*. However, it was sufficient for characterizing the Saaz type lager strains. The differences in fragment profile seen for the different groups of *S. pastorianus* (lager) strains are likely to reflect the hybridization events that created them. Frohberg type strains contain a higher proportion of the *S. cerevisiae* genome, which is likely the primary reason why they yield the same restriction profiles as *S. cerevisiae* strains. In contrast, the Saaz type yeast (CBS1174) contain a higher proportion of *S. eubayanus* genome, which may lead to divergence in DNA restriction fragment pattern (Baker *et al.*, 2015).

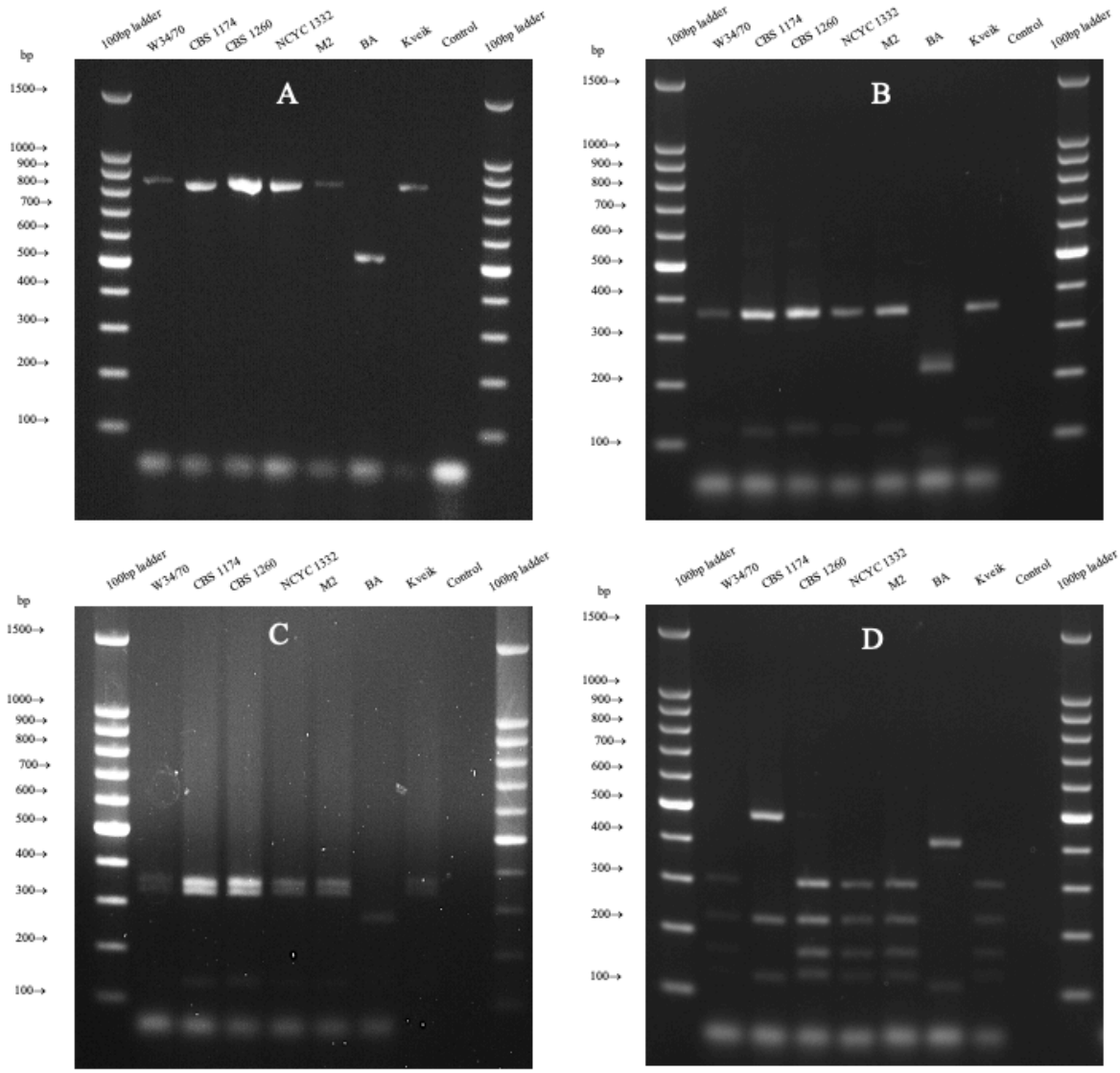

Figure 3.2. ITS PCR amplification and further RFLP analysis for the identification of the studied yeast strains. A, PCR amplified ITS regions. B, *HinfI* restriction fragments. C, *CfoI* restriction fragments. D, *HaeIII* restriction fragments. Each image displays the gel electrophoresis images with 100bp reference ladders in lane 1 and 10, and negative control in lane 9.

3.4 Identification of yeast strains by DNA fingerprinting (interdelta PCR)

Further to genus and species identification, each yeast strain was genetically fingerprinted by analysis of yeast interdelta regions. These repetitive sequences are associated with the terminal repeats of the retrotransposons TY1 and TY2. The position of such repeats vary in both number and genomic position between strains and therefore provides a useful tool for genetic fingerprinting and differentiation of brewing yeast strains (Legras and Karst, 2003; Xufre *et al.*, 2011). Consequently, this technique was used to fingerprint each strain as described in Section 2.7.1.

Using interdelta PCR it was possible to produce a genetic fingerprint for each yeast (Figure 3.3). From this data it was evident there were distinct fingerprint patterns which allowed for the accurate differentiation of strains. It should be noted that there were some commonalities seen between strains. For example, fragments of ~1950,1750, 1100 and 490bp were all found in both strains W34/70 and CBS 1260. This is most likely a result of their close genetic heritage, both being Frohberg lager type yeasts. However, despite these similarities, there were also significant differences that allowed clear differentiation, for example, large DNA fragments at ~5000bp and ~900bp were present for W34/70, but absent from the CBS 1260 fingerprint. This trend was also seen in strains NCYC 1332 and M2 which also showed common bands (for example, 2 bands just under 1500bp and a further cluster above 1000bp), perhaps indicative of them being ale (*S. cerevisiae*) strains. However, for these yeasts, observable differences were observed at ~600bp and ~6000bp. Interestingly, the Kveik strain appeared to show some commonalities with the other *cerevisiae* strains, but also showed the most distinct profile, likely to reflect the heritage of this group of organisms, certainly in relation to more traditional ale strains such as M2 and NYCY 1332. Furthermore, the Saaz lager strain CBS 1174 also yielded a fingerprint that shared few similarities to the other lager strains analysed and the ale yeasts. It should be recognized that although the size and the number of amplicons is likely to be somewhat related to genetic origin, it may also be impacted by ploidy and the frequency of viable mutations. Irrespective, the inter delta PCR fingerprinting

results shown here provide an accurate and reproducible methodology for strain differentiation, demonstrating that all strains investigated were unique.

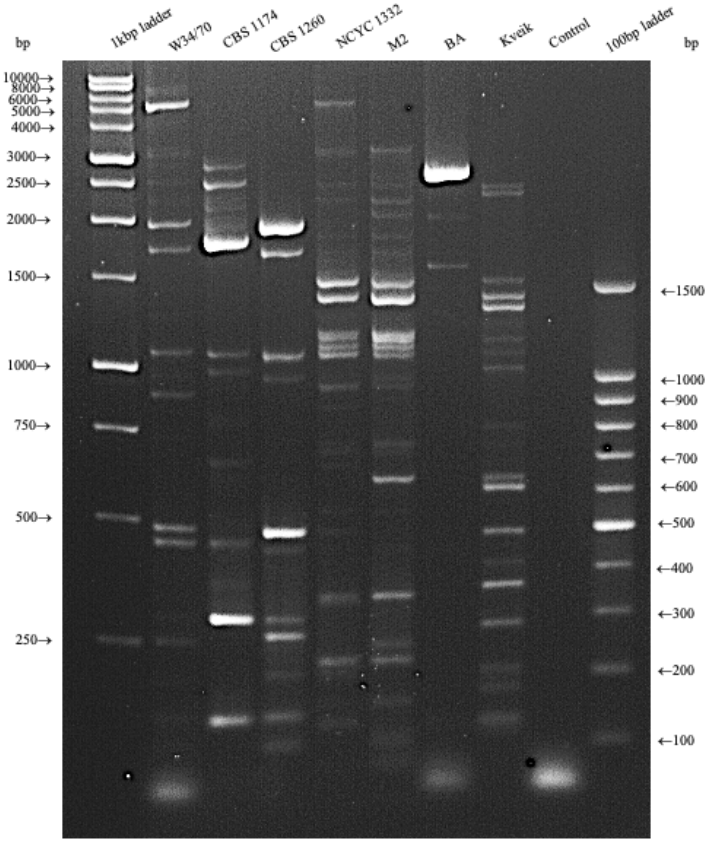

Figure 3.3. Gel electrophoresis image of PCR amplified interdelta regions found in the studied strains. The number and size of bands for each strain is determined by the interdelta regions present in the individual yeast genome and the subsequent amplification of these regions. The resultant fingerprint type image allows accurate visual differentiation between all studied strains. Lane 1: 1kbp ladder, lane 9: control, lane 10 100bp ladder.

3.5 Strain characterization based on growth kinetics

All brewing yeasts replicate by asymmetric division, whereby daughter cells bud from their respective parents, maintaining genetic uniformity and increasing biomass (Müller *et al.*, 1980; Kennedy et al., 1994; Austriaco Jr, 1996). While this method of replication and growth is conserved for brewing yeasts, specific growth kinetics can vary widely. This variation can in part be a result of differences in metabolic characteristics and the ability to utilize sugars such as melibiose, all of which centers around the genetic constitution of the yeast strain (Enevoldsen, 1981; Turakainen *et al*., 1993; Hazell and Attfield, 1999; Vincent *et al.*, 1999; Sanchez *et al*., 2012). Therefore, when comparing growth kinetics of different yeast strains, it is highly unlikely to see identical patterns. As such strain characterization and differentiation can be achieved by simply comparing growth dynamics under various conditions.

Growth kinetics were measured by inoculating each strain into 200µl of YPD in a 96-well plate. Following this the cultures were incubated at 25°C and the optical density measured at 600nm every 15 mins, in order to produce growth curves as described in section 2.4. In Figure 3.4 it can be seen that each strain showed significant variation in the shape of the resulting sigmoidal growth curves, representing differences in metabolism and growth kinetics. Despite this, each strain does follow the same general configuration, consisting of an initial lag phase, a logarithmic growth phase and finally a stationary phase.

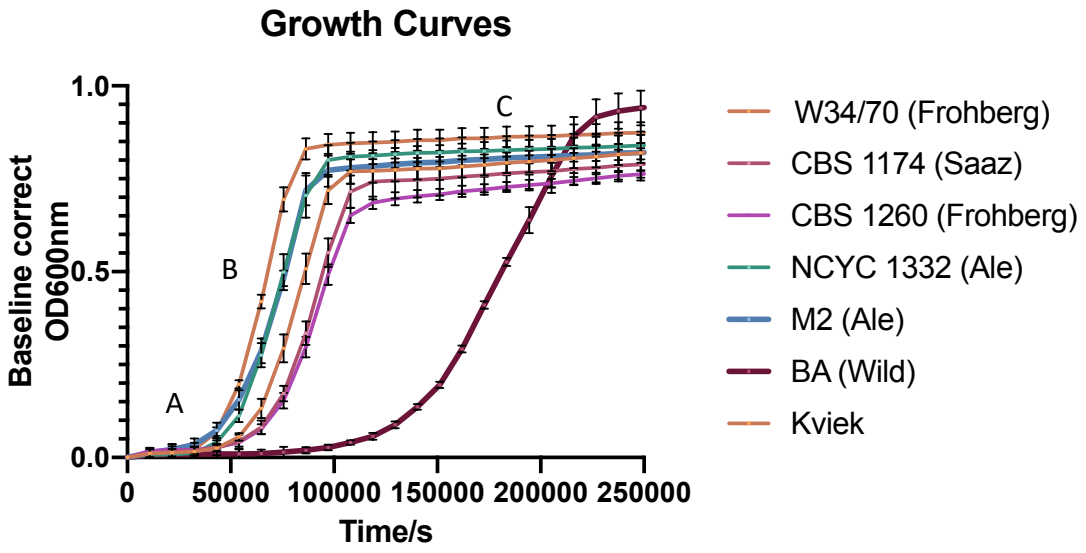

Figure 3.4. Growth curves for all studied yeast strains based on increasing OD600nm with time, when cultured at 25°C in YPD. The three distinct growth curve phases A, B and C are present: lag phase, exponential growth phase and stationary phase respectively. Measurements were taken at 15min intervals, however for clarity only readings every 3hrs are displayed. Error bars are representative of standard deviation of triplicate repeats.

As indicated in Figure 3.4, there were discernable differences in growth kinetics observed between the studied strains. The time required to complete the lag phase varied between ~32000s- 110000s, with the Kveik strain entering exponential growth phase the fastest and BA displaying the longest lag time. The second phase of growth (exponential growth) also showed large differences between strains. Analyzing this phase of growth by interpreting slope gradient data revealed that strains Kveik and NCYC 1332 had the fastest growth rate, while BA and CBS 1260 displayed the slowest. Interestingly, aside from BA, the lager strains entered stationary phase later than ale strains and also failed to achieve as high a population limit, as indicated by a lower final optical density. The large differences observed in the length of time each of the strains spent in each growth phase also further demonstrates the physiological variation present among brewing strains.

Investigation into growth kinetics allowed further strain characterization that broadly correlated with the previously performed genotypic analyses. The strains that spent the least time in lag phase exhibited the fastest growth rate and subsequently entered stationary phase the quickest were all ale strains (*S. cerevisiae*). In contrast the lager yeasts showed the opposite profile. The wild strain (BA) had a distinctly unique growth curve, allowing easy differentiation. The data presented here gives greater understanding into the specific growth rate of each of the studied yeast strains, demonstrating their natural rapid growth under unstressed conditions. This provides a useful reference for comparing data obtained in the subsequent brewing stress experiments described below. This is primarily because understanding the basal growth dynamics of each strain is important in determining the effects of brewing related stress factors on yeast cell proliferation.

3.6 The effect of stress on yeast cell growth

As previously discussed in Section 1.8, it is important to note that during beer fermentations yeast experience stress from environmental challenges such as the presence of ethanol, the emergence of ROS and high sugar osmolality. These can lead to significant pressures being exerted on yeast cells, resulting in cellular damage and ultimately cell death. The degree of tolerance exhibited by yeast varies widely from strain-to-strain and therefore offers an additional mechanism for differentiating, while also providing an understanding of the capacity of yeast strains to survive under adverse conditions (Carrasco *et al*., 2001; Lewis *et al.*, 2010). This is significant because the way in which different yeast strains respond to brewing stress factors can impact fermentation and, as a result, the finished product. This can have an impact on events such as strain selection for particular types of brewing fermentations; for example ethanol tolerant strains are likely to be preferentially used in high gravity brewing. Therefore, to optimize efficiency in brewing fermentations, understanding the stress response capabilities and dynamics of a range of yeast cells can provide useful information. In order to test the stress response capabilities of a range of yeast cells, yeast ‘spots’ of different cell concentrations were grown on YPD plates containing

increasing concentrations of the stressor's ethanol, hydrogen peroxide, sorbitol, zinc and copper as described in Section 2.8. As well as the conventional brewing related stressors, zinc and copper were also included as they have been applied in other similar studies (Holland *et al.*, 2014). Hence it was anticipated that their inclusion may provide a more detailed view on the stress response capabilities of brewing yeast strains. The resulting generation of colony forming units (CFUs), derived from viable cells on these stress plates, were used visually to indicate tolerance to each stress factor. It is worth noting here, that throughout this thesis unstressed conditions should be considered as a lack of deliberate stress, as such there may still be low levels of stress inducing compounds in the control media (YPD).

3.6.1 Ethanol stress tolerance

The production of ethanol by yeast is a key aspect of traditional brewing, crucial for the balance of flavours and mouthfeel, and contributing towards the sterility of the final product. It is typical for packaged beer to contain between 3-7% ethanol (v/v), however it is not uncommon for fermentations to finish in excess of 10% (v/v) (Odumeru *et al.*, 1992; Briggs *et al.*, 2004). It is known that the ethanol produced by yeast can cause damage to several components of the yeast cell, primarily the plasma membrane (Piper, 1995; Mizoguchi and Hara, 1998), leading to disruption of solute balance within the cell (Madeira *et al.*, 2010), reduced cell size (Lentini *et al.*, 2003; Canetta *et al*., 2006), DNA damage (Jenkins *et al.*, 2009), reduced growth rate (Yoshikawa *et al.*, 2009) and the occurrence of petite mutations (Piper, 1995; Chi and Arneborg, 1999; Canetta *et al*., 2006; Gibson *et al.*, 2007).

In order to test the sensitivity of yeast strains to ethanol, each yeast strain was exposed to increasing concentrations of ethanol, using bespoke 'stress plates' (Section 2.8). Specifically, 5µl aliquots of each yeast strain, serially diluted from 10^6 cells/ml to 10^2 cells/ml were spotted onto YPD agar plates containing ethanol ranging from 0% (v/v) to 30% (v/v). Subsequently, cultures were incubated at 25°C for 14 days to ensure sufficient time for growth to become visible. Stress tolerance was determined by visual inspection of growth on each plate as shown in Figure 3.5.

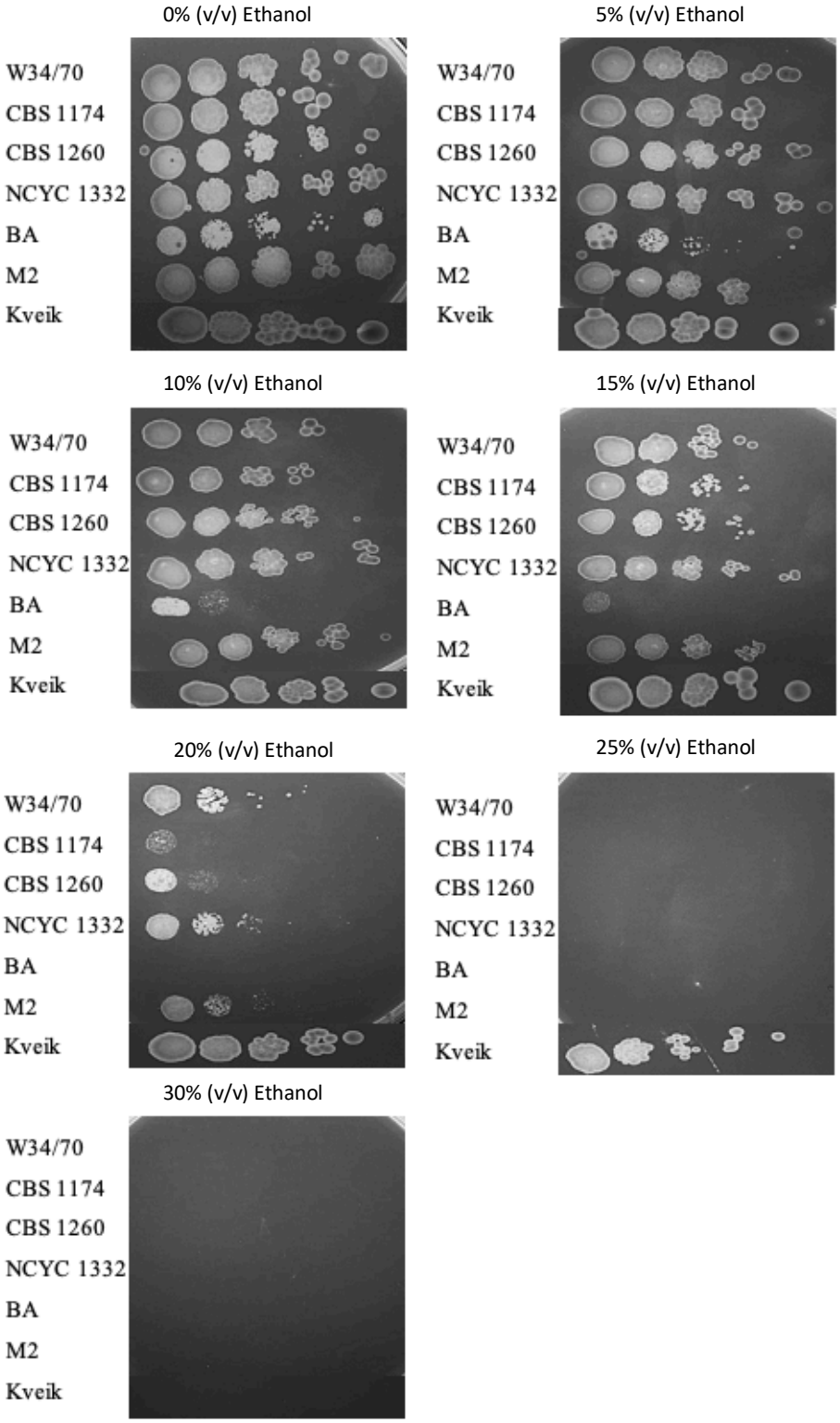

Figure 3.5. The effect of ethanol stress on yeast growth. Aliquots of 5µl of cells taken from cell stocks of $1x10^6$-$1x10^2$ cells/ml (left to right) were added to YPD agar plates containing 0-30% (v/v) ethanol. Plates were incubated aerobically at 25 °C for 14 days and analysed visually for the presence of growth.

It can be seen that all of the studied yeast strains were susceptible to increasing concentrations of ethanol, ultimately leading to an absence of observable growth when toxic concentrations were reached (Figure 3.5). The threshold at which this occurred varied widely between each strain. Across the brewing strains there was no detectable inhibition of growth from 0-10% (v/v) ethanol, whereas for the *Brettanyomces strain* (BA) there was a reduction in spot size at 5% (v/v) ethanol irrespective of initial cell concentration. This inhibition became more pronounced as ethanol concentration was increased and was more common across strains. At 15% (v/v) ethanol, growth inhibition was visible in W34/70, CBS 1174 and CBS 1260, particularly at lower initial cell concentrations. A similar trend was observed at 20% (v/v); all strains apart from the Kveik yeast displayed a significant reduction in CFUs (spot size) at cell concentrations below 10^6 cells/ml, with more pronounced growth inhibition in the strain CBS1174. On plates containing $\geq$ 25% (v/v) ethanol (v/v) only the Kveik yeast was able to show growth, displaying only a small degree of inhibition at low cell concentrations. However, at 30% (v/v) ethanol, no growth was detected.

In summary, it is apparent that the most susceptible strains to ethanol toxicity were the wild yeast, BA, and CBS 1174 from among the brewing strains. The yeast most tolerant to ethanol was the Kveik strain. Interestingly there did appear to be greater ethanol resistance in the *S. cerevisiae* (ale) strains (NCYC 1332, M2 and Kveik) compared to the lager strains CBS 1174 and CBS 1260 which showed greater susceptibility. However, with W34/70 being the most tolerant of the lager strains, there was very little evidence to indicate a relationship between lager yeast type (Saaz/Frohberg) and ethanol tolerance, perhaps indicative that stress tolerance is strain- rather than category-specific in traditional brewing yeasts.

3.6.2 Osmotic stress tolerance

Osmotic stress in brewing fermentations is caused by high concentrations of sugars and FAN in wort, leading to movement of both sugar and water into and out of the cell (D'Amore *et al*., 1987). The resulting affect is turgor pressure, causing the cell to use energy to prevent damage. However, even though a cell is capable of responding to osmotic stress, under intense conditions this can lead to cell shrinkage, loss in ability to take up sugars, and ultimately a loss in viability. In such scenarios growth rate and fermentation performance are significantly reduced (D'Amore *et al*., 1987; Pratt *et al*., 2003; Briggs *et al.*, 2004). To measure and compare the maximum tolerance limits for the studied strains, stress plates were utilized supplemented with sorbitol as an osmotic stressor (Pratt *et al.,* 2003). In each instance, 5µl aliquots of a yeast strain, serially diluted from 10^6 cells/ml to 10^2 cells/ml, were spotted onto YPD agar plates containing sorbitol ranging from 0% (w/v) to 60% (w/v). Subsequently, cells were incubated at 25°C for 14 days to ensure sufficient time for growth to become visible. Stress tolerance was determined by visual inspection of growth as shown in Figure 3.6.

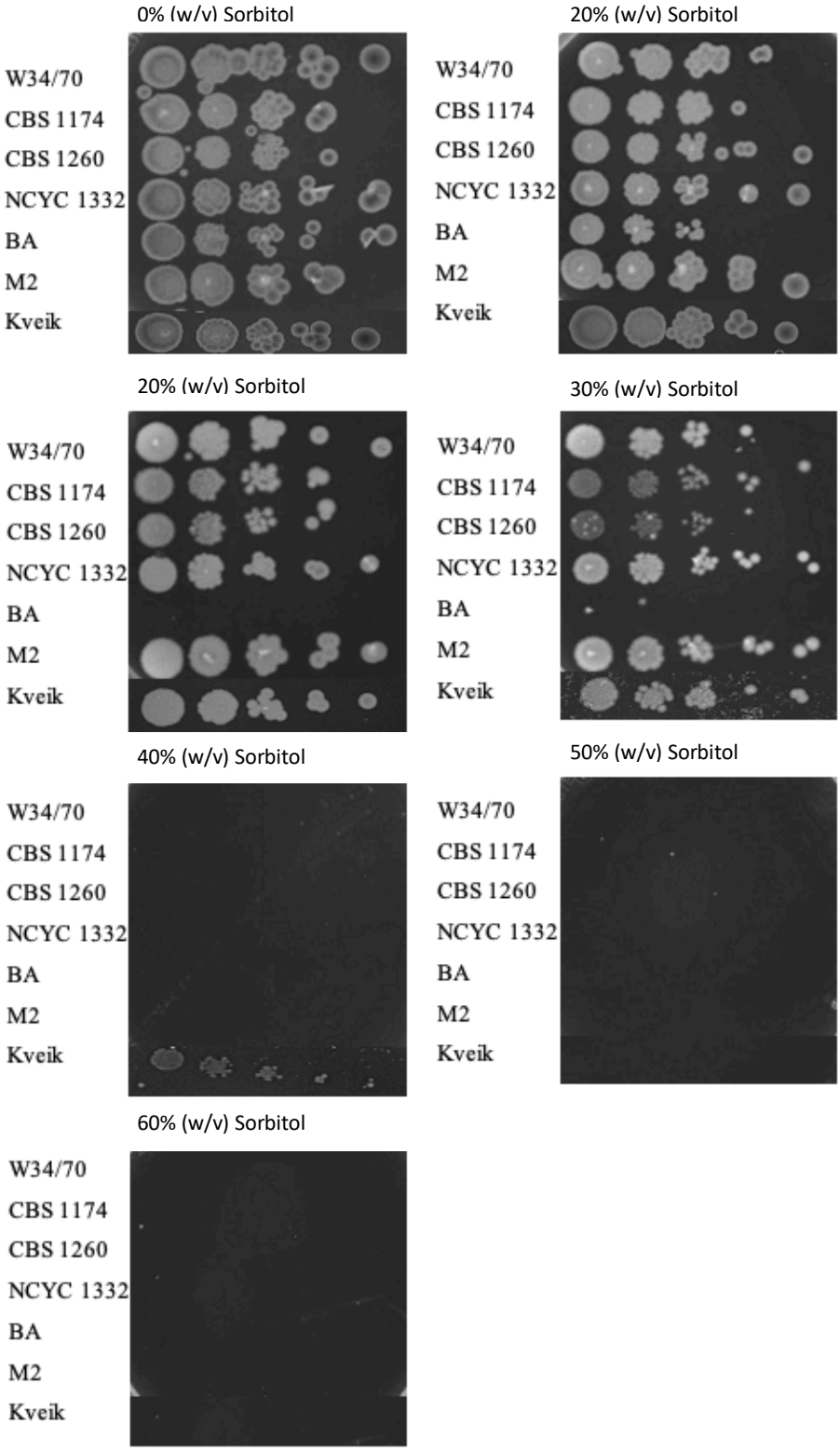

Figure 3.6. The effect of osmotic stress on yeast growth. Aliquots of 5µl of cells taken from cell stocks of $1x10^6$-$1x10^2$ cells/ml (left to right) were added to YPD agar plates containing 0-60% (w/v) sorbitol. Plates were incubated aerobically at 25 °C for 14 days and analysed visually for the presence of growth.

As expected, the brewing yeast strains showed a high tolerance to osmotic stress, demonstrated by a limited reduction in growth and CFU 'spot size' between 0-20% sorbitol (w/v) (Figure 3.6). However, the wild yeast strain, BA, displayed growth inhibition at just 10% (w/v) sorbitol, with a complete loss of observable growth when stress was increased to 20% (w/v). At 30% sorbitol (w/v) the first signs of growth inhibition appeared for the remaining strains, in particular CBS 1174 and CBS 1260, which displayed a reduced spot size at notably lower cell concentrations. Interestingly, at 40% sorbitol (w/v), growth in all strains was restricted, except in the Kveik yeast, where growth was seen at all cell concentrations, albeit with minor reduction in some instances (Figure 3.6). At 50% (w/v) sorbitol growth was restricted in all strains, including the Kveik yeast. Since 40% (w/v) sorbitol represented the limit of tolerance for conventional brewing strains, it was difficult to reveal any specific relationships between strain 'type' and osmotic response. However, based on the data obtained at 30% sorbitol (w/v), it did appear that the lager type yeast strains were slightly more susceptible to osmotic stress than the ale strains.

3.6.3 Oxidative stress tolerance

Wort oxygenation prior to fermentation is commonplace in the brewing industry (O'Connor-Cox and Ingledew, 1990; P J Verbelen *et al.*, 2009). This is because there is a requirement for yeast to assimilate available oxygen for the production of fatty acids and sterols, allowing cells to reproduce and create biomass needed for efficient fermentation (Briggs *et al.*, 2004; White and Zainasheff, 2010). However high oxygen concentrations can lead to the production of reactive oxygen species (ROS) such as H_2O_2 and $O_2^{\bullet-}$. The presence of ROS can lead to irreparable damage to DNA, proteins and the synthesis of lipids, ultimately leading to cell death and a significant decrease in population viability (Slupphaug *et a*l., 2003; Gibson *et al.*, 2007; Verbelen *et al.*, 2009). To determine the tolerance limits of the studied yeast strains to increasing levels of oxidative stress, hydrogen peroxide was applied in conjunction with bespoke stress plates as described in Section 2.8. In each instance, 5µl aliquots of yeast, serially diluted from 10^6 cells/ml to 10^2 cells/ml, were spotted onto YPD agar plates containing

H_2O_2 ranging from 0mM to 8mM. Subsequently, cells were incubated at 25°C for 14 days to ensure sufficient time for growth to become visible. Stress tolerance was determined by visual inspection of growth on each plate as shown in Figure 3.7.

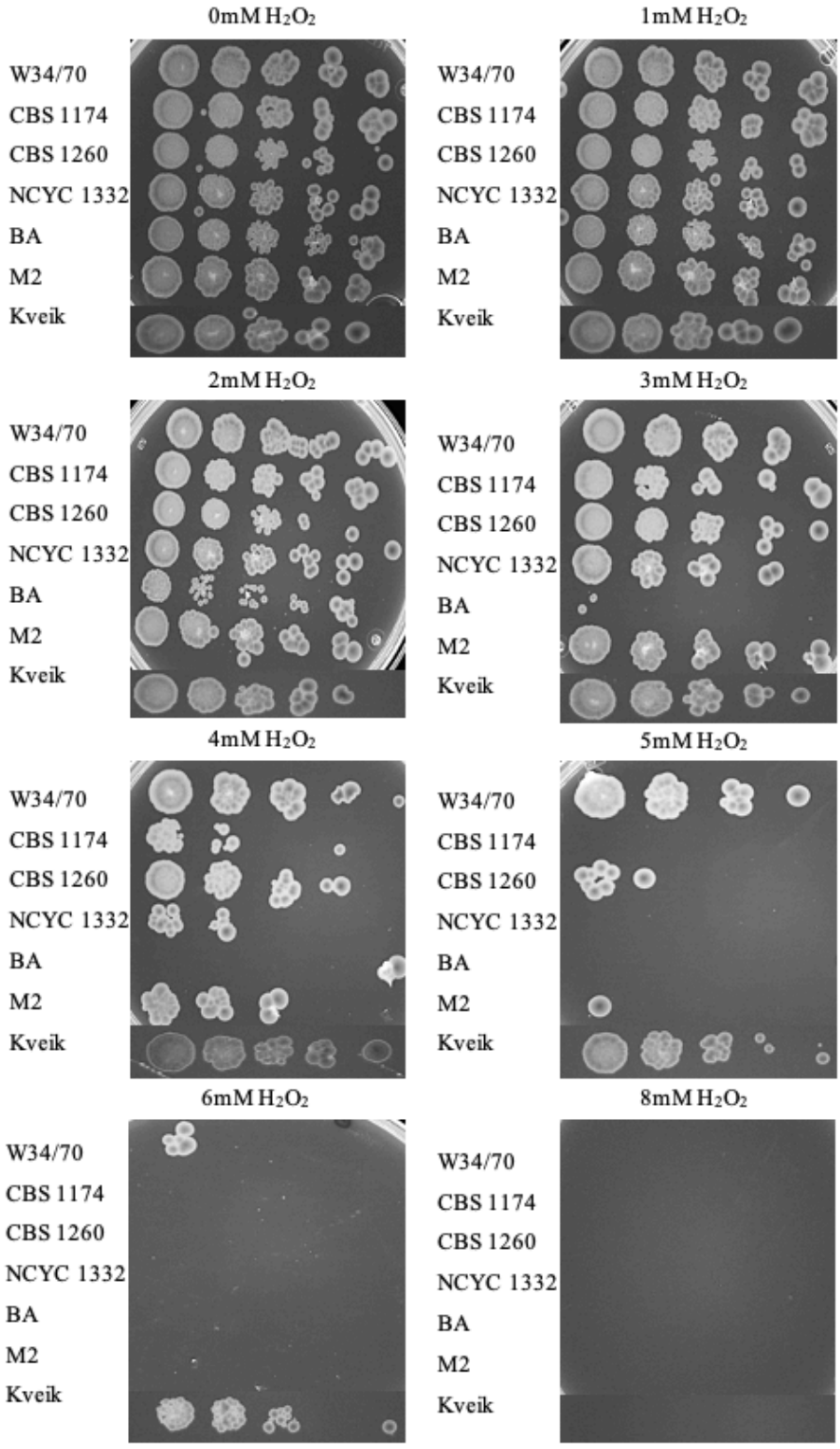

Figure 3.7. The effect of oxidative stress on yeast growth. Aliquots of 5µl of cells taken from cell stocks of $1x10^6$-$1x10^2$ cells/ml (left to right) were added to YPD agar plates containing 0-8mM H_2O_2. Plates were incubated aerobically at 25 °C for 14 days and analysed visually for the presence of growth.

It can be seen from Figure 3.7 that the studied yeast strains exhibited a high resistance to stress induced by hydrogen peroxide, with most strains displaying minimal inhibition of growth from between 0-3mM H_2O_2. However, correlating with the response to previous stresses, the wild yeast strain BA exhibited significant growth inhibition at 2mM H_2O_2, which was the lowest concentration applied. This effect was exacerbated at 3mM H_2O_2, where only 2 small colonies were observed even at the highest initial cell concentration applied, signifying significant growth inhibition due to oxidative stress. At 4mM H_2O_2, CBS 1174 and NCYC 1332 were the first of the other strains to show significant reduction in growth across all cell concentrations, with M2 only displaying growth inhibition at lower cell concentrations. Interestingly, in contrast, at both 4 and 5mM H_2O_2, strains W34/70 and the Kveik yeast were seemingly unaffected by the stress. CBS 1174 and NCYC 1332 demonstrated complete growth inhibition at 5mM, and CBS 1260 and M2 were only able to grow when high cell concentrations were applied. The Kveik yeast strain began to show growth inhibition and reduction in spot size at 6mM, while W34/70 also showed susceptibility to this level of stress, with only a few small CFUs growing when 10^6 cells were applied. Ultimately no strain displayed growth at 8mM H_2O_2.

Interestingly, it appears that among the lager strains, the Frohberg type yeast exhibited a superior resistance to oxidative stress. It is possible that this could be a result of the larger portion of *cerevisiae* genome compared to the Saaz type. However, this is perhaps not the case since the *S. cerevisiae* ale strains NCYC 1332 and M2 showed similar levels of resistance to CBS 1174 (Saaz type lager strain) and not the Frohberg strains. Consequently, it is perhaps more likely that it is simply a strain specific phenomenon.

Interestingly, even though the lager yeast W34/70 and the Kveik strain are genetically divergent, they both showed the highest tolerance to H_2O_2. This implies that the attributes required for oxidative stress resistance may be a result of acute genetic variation, in combination with long term adaptive evolution. For example, historically the use of Kveik yeast was observed primarily in open vessel farmhouse ales, where the brewing wort and yeast culture is constantly exposed to an aerobic environment.

It is possible therefore that an adaptation to ROS generated by aerobic metabolism may have played an evolutionary role allowing for these types of fermentation to prosper. Further analysis of the Crabtree effect and metabolic flux in Kveik yeast would be required to fully investigate this theory.

3.6.4 Zinc and copper tolerance

Both zinc and copper are heavy metals found in brewing wort, albeit in small concentrations significantly of less than 1mM. Consequently these are unlikely to procure a stress to yeast cells and indeed are essential to ensure a healthy yeast population due to their role as enzyme cofactors (Helin and Slaughter, 1977; Walker *et al.*, 2006; White and Zainasheff, 2010). However, heavy metal toxicity has been the focus of a previous stress related study (Holland *et al.*, 2014) and was chosen here to provide a more holistic analysis of the way in which brewing yeast populations respond to stress. In order to determine the concentrations at which zinc and copper became toxic for the yeast strains investigated here, zinc sulphate heptahydrate and copper sulphate pentahydrate were used to create stress plates as described in Section 2.8. In each instance, 5µl aliquots of yeast, serially diluted from 10^6 cells/ml to 10^2 cells/ml were spotted onto YPD agar plates containing $ZnSO_4 \bullet 7H_2O$ ranging from 0mM to 8mM, and from 0mM to 6mM CuSO4.5H2O respectively. Subsequently, cells were incubated at 25°C for 14 days to ensure sufficient time for growth to become visible. Stress tolerance was determined by visual inspection of growth on each plate as shown in Figures 3.8 and 3.9.

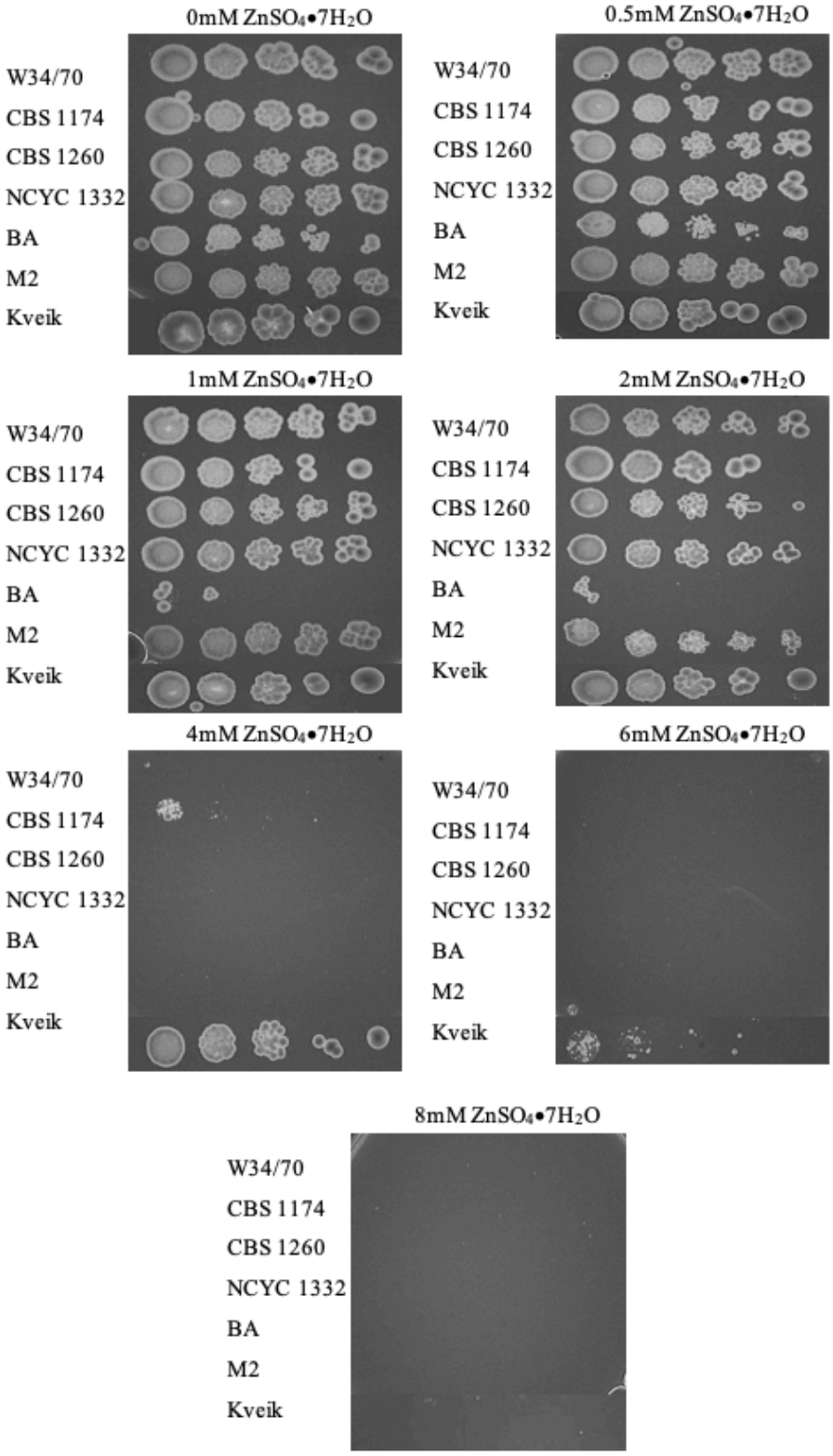

Figure 3.8. The effect of zinc toxicity on yeast growth. Aliquots of 5µl of cells taken from cell stocks of $1x10^6$-$1x10^2$ cells/ml (left to right) were added to YPD agar plates containing 0-8mM Zinc sulphate pentahydrate. Plates were incubated aerobically at 25 °C for 14 days and analysed visually for the presence of growth.

As expected, the brewing yeast strains showed a high tolerance to zinc, with no visible growth inhibition or CFU spot size reduction on agar plates containing 0-2mM $ZnSO_4 \bullet 7H_2O$. However, the wild yeast BA showed a reduced spot size at lower cell concentrations when subjected to 0.5mM stress. This effect was exacerbated at 1mM and 2mM, where only a few colonies were visible at 10^6 cell concentrations. At 4mM $ZnSO_4 \bullet 7H_2O$ only 2 strains showed any growth at all: CBS 1174 which displayed growth albeit with a small colony size, and the Kveik strain which remained unaffected by the presence of high concentrations of zinc. At 6mM only the Kveik yeast displayed any growth, but at this concentration reduced spot size was observed with only a small number of CFUs growing at lower cell concentrations. When zinc was applied at 8mM, no strains were able to grow.

Interestingly the tolerance to increasing concentrations of copper sulphate pentahydrate followed the opposite pattern to that of zinc (Figure 3.9). At 1mM, all strains began to show signs of growth inhibition, with a visible reduction in spot size and reduced growth particularly at lower cell concentrations. This was especially true for strain CBS 1774, which was reduced to only 2 small colonies at 2mM concentrations. However, the Kveik strain and the wild BA yeast remained largely unaffected. The brewing strains W34/70, CBS 1260 and NCYC 1332 showed signs of significant growth inhibition at 2mM, with M2 growth completely inhibited. Aside from a small number of colonies for W34/70, only BA and the Kveik yeast exhibited growth at 3mM, with limited signs of growth reduction. At 4mM $CuSO_4.5H_2O$ these two remaining strains began to show CFU spot size reduction at all cell concentrations as a result of copper induced growth inhibition, while at 6mM no growth was visualised.

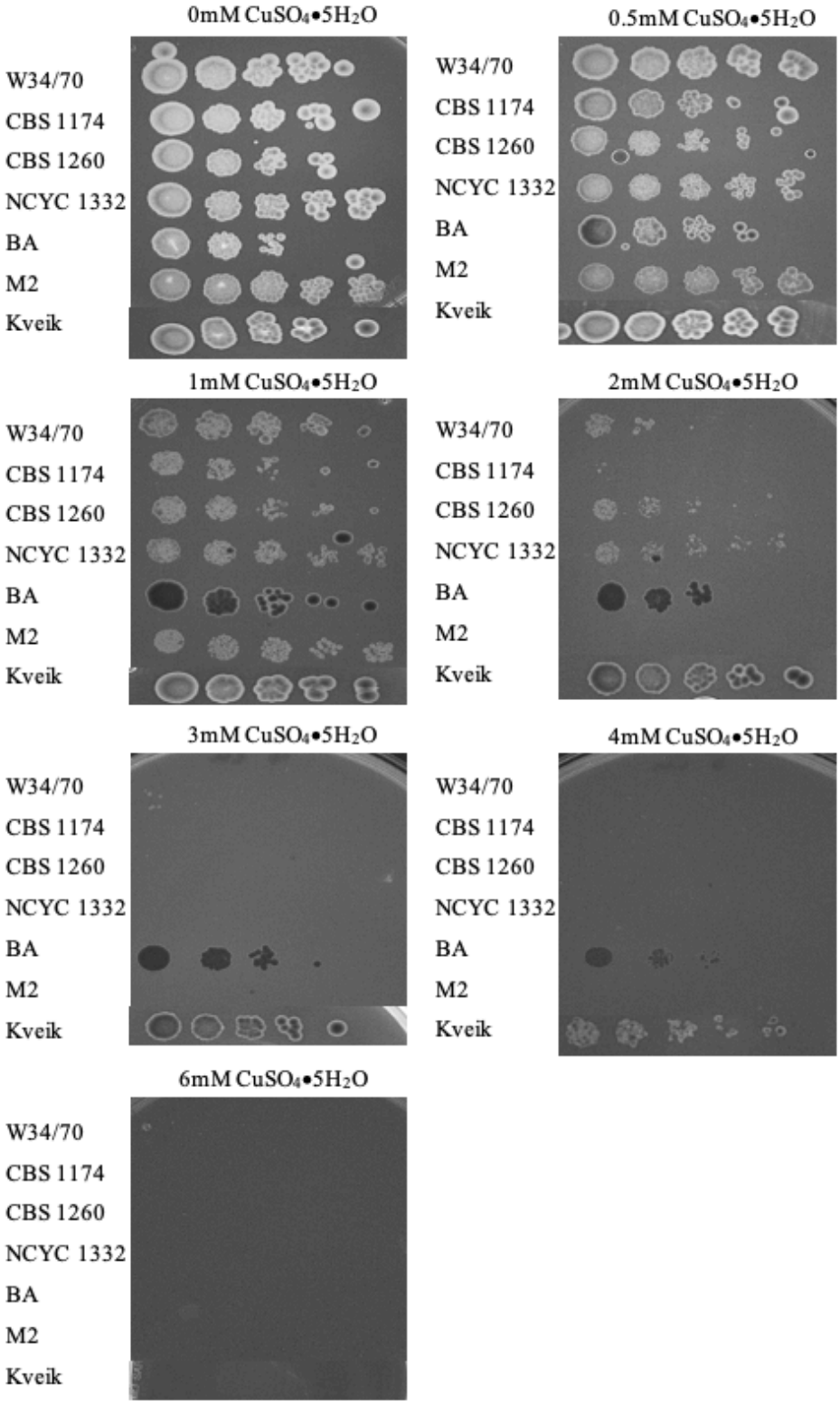

Figure 3.9. The effect of copper toxicity on yeast growth. Aliquots of 5µl of cells taken from cell stocks of $1x10^6$-$1x10^2$ cells/ml (left to right) were added to YPD agar plates containing 0-6mM copper sulphate pentahydrate. Plates were incubated aerobically at 25 °C for 14 days and analysed visually for the presence of growth.

Interestingly the two stressors used here presented contrasting patterns of tolerance. CBS 1174 was the most tolerant strain to zinc, while one of the most susceptible to copper. Conversely, the wild BA yeast was the least tolerant to zinc but possessed one of the highest tolerance levels to copper. Only Kveik showed high levels of tolerance to both stressors. These results are partially unsurprising, since the requirement for zinc in brewing fermentations is essential for yeast cell health, growth and to achieve the desired final product (De Nicola and Walker, 2011). Hence, the wild yeast strain BA may not have encountered any selective pressure required to evolve a tolerance to zinc, due to lack of exposure. Related to this, it is known that ethanol yields are lower in *Brettanomyces* species than other ethanolic yeast, since acetic acid production forms a major route for redox balance rather than ethanol (Aguilar Uscanga *et al*., 2003; Serra Colomer *et al*., 2019). Conversely, aside from the Kveik strain, the traditional brewing yeasts displayed a lower tolerance to copper than the wild strain. Copper is found in trace amounts in brewing wort and is a heavy metal only rarely used as a supplement in aid of yeast performance. However, from studies investigating environmentally polluted habitats, it is known that copper is a common pollutant (Holland *et al.*, 2014) more likely to be present in 'wild' habitats, which may partly account for the superior tolerance of the wild BA strain to copper.

3.7 Conclusion

The aim of this chapter was to investigate genotypic and phenotypic differences between a group of selected yeasts, providing greater detail into both the stress response and individual growth characteristics. The selected strains (2 ale type, 3 lager type, a Kveik yeast and 1 wild *Brettanomyces anomolus* (BA) strain) were chosen to represent the current diversity used in industry. Subsequently these strains were subjected to a range of analyses including PCR, growth kinetics and stress tolerance.

When considering the amplification of ITS regions found in yeast, each of the brewing strains displayed ITS regions of the same size when separated by gel electrophoresis, and the same patterns when ITS fragments were digested using the restriction endonucleases *HinfI* and *CfoI.* However, the ITS region of wild strain *B. anomalus*

differed, and subsequently the band patterns from the *HinfI* and *CfoI* digestion were also different. The *HaeIII* digestion of ITS regions allowed for more clarity on the brewing isolates, allowing differentiation of *S. cerevisiae* (ale) and Saaz type *S. pastorianus* (lager) strains. However, the ITS and RFLP band patterns of the Frohberg lager strains were similar to ale yeasts, likely due to inheritance of this region from the *S. cerevisiae* parental strain. Evidence for this stems from the fact that Frohberg yeasts have a higher ratio of *S. cerevisiae* to *S. eubayanus* in their genome than Saaz strains (Dunn and Sherlock, 2008; Pham *et al.*, 2011; Gibson *et al.*, 2013; Baker *et al.*, 2015). Irrespective, a combination of ITS-PCR and permissive growth temperature analysis were able to accurately classify each yeast, providing assurance as to their speciation and brewing classification. To determine if each of the yeast strains employed were unique, PCR amplification of interdelta regions was performed. The fingerprints produced showed unique patterns for each strain, thus proving that the group represented a diverse collection of yeasts. It is known that variation in fingerprint patterns observed between strains relates to their distinct genotypic differences (Legras and Karst, 2003; Xufre *et al.*, 2011), however given that it is a non-specific form of fingerprinting little can be revealed about phenotypic differences between strains using this approach.

To complement the genetic differentiation, each yeast strain was also studied for growth kinetics in strictly controlled conditions. This analysis highlighted significant variation in kinetics between strains, with disparity observed in all 3 growth phases. The lag phases of the Kveik yeast and the wild BA strain displayed the biggest difference, which was unsurprising since Kveik strains are known to be display particularly rapid growth, while wild yeast strains are typically significantly slower (Suzuki *et al.*, 2008; Shimotsu *et al.*, 2015). Within the brewing strains there was also distinct variation in length of time spent in lag phase, with a clear link to genetic origin. It appeared that ale strains were quicker to exit lag phase, since they resided in lag phase for a shorter duration than all of the lager strains analysed. This pattern of rapid growth was also seen in entry/exit to exponential and stationary phase; ale strains showed a steeper exponential growth curve gradient, and entered stationary phase earlier than lager strains at 25°C. This investigation into growth kinetics was a useful

measure of variation between yeast strains based on obtaining 'mean population' data, giving insight into how the cell metabolism of a population manifests itself in a controlled growth environment.

Once strain identification and growth characteristics had been determined, yeasts were investigated for their capacity to tolerate stress factors associated with industrial fermentations. Although stress tolerance is a key attribute required in all industrial yeasts, in brewing this is particularly important to ensure product quality and consistency. The ability to withstand stress is increasingly important as brewing intensification (the practices of high and very high gravity fermentations) becomes more widely adopted. This practice elevates key stress factors, including osmotic, oxidative and ethanol challenges, and is further exacerbated for the reason that brewing yeast cultures are re-used (Odumeru *et al.*, 1992; Majara, *et al.,* 1996). The most basal and perhaps most crucial attribute of stress tolerance is a yeast cells ability divide under duress. Hence the ability to produce CFUs when challenged with stress was determined to provide a useful insight into population stress dynamics. The maximum limits for growth were ascertained by using spot plate analysis to determine the capacity to divide once subjected to a range of brewing related stresses. Analysis of ethanol and osmotic stress sensitivity indicated that the ale strains had a higher degree of tolerance compared to the lager strains, with the Saaz type lager strain (CBS 1174) possessing the lowest tolerance of all the brewing strains, particularly to ethanol. These findings were not especially surprising, given the diverse nature of *S. cerevisiae* strains, their well-documented high ethanol tolerance (D'amore *et al.*, 1989) and their regular use in high gravity brewing, wine, sake and spirit production. Similarly, although *S. pastorianus* lager yeast are hybrid organisms with a significant proportion of the *S. cerevisiae* genome, it is interesting to note that CBS 1174 is a Saaz type yeast, with a significantly smaller portion of the *cerevisiae* genome than any of the other brewing strains analysed (Gibson *et al.*, 2013; Gallone *et al.*, 2019). This pattern was partially repeated in response to hydrogen peroxide as an oxidative stressor, however in this instance the Frohberg type lager strains appeared the most resistant. The lower tolerance of ale strains to oxidative stress when compared to previous stressors is perhaps harder to explain, however this may be a consequence

of a number of factors such as their shorter lag and faster exponential growth phases. This may result in quicker uptake of oxygen and faster production of ROS, without the required time to accumulate intracellular ROS protectants such as glutathione (Izawa *et al.,* 1995). It is known that the oxidative stress response is largely dictated by a complicated general stress response which differs between yeast strains, so the true cause of these differences in tolerance is difficult to ascertain and unlikely to be a result of one factor (Gibson *et al.*, 2008). Finally, all of the brewing yeast strains analysed showed similar tolerance levels in response to copper and zinc stress, although the Saaz type lager strain (CBS 1174) displayed high tolerance to zinc but low tolerance to copper. Interestingly the opposite was true for the wild yeast strain (BA) with good tolerance to copper but not zinc, likely due to evolutionary and selective pressures associated with wild vs commercial environments. Finally, a key finding related to the Kveik yeast strain indicated that this organism was extremely tolerant to most of the stress factors applied, which offers opportunities for the future in mainstream brewing. This robustness is perhaps un-surprising considering its origin; such strains were historically used almost exclusively in the production of high strength Norwegian farmhouse ales through warm and rapid fermentations (Preiss *et al.*, 2018; Foster *et al.*, 2021).

As discussed above, the data obtained here was important to provide key information regarding the tolerance limits of industrially relevant brewing strains. However, it should be noted that the experimental approach applied thus far was not particularly novel; there have been a number of previous studies investigating the stress response in yeast populations (Gibson *et al.*, 2007, 2008; Verbelen *et al.*, 2009; Bleoanca *et al.*, 2013). While valuable, one shortcoming of this form of analysis is that using population-wide data (i.e. obtaining an 'average' value for a strain) can mask the true growth kinetics/vitality of a yeast strain, which can only be revealed when considering and comparing individual cells within a population (Junker and van Oudenaarden, 2014; Mouton *et al.*, 2016). Interestingly, at the fundamental level, the spot plate analysis demonstrated that when an imposed stress becomes toxic a small number of colonies were able to survive, indicating variation in limits within a population. This evidence leads to the expectation that there are sub-populations of cells better

adapted to stress, as well as those that are more susceptible. This has the potential to result in a non-binary spectrum of tolerance characteristics from cell-to-cell, which is perhaps surprising given that the budding nature of industrial strains should yield theoretically genetically identical cells. Conversely, this implies that cell-to-cell variation may be a purely phenotypic trait, which raises the notion of physiological heterogeneity in microbial cell populations. This phenomenon has not previously been investigated in industrial brewing yeast strains and forms the basis of the following Chapters.

Chapter 4. Phenotypic heterogeneity in the yeast stress response

4.1 Introduction

The success of a brewing fermentation is largely defined by process and product consistency. To some extent this can be assured through the use of standard raw materials, however batch-to-batch variation can be observed due to the quality, activity and consistency of the yeast culture employed. At the current time, most industrial strains are stored under strict conditions (typically liquid nitrogen at -196°C) to prevent or restrict genetic change. Working yeast cultures are then produced from isolated yeast colony(ies) grown on solid media and then propagated to gain the required cell mass for pitching (White and Zainasheff, 2010).

Despite efforts to ensure genetic stability in brewing yeasts, genetic variants can still arise within a population. Typically these occur as a result of exposure to brewing related stress factors such as ethanol, osmotic stress, oxidative stress and starvation (Smart, 2007; Gibson *et al.*, 2008). For example, prolonged exposure to high concentrations of ethanol is known to instill point mutations, ploidy changes and copy number variation, resulting in a wide range of phenotypes within a population and significant differences in stress tolerance between lineages (Voordeckers *et al.*, 2015; Gallone *et al.*, 2016). These are not only related to the nuclear DNA, but also arise from changes to the mitochondrial DNA (mtDNA), specifically the emergence of mitochondrial deficient (petite) mutants. Irrespective of the origins and form of mutation, changes can lead to genetic drift within a population, resulting in populations of cells with undesirable brewing qualities (Bandas and Zakharov, 1980; Heidenreich and Wintersberger, 1997; Powell, *et al*., 2000; Gibson, *et al*., 2008). While genetic mutations are relatively easy to quantify in terms of the underpinning causes and impacts of change, it is important to note that natural variation can also be observed within isogenic populations of cells. This phenomenon occurs due to non-genetic (phenotypic) variation and is referred to as population heterogeneity. This form of variation is complex and challenging to monitor and quantify, since many

analytical methods rely on assessments of populations of cells. Furthermore, while there have been previous studies focused on the analysis of yeast heterogeneity in yeast strains (Sumner and Avery, 2002; Bishop *et al.*, 2007; Carlquist *et al.*, 2012; Holland *et al.*, 2014; Gasch *et al.*, 2017), there have been only limited reports on the extent and impact of phenotypic variation in industrial systems, such as brewing.

Yeast phenotypic heterogeneity can be partially attributed to basic somatic factors including differences in cell size, age and morphology (Ackermann, 2015). These superficial forms of variation can themselves lead to more intricate manifestations of heterogeneity, for example cell age itself has been linked to important yeast functional properties such flocculation efficiency (Powell *et al.*, 2000; Jin *et al.*, 2001; Powell, *et al*., 2003). Phenotypic heterogeneity can also be derived from more complex sources in yeast, including the various stress response pathways. These are known to be interlinked with both general and specific mechanisms, caused by a dynamic and convoluted gene expression network within yeast. The complex nature of gene expression, consisting of feedback loops, transcription factors, repressors and signaling cascades, instills a degree of 'expression noise' between cells (Gasch, 2003; Levy *et al*., 2001; Gasch *et al.*, 2017) leading to difference in response. However irrespective of the underlying mechanism, cell-to-cell heterogeneity within a population is believed to form the basis for population survival strategies, including 'bet-hedging' and 'division of labour', particularly in response to a changing environment (Section 1.10). The extent to which this occurs is likely to lead to sub-populations of cells with different traits and abilities, particularly in relation to stress, but also to connected phenotypes important in fermentation. There is an argument to suggest that from an industrial perspective, sub-populations may lead to inconsistencies in performance and affect the versatility and suitability of strains for specific industrial processes. However, as previously discussed, the occurrence of sub-populations with differing abilities to perform, may allow a cell population to survive in stressful conditions. Furthermore, they may also provide the opportunity to isolate and develop a new cell population consisting of cells with improved performance and process specific qualities. As such, to understand the potential impact of yeast phenotypic heterogeneity on brewing performance, the degree of heterogeneity in a

spectrum of brewing yeast strains was determined. To achieve this, single cell flow cytometry was used in conjunction with cell cytotoxicity assays to ascertain population heterogeneity in response to stress for ale, lager and non-conventional production yeast strains. Simultaneously, physiological variation between cells was determined by analysis of key organelles (cell membrane fluidity, mitochondria and vacuolar structures), lipid and sterol content, and overall cell size and age distribution within the population.

4.2 Phenotypic Heterogeneity in stress response

Environmental stress factors exert a range of adverse effects on yeast cells, causing damage to organelles and DNA, and ultimately cell death. It is well known that the effect of individual stress factors on yeast cells is strain specific (Jenkins *et al.*, 2003; Gibson *et al.*, 2008; Bleoanca and Bahrim, 2013) and that industrial strains are typically more robust than their laboratory and wild counterparts (Sanchez *et al*., 2012; Qiu *et al.*, 2019). However, previous analyses of the sensitivity and/or tolerance of strains to stress factors has typically been focused on analysis of whole populations (Section 3.6) (Gibson *et al.*, 2008; Zhao and Bai, 2009; Kitagaki and Takagi, 2014). There is value in this approach as it enables the limits of a strain to be identified, however a large amount of information can be masked by this form of analysis. For example, it is possible that two yeast strains may both exhibit a maximum ethanol tolerance of 10%, however for one strain this may be restricted to a small sub-population rather than being a reflection of the entire culture. Consequently, this could have industrial implications related to the unexpected death of the main population, particularly when exposing yeast cells to high levels of stress such as those found in high gravity brewing.

In order to gain a more complete understanding of the brewing yeast stress response, the presence and extent of phenotypic heterogeneity related to both fermentation (ethanol, osmotic and oxidative) and non-fermentation (zinc and copper) stress factors was determined in a selection of *Saccharomyces cerevisiae* ale yeast, *S.*

pastorianus lager yeast, a wild *Brettanomyces anomalus* (BA) strain and a *S. cerevisiae* Kveik yeast. This was achieved using a cell cytotoxicity assay that relies on the ability of healthy, metabolizing yeast cells to reduce the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) into discernible formazan crystals (Section 2.9.2.) that can be quantified.

As described in Section 2.9.2, samples of 5000 cells were incubated for 3 days in the presence of environmental stressors and the MTT assay was used to determine the activity of the surviving cells as a measure of stress tolerance. Data was formatted to produce sigmoidal dose response curves for each strain/stress combination. The gradient of each curve was then calculated and used as a direct indication of population heterogeneity as described in Section 2.9.4. Curves displaying a gradual hillslope are the result of the upper and lower asymptotes of the dose response curve being further from the point of inflection, representing a steady decline in viability as the stressor concentration is increased. Steeper gradients form as a result of the upper and lower asymptotes being on a tighter plane with the point of inflection and represent a more sudden decline in viability. These hillslopes and their respective gradients therefore indicate the degree of heterogeneity observed in response to stress for each strain, where lower hillslope gradients indicate the presence of divergent heterogeneous sub-populations of cells, and higher hillslope gradients represent less heterogeneous cultures, where cells in a population possess similar levels of tolerance to stress. In addition, the same dose response curves were used to calculate IC50 values, defined as the stressor concentration at the point at which population viability is reduced to 50%. Smaller IC50 values shift the dose response curve horizontally towards the y-axis and these values are representative of lower stress tolerance, while the opposite is true for those measuring higher IC50 values.

4.2.1 Phenotypic heterogeneity of yeast populations in response to ethanol stress.

Based on the data obtained in Chapter 3, it is evident that exposure to increasing concentrations of ethanol (0-25% v/v) is likely to be a candidate technique to reveal differences in the survival dynamics between yeast strains. When tolerance to ethanol was assessed using the MTT assay, it became clear that all strains follow the same general sigmoidal curve shape, as expected (Figure 4.1). However the horizontal positioning and gradient was observed to vary from strain-to-strain, giving insights into both the maximum tolerance, as well as the presence of heterogeneity. The horizontal positioning of the maximum asymptotes are synonymous with the point at which the cells in the population become susceptible to the effects of ethanol stress and begin to lose viability. Strains with maximum asymptotes further along the x-axis are those with a superior tolerance to those closer to the y-axis. As mentioned previously, the value of IC50 provides a measure of tolerance, representing the stressor concentration at which 50% population viability is achieved, and is similarly related to the position of the maximum asymptotes, allowing tolerance to be quantified. The comparative analysis of IC50 values in Figure 4.2 shows that the Kveik yeast had the highest tolerance to ethanol with an IC50 value of 11.2% (v/v) while the wild *Brettanomyces* (BA) yeast was the lowest with 3.7% v/v, broadly matching previous data (Chapter 3). The IC50 of the remaining brewing strains varied from 6.1% ethanol v/v (CBS 1260) to 9.7% ethanol v/v (W34/70, M2 and NCYC 1332), thus reinforcing the wide range of ethanol stress tolerances between these yeast strains. The IC50 values presented in Figure 4.2 correlate closely with the stress tolerance results observed in the spot plate analysis (Chapter 3). Among the traditional brewing strains, the lager strains (CBS 1174, CBS 1260) exhibited a lower IC50 than the ale strains, aside from W34/70 which appeared to display an IC50 closer to that of the ale strains.

In addition to the IC50 values, the hillslope gradients of each dose response curve were calculated as shown in Figure 4.3. These give the principal information related to the degree of heterogeneity present in each strain, in this case in response to ethanol stress. The hillslope gradient data presents clear differences between strains,

with some strains producing larger negative values, indicating a dose response curve with a steeper gradient, and some with the opposite effect. It should be noted that smaller values for a strain are indicative of cells with a broader range of tolerances (greater population heterogeneity) compared to those with larger values, which indicate a narrow range of tolerance (less population heterogeneity). As expected from visual inspection of the dose response curves (Figure 4.1), the wild BA yeast had the smallest negative hillslope gradient of -3.9 and therefore possessed the highest degree of heterogeneity, while NCYC 1332 had the largest negative hillslope gradient of -92 and the lowest observed population heterogeneity (Figure 4.3). Interestingly (as with IC50 values), it appeared that the lager strains exhibited a higher degree of heterogeneity than the *S. cerevisiae* ale strains (M2 and NCYC 1332). However, large differences in heterogeneity were still observed within each yeast type.

Generally the data appeared to follow a pattern, such that a higher observed ethanol tolerance coincided with a lower degree of population heterogeneity. This was true for all strains except the Kveik yeast, where the observed degree of heterogeneity did not directly correlate with tolerance (IC50). The Kveik strain presented one of the highest heterogeneities (-12.2) whilst also showing the greatest tolerance to ethanol stress (IC50-11.2% ethanol v/v). The hillslope gradient (and therefore degree of heterogeneity) were then compared between strains using Tukeys multiple comparison test, in order to determine significance in the observed heterogeneity profiles. These comparisons can be seen in Table 4.1, and values highlighted in green indicate significantly contrasting heterogeneities ($P<0.05$).

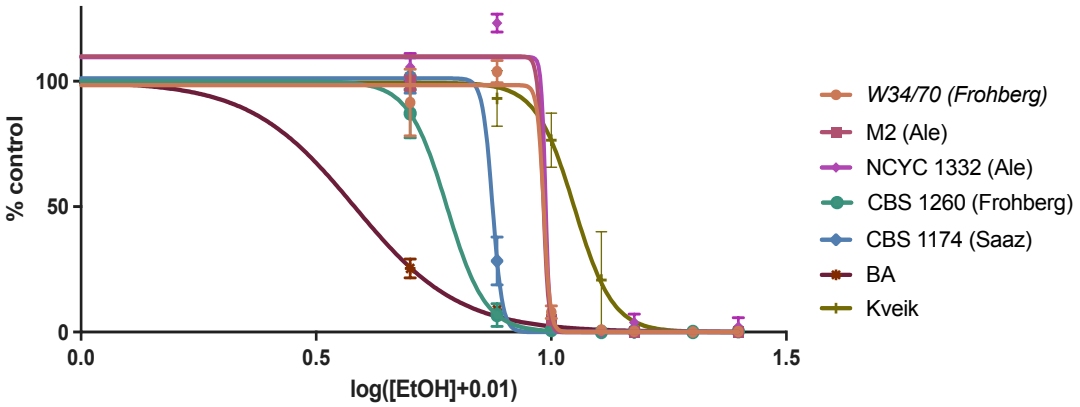

Figure 4.1. Dose response curves in response to ethanol stress for each strain, obtained from the MTT cell cytotoxicity assay. Yeast strains were exposed to increasing concentrations of ethanol (0-25% v/v) and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

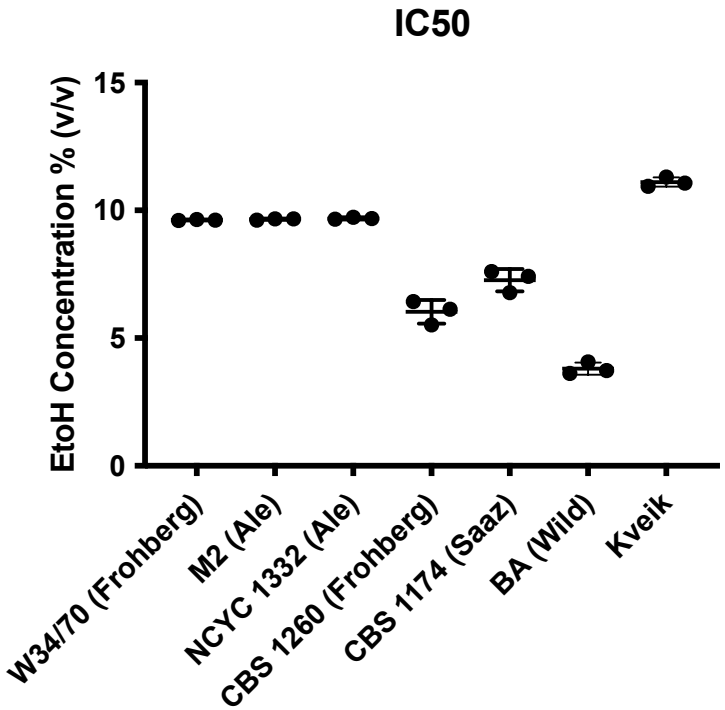

Figure 4.2. IC50 values generated from the ethanol stress dose response curves. The data displayed represents the ethanol concentration at which 50% viability is achieved. Values are a mean of triplicate data with error illustrating the standard deviation.

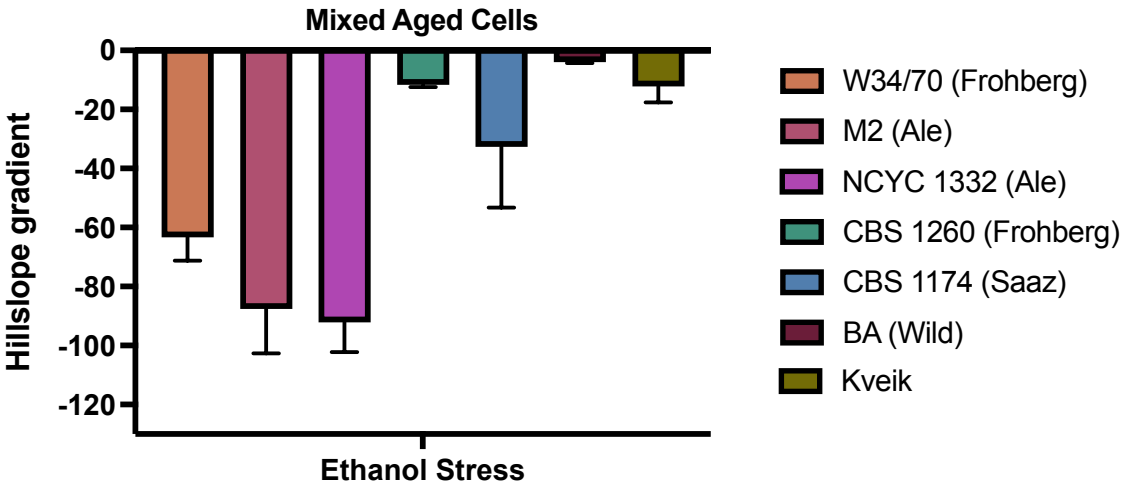

Figure 4.3. Hillslope gradient data obtained from dose response curves generated by exposing yeast to increasing concentrations of ethanol. Smaller negative values (BA) are representative of a more gradual decline viability and therefore indicate the presence of a higher degree of phenotypic heterogeneity. While larger negative values (NCYC 1332) represent a sudden decline in viability due to a lower degree of heterogeneity.

Table 4.1. Tukeys multiple comparison test for differences in ethanol stress heterogeneity (hillslope gradient) between the studied strains. (P<0.05). Green indicates significant differences and yellow corresponds to differences in heterogeneity that did not reach statistical significance.

	M2	CBS 1260	NCYC 1332	CBS1174	BA	Kveik
W34/70	0.1213	<0.0001	0.0376	0.0221	<0.0001	<0.0001
M2		<0.0001	0.9988	<0.0001	<0.0001	<0.0001
CBS1260			<0.0001	0.2712	0.9777	>0.9999
NCYC 1332				<0.0001	<0.0001	<0.0001
CBS 1174					0.0443	0.2976
BA						0.9696

4.2.2 Phenotypic heterogeneity of mixed aged yeast populations in response to osmotic stress.

Similar to the analysis of ethanol stress, populations of cells were subjected to increasing concentrations of osmotic stress through the use of sorbitol, and phenotypic heterogeneity was determined using MTT staining as described in Section 2.9.2. The survival and growth of cells from an initial seed of 5000 cells was used to stress dose response curves for each strain (Figure 4.4). After exposure to increasing concentrations of sorbitol (0-60% w/v), each brewing strain displayed similar survival dynamics, shown by the similar shapes of the dose response curve obtained, with only the wild BA yeast showing visual differences. It was also evident that the brewing strains followed the same general sigmoidal curve shape, with similarly positioned maximum and minimum asymptotes, indicating a closely related osmotic stress response between strains. Although the wild BA yeast also showed similarities in curve shape, the horizontal positioning of the asymptotes suggests that this strain had a much lower sorbitol tolerance than the others analysed. As expected, the BA yeast displayed the lowest IC50 (9.2% sorbitol w/v), while the remaining strains all showed similar IC50s ranging from 41-45% sorbitol (w/v) with only W34/70 among the conventional brewing strains to show a reduced tolerance at 35% w/v (Figure 4.5). Furthermore, there appeared to be little evidence suggesting a link between strain type and osmotic stress tolerance among brewing strains; similar dynamics of tolerance were observed for each strain. The IC50 data presented in Figure 4.5 also correlated with the stress tolerance observed in spot plate analysis in Chapter 3, with the wild BA strain displaying higher susceptibility to osmotic stress, and the conventional brewing strains showing high, and relatively similar, tolerance limits.

From a visual inspection of the dose response curves in Figure 4.4, it was difficult to ascertain differences in the extent of heterogeneity within each strain. Therefore, the hillslope gradient of each curve were calculated at the point of inflection in an attempt to gain insights into the heterogenous nature of each strain. The hillslope gradients displayed in Figure 4.6 range between -6.1 (1174) and -21.6 (M2), indicating a relatively gradual hillslope in all of the studied strains symptomatic of a steady decline

in population viability as stressor concentration was increased. These small gradients indicate the presence of divergent sub-populations and a high degree of heterogeneity in all strains. The similarity of hillslope gradients was confirmed using the Tukeys multiple comparison test (Table 4.2), which indicated that there were no significant differences between any of the strains. As such, there is no evidence to suggest a link between the degree of heterogeneity, yeast strain and overall and tolerance to osmotic stress, at least for the brewing strain investigated.

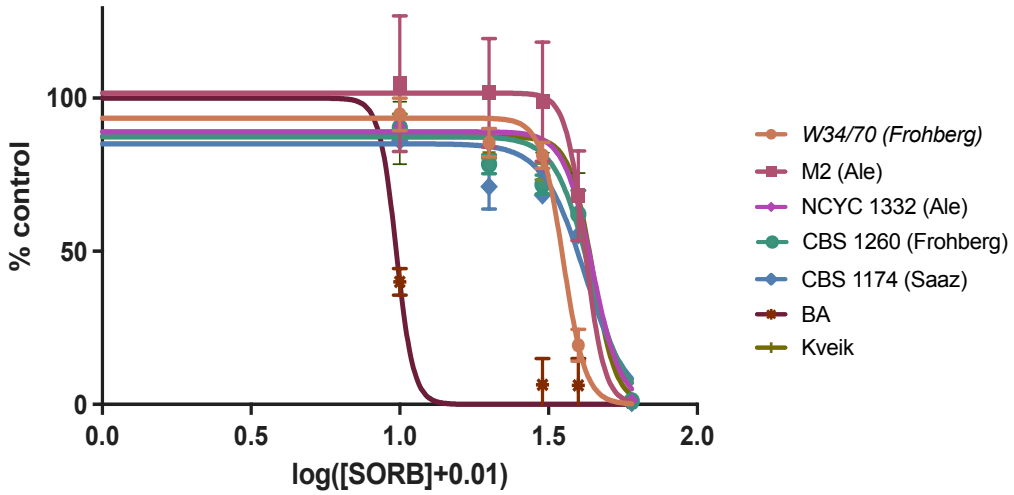

Figure 4.4. Dose response curves in response to sorbitol induced osmotic stress for each strain, obtained from the MTT cell cytotoxicity assay. Yeast strains were exposed to increasing concentrations of sorbitol (0-60% w/v) and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

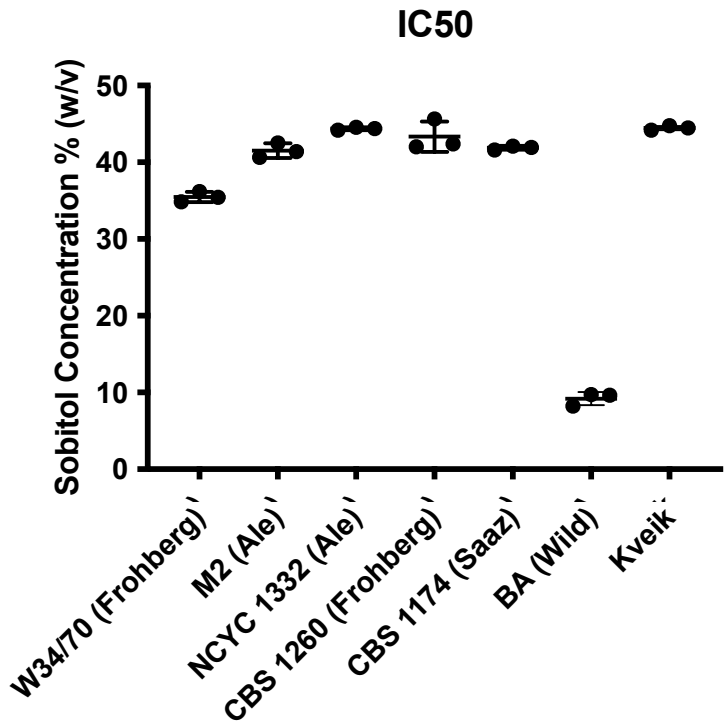

Figure 4.5. IC50 values generated from the osmotic stress dose response curves. The data displayed represents the sorbitol concentration at which 50% viability is achieved. Values are a mean of triplicate data with error illustrating the standard deviation.

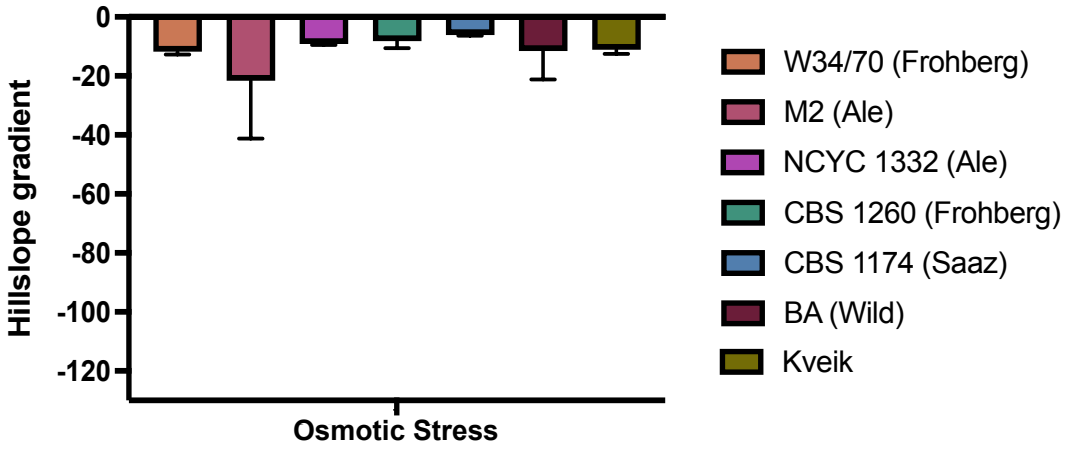

Figure 4.6. Hillslope gradient data obtained from dose response curves generated by exposing yeast to increasing concentrations of sorbitol. Smaller negative values are representative of a more gradual decline viability and therefore indicate the presence of a higher degree of phenotypic heterogeneity. While larger negative values represent a sudden decline in viability due to a lower degree of heterogeneity.

Table 4.2. Tukeys multiple comparison test for differences in osmotic stress heterogeneity (hillslope gradient) between the studied strains. (P<0.05). Green indicates significant differences and yellow corresponds to differences in heterogeneity that do not reach statistical significance.

	M2	CBS 1260	NCYC 1332	CBS 1174	BA	Kveik
W34/70	0.7754	0.9983	0.9997	0.9810	>0.9999	>0.9999
M2		0.4496	0.5428	0.2835	0.7553	0.7216
CBS1260			>0.9999	>0.9999	0.9989	0.9995
NCYC 1332				0.9993	0.9999	>0.9999
CBS1174					0.9849	0.9898
BA						>0.9999

4.2.3 Phenotypic heterogeneity of mixed aged yeast populations in response to oxidative stress.

Similar to previous analyses, the dynamics in oxidative stress response were evaluated for yeast populations through exposure to increasing concentrations of hydrogen peroxide (0-6mM) and assessed using the MTT assay. By analysing the sigmoidal dose response curves produced for each strain (Figure 4.7), significant differences in survival mechanics could be observed. Each curve generally followed a similar sigmoidal shape, however the horizontal positioning of both the maximum and minimum asymptotes varied largely between strains. This indicates that while each strain acts in a similar fashion to oxidative stress, their maximum tolerances differ significantly. Those with maximum asymptotes positioned further along the x-axis represent strains that are tolerant to oxidative stress. From this it can be deduced that the wild yeast strain BA was the most susceptible to oxidative stress, while strain W34/70 and the Kveik yeast possessed high levels of tolerance. Figure 4.8 shows that the IC50 value for each strain varied widely from 0.92mM hydrogen peroxide (BA) to 4.82mM (Kveik). A comparative analysis showed that the remaining strains varied

between these two limits, such that any strong link between species type and tolerance limit was not evident. However, from the small sample set studied, it did appear that *S. cerevisiae* strains generally had a higher tolerance to oxidative stress than the other strains analysed. The IC50 data presented here mostly agrees with the spot plate analysis (Chapter 3), in that wild BA strain was the least tolerant and Kveik the most tolerant, although the placing of CBS 1174 was different in this current study, previously appearing to be more sensitive to oxidative stress than suggested here.

Dose response curves were also used to quantify and compare population heterogeneity observed between strains as shown in Figure 4.9. Visually, when comparing the dose response curves, the gradient of each hillslope appeared similar, with only the Kveik strain presenting a differing, steeper gradient and a more sudden decline of population viability as the stressor concentration was increased. The low degree of heterogeneity demonstrated by the Kveik strain in response to oxidative stress appears to be a unique characteristic among the studied strains. The remaining yeasts all exhibited similarly high levels of heterogeneity, based on values ranging between -40 (W34/70) and -11 (BA). This demonstrates an alternative survival dynamic for these yeasts, with viability declining more gradually with increasing concentrations of oxidative stressor. By comparing the hillslope gradients using Tukeys multiple comparison test displayed, only the Kveik strain was identified as an outlier (Table 4.3). The absence of any significant difference between the remaining strains suggests that there is no link between strain type and the degree of observed heterogeneity, further emphasising the singular nature of the Kveik strains survival dynamics. In addition, when comparing the IC50 tolerance data to the hillslope gradient data for each strain, there did not appear to be any significant link between tolerance and population heterogeneity. This implies that the degree of heterogeneity and maximum tolerance limits are two independent characteristics, at least with regard to oxidative stress.

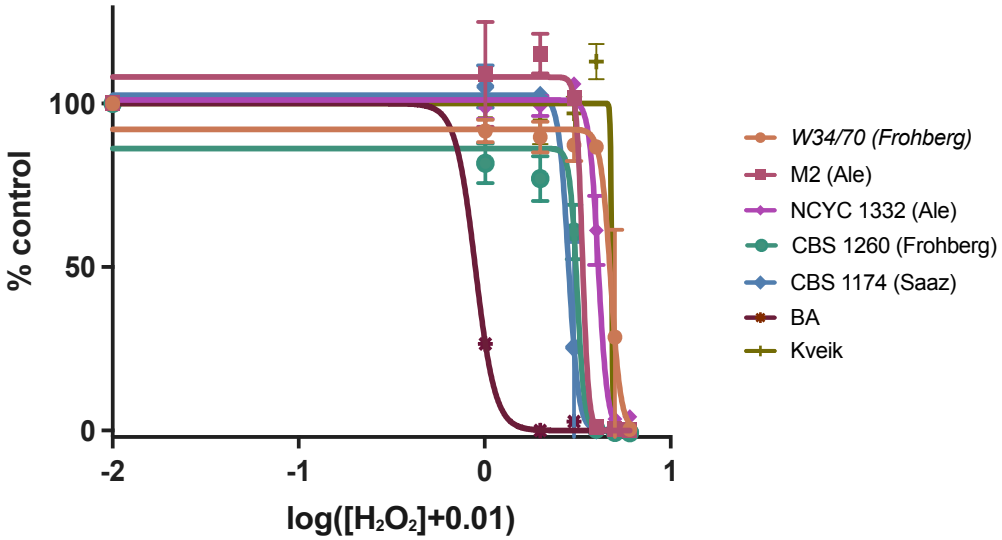

Figure 4.7. Dose response curves in response to hydrogen peroxide induced oxidative stress for each strain, obtained from the MTT cell cytotoxicity assay. Yeast strains were exposed to increasing concentrations of hydrogen peroxide (0-6 mM) and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

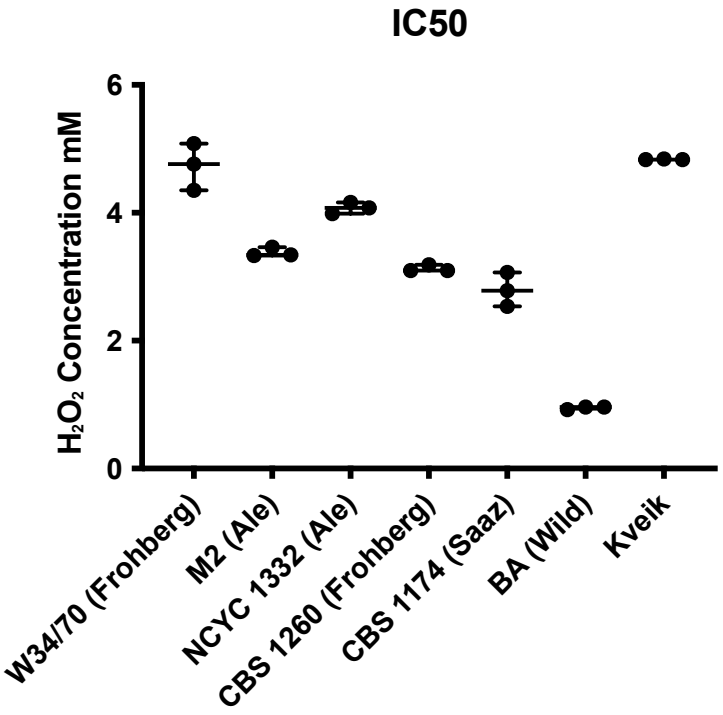

Figure 4.8. IC50 values generated from the oxidative stress dose response curves. The data displayed represents the hydrogen peroxide concentration at which 50% viability is achieved. Values are a mean of triplicate data with error illustrating the standard deviation.

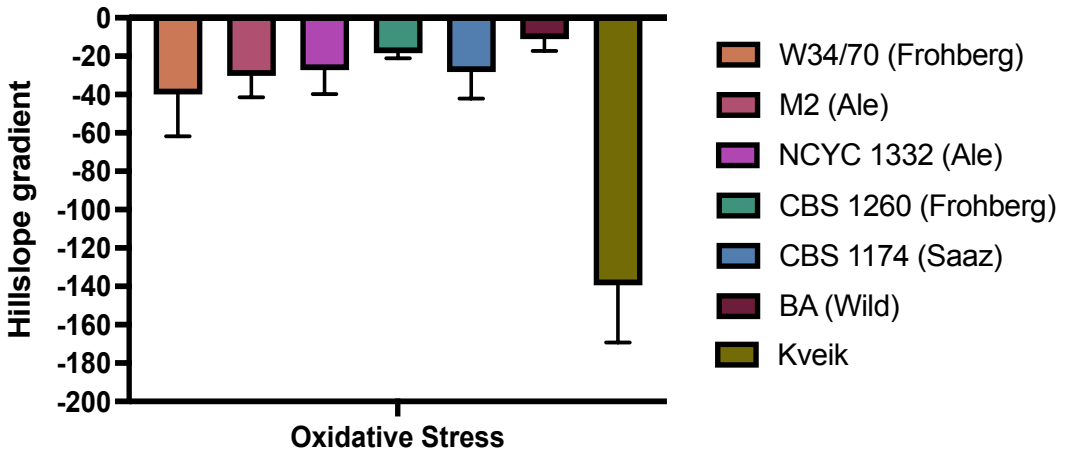

Figure 4.9. Hillslope gradient data obtained from dose response curves generated by exposing yeast to increasing concentrations of hydrogen peroxide. Smaller negative values are representative of a more gradual decline viability and therefore indicate the presence of a higher degree of phenotypic heterogeneity. While larger negative values represent a sudden decline in viability due to a lower degree of heterogeneity.

Table 4.3. Tukeys multiple comparison test for differences in oxidative stress heterogeneity (hillslope gradient) between the studied strains. (P<0.05). Green indicates significant differences and yellow corresponds to differences in heterogeneity that do not reach statistical significance.

	M2	CBS 1260	NCYC 1332	CBS 1174	BA	Kveik
W34/70	0.9907	0.6794	0.9616	0.9755	0.3421	<0.0001
M2		0.9726	>0.9999	>0.9999	0.7803	<0.0001
CBS 1260			0.9943	0.9892	0.9979	<0.0001
NCYC 1332				>0.9999	0.8893	<0.0001
CBS 1174					0.8544	<0.0001
BA						<0.0001

4.2.4 Phenotypic heterogeneity of mixed aged yeast populations in response to heavy metal (copper) toxicity.

Although copper is seldom present at toxic concentrations in brewing fermentations, this metal ion has been the focus of previous yeast phenotypic heterogeneity studies (Bishop *et al.*, 2007; Holland *et al.*, 2014). As such it was also applied here in order to obtain a comprehensive and rounded view on the stress response of brewing yeast populations. As before, by analysing the sigmoidal stress dose response curves obtained through the exposure of yeast populations to increasing concentrations of copper, insights into the tolerance dynamics could be discovered. Analysis of the dose response curves obtained using the MTT assay showed similar growth kinetics across the studied strains (Figure 4.10). However, from visual inspection of the dose response curves, there were clear differences between the strains, since the maximum asymptotes varied largely in their horizontal positioning. Strains M2 and NCYC 1332 presented maximum asymptotes closer to y-axis, as opposed to the wild BA strain which was shifted furthest to the right. The IC50 values also varied considerably, ranging from between 1.1mM copper (M2 and NCYC 1332) to 4.4mM copper (BA), with the other remaining strains distributed between these values (Figure 4.11). The conventional brewing *S. cerevisiae* strains M2 and NCYC 1332 displayed low IC50 values, indicating that these strains were more susceptible to copper than the other strains analysed. Interestingly, this was also observed in the Frohberg type lager strains (CBS 1260 and W34/70), which have a larger proportion of the *S. cerevisiae* genome than Saaz type lager yeasts, which showed greater tolerance. Interestingly this pattern was not seen in the *S. cerevisiae* Kveik strain, which demonstrated a high degree of tolerance, similar to the wild strain BA. Irrespective, this IC50 data closely correlates to that observed previously using spot plate analysis (Chapter 3), where M2, NCYC 1332 and CBS 1260 all showed low tolerance to copper sulphate, and the BA and Kveik strains the highest. However, similar to that seen with oxidative stress, data for the yeast strain CBS 1174 differed to that previously obtained. Using the current methodology, based on the MTT assay in liquid media, a higher tolerance was observed that on solid-based media.

Additional detail could be derived from the dose response curves through calculation of hillslope gradients as discussed above. From Figure 4.12 it can be seen that the strains investigated in this study all displayed a relatively small hillslope gradient value, ranging from -2.0 (NCYC 1332) to -11 (CBS 1174). This data is indicative of a high degree of population heterogeneity and a gradual decline in population viability as copper concentration increased. Although all of the strains presented a high degree of heterogeneity in response to copper, there were some significant differences between them. For example, the Tukeys multiple comparison test indicated that strains NCYC 1332 and CBS 1174 were significantly different in hillslope gradient (Table 4.4), albeit in the context of population heterogeneity both values indicated a relatively high degree of heterogeneity. This was reflected in other comparisons, with a highly conserved degree of heterogeneity between strains, indicating that there is no evidence to suggest any link between yeast strain and population heterogeneity in response to copper toxicity. Similarly, there was no obvious link between tolerance values (IC50) and heterogeneity in response to copper in general.

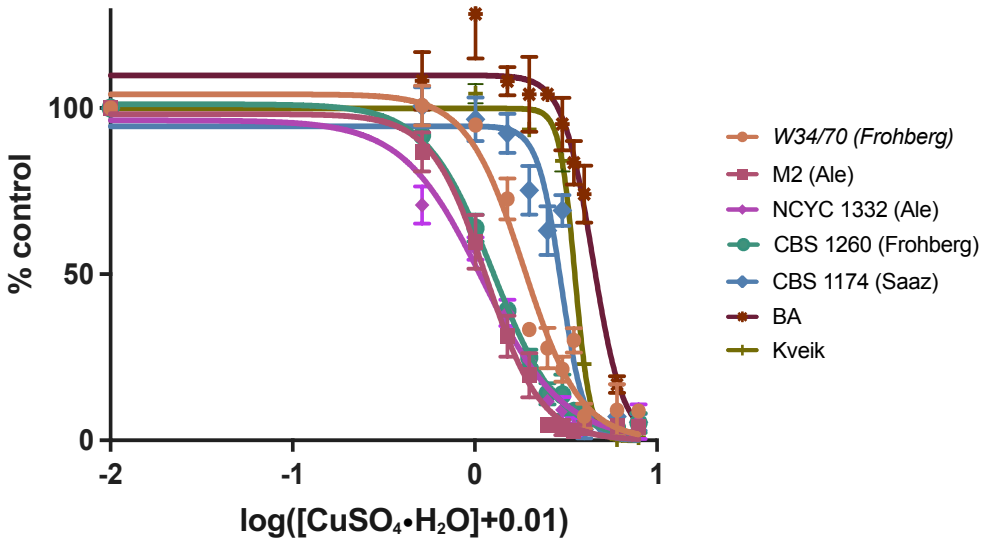

Figure 4.10. Dose response curves in response to copper sulphate pentahydrate for each strain, obtained from the MTT cell cytotoxicity assay. Yeast strains were exposed to increasing concentrations of $CuSO_4 \bullet H_2O$ (0-8 mM) and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

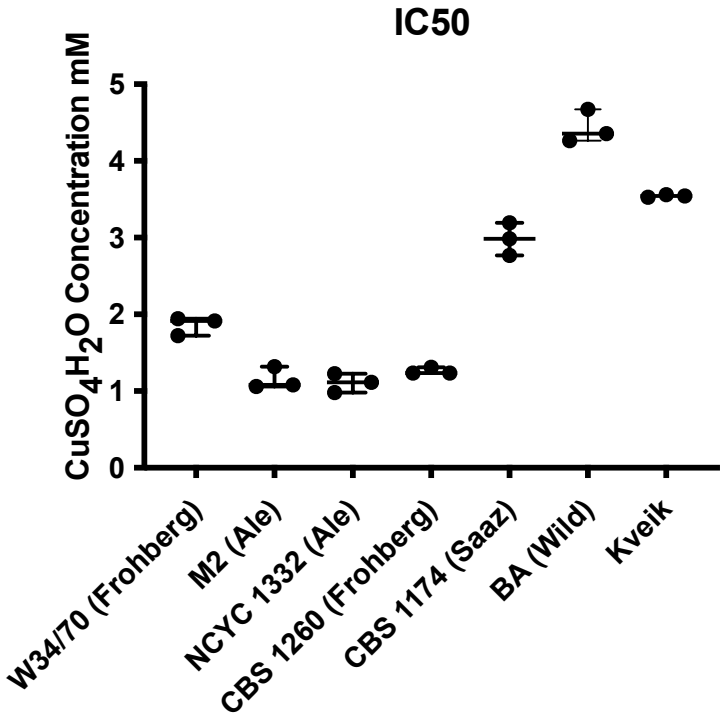

Figure 4.11. IC50 values generated from the $CuSO_4 \cdot H_2O$ dose response curves. The data displayed represents the copper sulphate pentahydrate concentration at which 50% viability is achieved. Values are a mean of triplicate data with error illustrating the standard deviation.

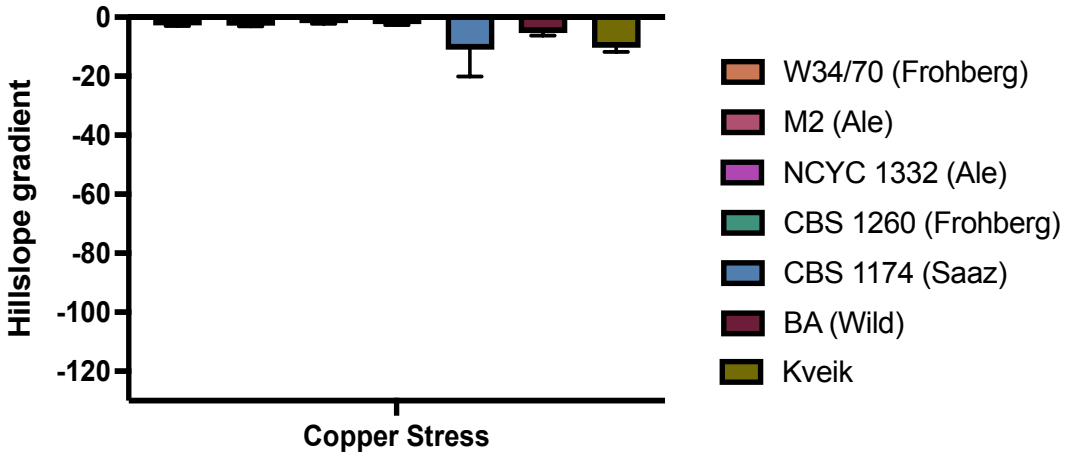

Figure 4.12. Hillslope gradient data obtained from dose response curves generated by exposing yeast to increasing concentrations of $CuSO_4 \cdot H_2O$. Smaller negative values are representative of a more gradual decline viability and therefore indicate the presence of a higher degree of phenotypic heterogeneity. While larger negative values represent a sudden decline in viability due to a lower degree of heterogeneity.

Table 4.4. Tukeys multiple comparison test for differences in copper sulphate pentahydrate stress heterogeneity (hillslope gradient) between the studied strains. (P<0.05). Green indicates significant differences and yellow corresponds to differences in heterogeneity that do not reach statistical significance.

	M2	CBS 1260	NCYC 1332	CBS1174	BA	Kveik
W34/70	>0.9999	>0.9999	>0.9999	0.0862	0.9745	0.1389
M2		>0.9999	>0.9999	0.0914	0.9780	0.1466
CBS 1260			>0.9999	0.0611	0.9456	0.1010
NCYC 1332				0.0442	0.9060	0.0747
CBS 1174					0.4364	>0.9999
BA						0.5753

4.2.5 Phenotypic heterogeneity of mixed aged yeast populations in response to heavy metal (zinc) toxicity.

Zinc, in the form of zinc sulphate heptahydrate, is commonly used in the brewing industry as a nutritional additive to ensure and improve yeast health and growth (de Nicola *et al.*, 2009). However, the role of zinc as an essential enzyme co-factor means that zinc is not typically considered to be a stressor in brewing yeast, particularly as the concentrations used rarely reach toxic levels (Vecseri-Hegyes *et al.*, 2005). However, similar to copper, this heavy metal has been the focus of previous investigations into yeast cell heterogeneity studies, and so was included here for a fully comprehensive view on population heterogeneity in response to stress (Sumner and Avery, 2002; Takahashi *et al.*, 2015). Typically, depending on yeast strain, 4-8µM of zinc ions can be added to yeast as a growth supplement (De Nicola and Walker, 2011). In this study, to achieve toxicity, a range of 0-5mM $ZnSO_4 \bullet 7H_2O$ was used, far exceeding the amounts associated with fermentations.

As before, by analysing the sigmoidal stress dose response curves obtained through the exposure of yeast populations to increasing concentrations of zinc, insights into the tolerance dynamics of each strain could be discovered. Analysis of the dose response curves obtained using the MTT assay showed similar growth kinetics across the studied strains (Figure 4.13). However, the horizontal position of dose response curve for the wild BA yeast indicated a much lower tolerance to zinc when compared to the other strains. From purely visual inspection there was little evidence to suggest other differences in tolerance between strains, demonstrated by the similar positioning of the maximum and minimum asymptotes, and similar hillslope gradients for each curve (Figure 4.13).

As described previously, the dose response curves generated were again used to calculate IC50 values (the concentration of stressor required to reduce viability to 50%). The IC50 values for each strain when exposed to zinc indicated that the wild strain BA had a significantly lower tolerance than the brewing strains (Figure 4.14). Among the brewing strains, M2 had the lowest IC50 (1.9mM zinc) and the Saaz type lager yeast CBS 1174 had the highest (2.9 mM zinc), with the remaining strains falling between 2.4-2.8mM zinc. From this IC50 data there appeared to be very little evidence suggesting a link between strain type and tolerance to zinc toxicity. Although the sample set is too small to make any firm conclusions based on species, it may be that *S. cerevisiae* brewing strains are more sensitive to this stress factor than lager yeasts. Irrespective, the IC50 values presented here correlate with previous findings using spot plate analysis (Chapter 3), such that the wild yeast BA presented the lowest tolerance and CBS 1174 one of the highest. However, the spot plate analysis found the Kveik strain had the highest tolerance to zinc, which was not reflected here, indicating that the growth matrix (liquid/solid) may impact toxicity.

Aside from the wild yeast BA, the IC50 data did not show any overwhelming differences between the strains in terms of maximum tolerance to zinc. However, the data was also assessed for heterogeneity in response based on the stress dose response curves obtained. As with the previous stressors, by obtaining the gradient of each curve at the point of inflection, an indication of population survival strategy could

be deduced. The hillslope gradient for each strain is presented in Figure 4.15, from which the data shows CBS 1260 had the largest negative gradient of -50, representing a steeper hillslope and therefore a less heterogeneous population. This was in contrast to strain M2 which yielded a hillslope gradient of -11.9, indicating the presence of a more heterogenous population of cells where viability declined more gradually as stress was increased. In order to fully ascertain differences in the degree of heterogeneity observed, Tukeys multiple comparison test was performed. It can be seen that despite the apparent visual similarities in hillslope gradient there were significant differences in heterogeneity between yeast between populations (Table 4.5). As might be expected from the data above, strains M2 and CBS 1260 presented significantly different survival strategies, and differences were also observed when comparing M2 and CBS 1174, and CBS 1260 and the Kveik yeast strain. This indicates that both CBS 1260 and CBS 1174 were significantly less heterogeneous than the other strains examined in response to zinc stress. The heterogeneity data indicated that there was no link between strain type and the degree of heterogeneity, however it is possible that there is a link between the degree of heterogeneity and tolerance (IC50) in this instance, as the strains with the weakest tolerance (M2 and BA) also had the highest degree of heterogeneity.

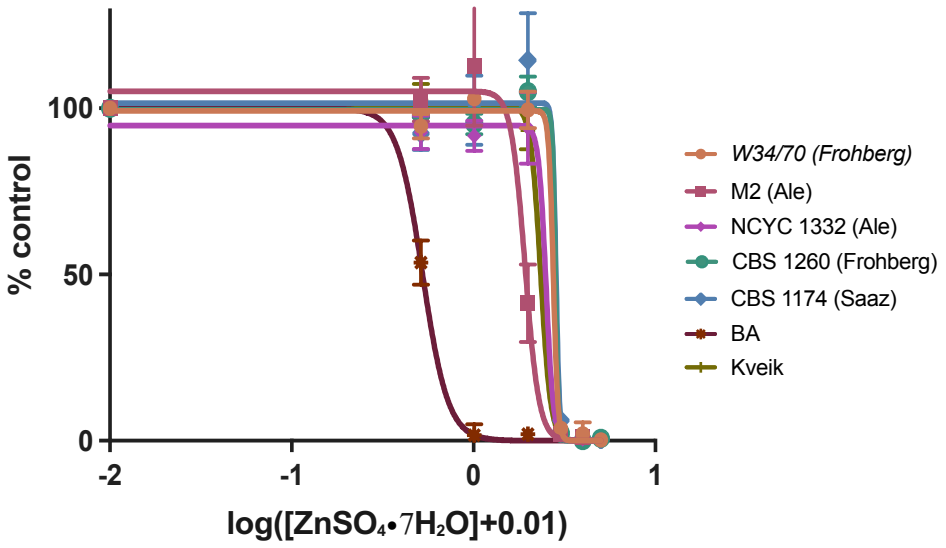

Figure 4.13. Dose response curves in response to zinc sulphate heptahydrate for each strain, obtained from the MTT cell cytotoxicity assay. Yeast strains were exposed to increasing concentrations of $ZnSO_4 \bullet 7H_2O$ (0-5 mM) and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

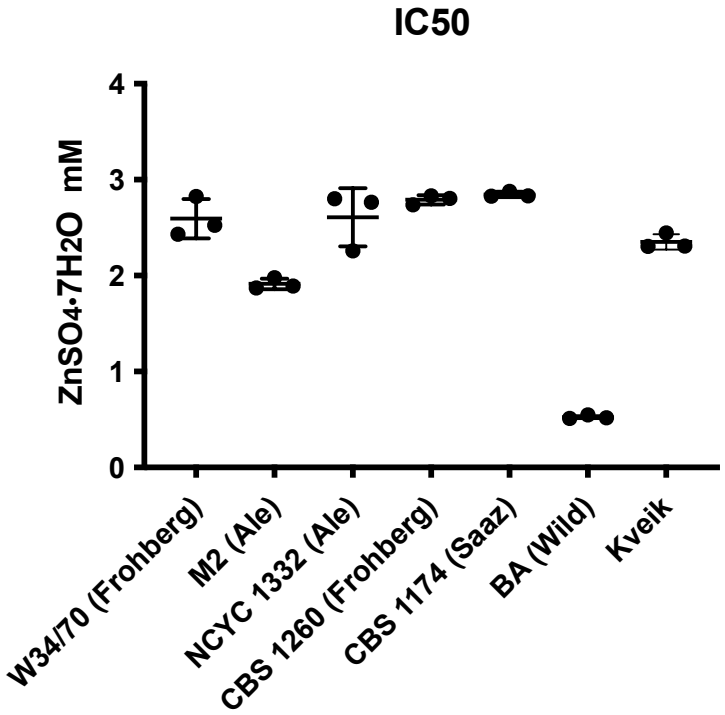

Figure 4.14. IC50 values generated from the $ZnSO_4 \bullet 7H_2O$ dose response curves. The data displayed represents the zinc sulphate heptahydrate concentration at which 50% viability is achieved. Values are a mean of triplicate data with error illustrating the standard deviation.

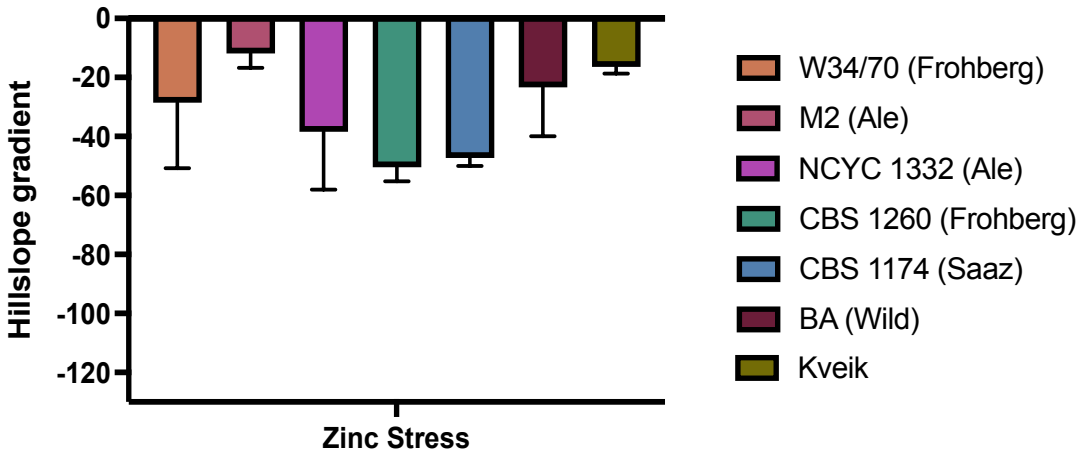

Figure 4.15. Hillslope gradient data obtained from dose response curves generated by exposing yeast to increasing concentrations of $ZnSO_4 \bullet 7H_2O$. Smaller negative values are representative of a more gradual decline viability and therefore indicate the presence of a higher degree of phenotypic heterogeneity. While larger negative values represent a sudden decline in viability due to a lower degree of heterogeneity.

Table 4.5. Tukeys multiple comparison test for differences in zinc sulphate heptahydrate stress heterogeneity (hillslope gradient) between the studied strains. (P<0.05). Green indicates significant differences and yellow corresponds to differences in heterogeneity that do not reach statistical significance.

	M2	CBS 1260	NCYC 1332	CBS 1174	BA	Kveik
W34/70	0.7112	0.4079	0.9683	0.5938	0.9989	0.9139
M2		0.0139	0.1973	0.0304	0.9333	0.9995
CBS 1260			0.9174	>0.9999	0.1782	0.0416
NCYC 1332				0.9807	0.8001	0.4015
CBS 1174					0.3050	0.0842
BA						0.9945

4.3 Analysis of physiological heterogeneity in stress-related cell targets

The data obtained in the previous section demonstrated that the survival dynamics of a yeast strain can vary in both maximum tolerance limits, and in terms of phenotypic heterogeneity. In addition, heterogeneity in stress tolerance was also observed to vary depending on the individual stress factor applied. While this data reflects the broad response of cells to stress in terms of survival, the physical difference between cells in a given population remain unclear. In order to investigate the potential sources of heterogeneity reported in the previous section, variations in physical and stress-related cell parameters were assessed. Specifically, fluorescent probes were used to target key cell components, including the mitochondria, internal membrane structure, neutral lipids, membrane fluidity and sterols in order to determine variation within populations.

Mitochondria were targeted since it is known that their activity is crucial in the tolerance to oxidative (Demasi *et al*., 2006; Müller and Reichert, 2011; Kitagaki and Takagi, 2014), osmotic (Pastor *et al*., 2009) and ethanol stress (Stanley *et al.*, 2010). Internal cell membranes were analysed since this provides insight into the structural integrity of organelles such as the endoplasmic reticulum (ER) (Sabnis *et al.*, 1997), which is also an essential asset in the yeast stress response. For example, ethanol stress can impair protein folding in the ER, an effect which can cause the ER to trigger the unfolded protein response (UPR) in order to maintain protein homeostasis and ER function (Miyagawa *et al.*, 2014; Wu *et al*., 2014). Neutral lipids such ergosterol and triacylglycerols are essential storage molecules (Koch *et al*., 2014), also important in responding to stress, since they can be hydrolysed and broken down into constituents for membrane construction, as well as for energy production. Their presence directly influences the ratio of sterols and fatty acids in the cell membrane, which has a key role in membrane organisation and correct membrane fluidity, crucial for tolerating external stressors (Alexandre *et al*., 1994; Valero *et al*., 2001; Rupčić and Jurešić, 2010). Similarly, membrane fluidity is a key parameter in the ability to tolerate ethanol, osmotic pressures and cold temperatures as response to these stresses relies

on an ordered gel phase lipid bilayer in the cell membrane (Thieringer *et al*., 1998; Beney and Gervais, 2001; Ding *et al.*, 2009; Ishmayana *et al*., 2017; Navarro-Tapia *et al*., 2018). Furthermore, the ability to switch between fluid states in the membrane can give a yeast cell an advantage in rapidly changing conditions (Ishmayana *et al*., 2017). Finally, membrane sterols, in particular ergosterol, also play a similar role since they are essential for maintaining membrane structure under periods of stress. The presence of ergosterol regulates the lipids and proteins in the membrane, and a greater ergosterol concentration essentially enhances membrane structure, allowing the cell to tolerate higher concentrations of stress such as ethanol, which act to cause the membrane to become more fluid and disordered (Swan and Watson, 1999; Bagnat *et al.*, 2000; Ding *et al.*, 2009).

In order to target mitochondria, the fluorescent probe Mitotracker Green FM (MTG) was utilised as a mechanism for quantifying mitochondrial mass. This probe permeates actively viable yeast cells and becomes oxidised. Subsequently the probe is sequestered in the mitochondria where the thiol reactive chloromethyl moiety reacts with mitochondrial proteins, thus allowing the fixed probe to be visualised by fluorescence imaging using an excitation wavelength of 490nm and emission of 516nm (Presley *et al*., 2003). The probe 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) functions to allow quantification of mitochondrial membrane potential (MMP), such that enhanced staining corresponds to greater MMP (Johnson *et al.*, 1981). Unlike the mitochondria specific stain MTG, this measure is a gauge of mitochondrial performance/health rather than their physical presence. Therefore, differences in the relative $DiOC_6$ fluorescence intensity (ex484nm/em501nm) from cell-to-cell provide insights into differences in the effect of stress on this organelle. Similarly, $DiOC_6$ can also be used to assess internal cell membranes in general, giving insight into the structural integrity of components such as the endoplasmic reticulum (ER). In order to target neutral lipid stores as a stress tolerance indicator, the fluorescent probe Nile red was utilised. Nile red is a hydrophobic, lipophilic stain emitting a yellow/red fluorescence when in a hydrophobic environment and has been successfully used previously to visualise and quantify neutral lipids in yeast cells (Kimura *et al*., 2004; Rostron and Lawrence, 2017). For analysis of cell membrane structure and function,

the fluorescent probe 6-Dodecanoyl-2-Dimethylaminonaphthalene (Laurdan) was used. This lipophilic stain is incorporated in the phospholipid bilayer and excited at ~400nm; the emission characteristics depend on the structure of phospholipid bilayer. Luardan emits fluorescence at ~440nm when incorporated in an ordered gel phase membrane, while a red shift in emission occurs when the probe is incorporated in a liquid disordered membrane and emits fluorescence at ~490nm (Parasassi *et al.*, 1998; Pilkington *et al.*, 2016; Ishmayana *et al*., 2017). By analysing the fluorescence of this membrane probe, insights in both membrane structure and the presence of heterogeneity from cell-to-cell can be determined through the use of flow cytometry. Finally, membrane sterols were analysed using the fluorescent stain filipin, which binds sterols, cholesterol and lipoproteins (Miller, 1984). Using an excitation of ~360nm the fluorescence of the targeted filipin stain can be detected and quantified at ~480nm emission (Maxfield and Wüstner, 2012).

4.3.1 Mitochondria heterogeneity determination

In order to assess the cell-to-cell variability in yeast mitochondrial content and integrity, cell populations were stained with Mitotracker Green FM (MTG) as described in Section 2.10.1. To confirm the accuracy of this procedure, stained cells were first visualised using fluorescence microscopy. Using W34/70 as an example, images of cell populations stained with MTG could be obtained (Figure 4.16a). From this visual data it was evident that the mitochondrial probe accurately stained yeast mitochondria, with a distinct 'string' of organelles clearly visible. After successful staining had been established, cells within each strain were assessed for mitochondria number using the MTG probe in conjunction with single cell flow cytometry. Stained cells were first visualised by flow cytometry based on how the source light was scattered from each cell, giving data for cell size (FSC1-Area) and cell granularity (SSC1-Area). This was performed in order to identify the main population of cells (Figure 4.16b). Using W34/70 as an example, the dot plot data can be seen to show each individual cell as coloured dots (Figure 4.16b). From this, the main population could be identified and gated, in this instance comprising 96.4% of the whole population. Cells in this identified population were subsequently visualised using the size

parameters FSC1-height and FSC1-Width to discriminate between singlets, doublets and triplet aggregates. This analysis allowed the desired population, comprising only single cells, to be gated and unwanted cell fragments and aggregates were discarded (Figure 4.16c). The final population selected was then assessed for MTG fluorescence, with data recorded for each individual cell (Figure 4.16d). By analysing the variation in MTG fluorescence intensity (FL-17 Area) between cells, heterogeneity in mitochondrial content could be assessed. By analysing the breadth of the fluorescence peak obtained, an insight into mitochondrial content variation could be made, with a broader peak indicative of greater cell-to-cell variation (Figure 4.16d).

Analysis of populations in this way resulted in fluorescence histograms being generated for each of the studied strains (data not shown), giving insight into the degree of variation in each yeast. In order to quantify the variation seen in these histogram peaks, the coefficient of variance (CV) was used and these could be compared between strains. For example, when comparing the MTG fluorescence CVs for the wild strain BA and the ale strain M2, significant differences were evident (Table 4.6). The wild BA yeast displayed the smallest population variation, with a CV of 75, while M2 exhibited a significantly higher variation in MTG with a CV of 316. The remaining strains produced values within the range of these extremes (Table 4.6). Further analysis indicated that there was little evidence to suggest a link between CV and strain type (ale/lager/wild). Interestingly, it appeared that those strains with a greater degree of variation exhibited two distinct peaks, due to the presence of sub-populations with higher MTG fluorescence (Figure 4.16d). This can also be seen to some extent in the fluorescence images shown in Figure 4.16a, where some W34/70 cells exhibited a much brighter fluorescence than others.

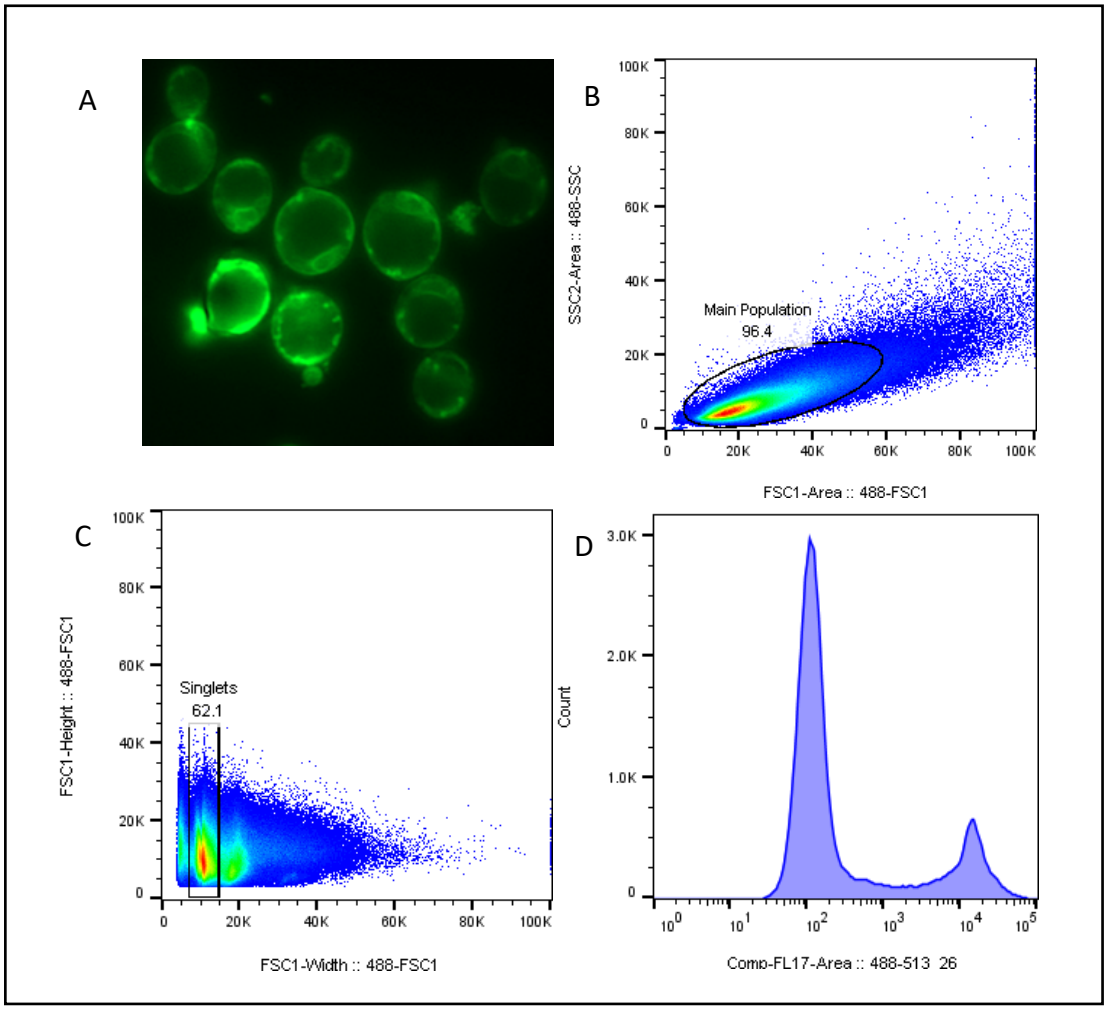

Figure 4.16. Assessment of mitochondrial mass in stained W34/70 cells using Mitotracker Green FM. A: Stained W34/70 cells visualised using 400X magnification, excited using UV source light and green fluorescence detected using a GFP emission filter. B: Flow cytometry dot plot for side scatter area against forward scatter area for W34/70 cells. Each coloured dot represents the light scatter detected for each cell event, the use of a heat map identified areas of greater cell concentration, designated the 'Main Population'. C: Flow cytometry dot plot for W34/70 cells analysed by forward scatter size parameters. This dot plot, demonstrates the scatter data for each cell of the 'Main Population' giving further detail in the size of each cell, revealing populations such as: Cell fragments, singlets, doublets etc. D: Fluorescence histogram peak for Mitotracker Green fluorescence among cells of W34/70. Histogram data represents the frequency (count) of cells based on MTG fluorescence increasing along the x-axis.

Table 4.6. Population variance data for mitochondrial mass based on MitoTracker Green fluorescence. Cell-to-cell variance was evaluated using the coefficient of variance for each of the studied strains.

Strain	CV- MitoTracker Green FM (Mitochondrial biomass)
W34/70 (Frohberg)	236
M2 (Ale)	316
NCYC 1332 (Ale)	203
CBS 1260 (Frohberg)	239
CBS 1174 (Saaz)	152
BA (Wild)	75
Kveik	191

4.3.2 Internal membrane integrity and mitochondrial membrane potential heterogeneity determination

Internal membranes structures, such as the endoplasmic reticulum (ER) and the mitochondria must have sound integrity in order for the yeast cell to tolerate fermentation related stress (Yang *et al.*, 2012; Wu *et al.*, 2014). To determine the cell-to-cell variability within each yeast strain with respect to these attributes, the fluorescent probe 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) was used. A greater $DiOC_6$ fluorescence intensity is a reflection of superior internal membrane integrity and greater mitochondrial membrane potential (MMP), compared to cells with a lower degree of fluorescence. Initially, staining efficiency was assessed by analysing cell populations using fluorescence microscopy and examples of the acquired images can be seen in Figure 4.17(a) using strain W34/70. From these images it is evident that the fluorescence intensity varies from cell-to-cell, implying the presence of heterogeneity in internal membrane integrity. In order to quantify this variation, $DiOC_6$ stained cell populations were analysed using flow cytometry. As with the previous protocols, only the desired single cell populations were investigated and

these were isolated as described above (Section 4.3.1). Once the desired singlet population had been identified, $DiOC_6$ fluorescence intensity was determined and, using strain W34/70 as an example, the data broadly indicated the distribution of fluorescence intensities between cells within a population (Figure 4.17b). From this data, distribution of fluorescence was quantified and strains exhibiting a wide $DiOC_6$ fluorescence peak (FL17-Area) were indicative of greater population heterogeneity when compared to those with a more narrow peak. Using the coefficient of variance to quantify the degree of variation, heterogeneity in membrane composition was determined for each strain, as shown in Table 4.8. When comparing the CV values for each strain there was evidence demonstrating large differences between the strains, for example NCYC 1332 has a low CV of 22, while the Kveik strain displays more variation with a CV of 86. This data suggests there are differing degrees of heterogeneity in internal membrane integrity and MMP between brewing yeast populations, this may be linked to heterogeneity in the yeast stress response. For example, the high degree of MMP/internal membrane integrity variation among cells of the Kveik strain may be a contributing factor to the high heterogeneity in response to ethanol and osmotic. As a result of the link between internal membrane integrity and superior tolerance to these stresses (Yang *et al.*, 2012; Wu *et al*., 2014).

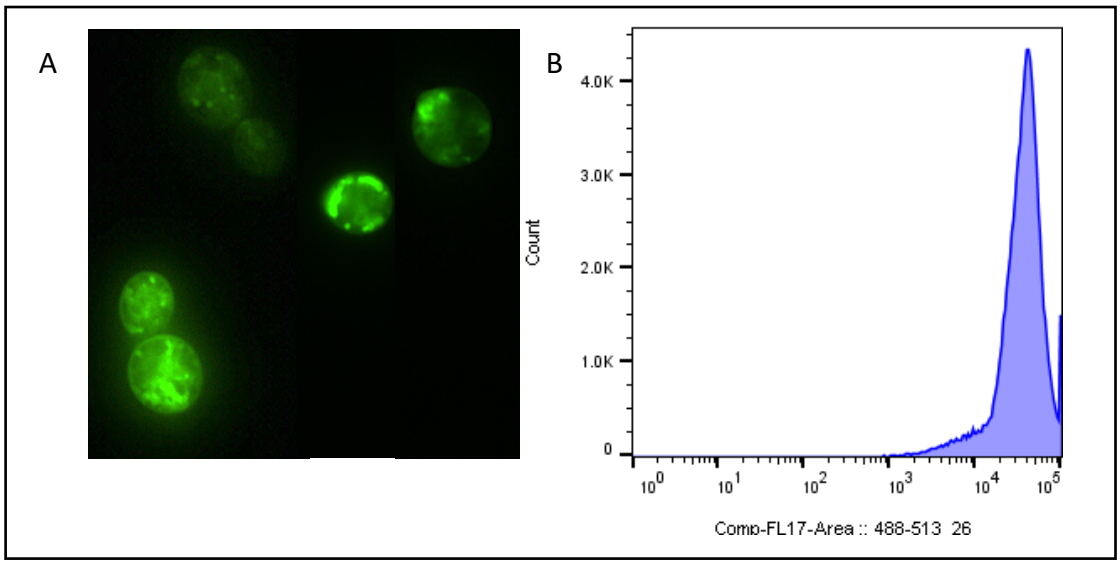

Figure 4.17. Assessment of mitochondrial membrane potential based on $DiOC_6$ fluorescence in stained W34/70 cells. A: Stained cells visualised using 400X magnification, excited using UV source light and green fluorescence detected using a GFP emission filter. B: Fluorescence histogram peak for $DiOC_6$ fluorescence among cells of W34/70. Histogram data represents the frequency (count) of cells based on $DiOC_6$ fluorescence (FL17- Area) increasing along the x-axis.

Table 4.8. Population variance data for mitochondrial membrane potential based on $DiOC_6$ fluorescence. Cell-to-cell variance was evaluated using the coefficient of variance for each of the studied strains.

Strain	**CV- $DiOC_6$**
W34/70 (Frohberg)	49
M2 (Ale)	37
NCYC 1332 (Ale)	22
CBS 1260 (Frohberg)	44
CBS 1174 (Saaz)	75
BA (Wild)	63
Kveik	86

4.3.3 Neutral lipid heterogeneity determination

As mentioned previously, neutral lipids are essential as a reserve carbohydrate store and for their role in membrane reorganisation when responding to stress (Alexandre *et al.*, 1994; Koch *et al.*, 2014). Consequently, the presence of cell-to-cell variation in neutral lipid content could be an influential factor in the heterogeneous stress response in yeast. To determine the cell-to-cell heterogeneity in neutral lipid content, the stain Nile red (NR) was used. Yeast cells stained with NR were analysed using fluorescence microscopy and visual examples of this can be seen in the fluorescence images shown in Figure 4.18(a). Using strain W34/70 as a representative example, the distinct brightly fluorescent neutral lipid droplets are readily visible and variable from cell to cell. To quantitatively assess the degree of variation in neutral lipids within the studied yeast strains, single cell flow cytometry was utilised. As with the MTG heterogeneity investigation, stained cells for each strain were first analysed by cell size (FSC1-Area) and granularity (SSC2-Area) to identify the main population, followed by FSC1-height against FSC1-width. From this the desired singlet population was identified, and these cells were analysed for their individual NR fluorescence intensities. The NR fluorescence in singlets of the main population was analysed for each strain, providing fluorescence histograms from which variation can measured and insights into heterogeneity could be obtained. The distribution of NR fluorescence intensity for individual cells of strain W34/70 is displayed in Figure 4.18(b). This demonstrates a variation in fluorescence from cell-to-cell with some individuals emitting a much higher fluorescence than others. In order to quantify this variation for each strain, the coefficient of variance was again used; a larger CV corresponds to a broader peak, and therefore a greater degree of heterogeneity in neutral lipid content, while smaller CVs correspond to a lower degree of heterogeneity. The CVs for each of the strains can be found in Table 4.7. This data revealed significant differences in neutral lipid heterogeneity between the strains, for example the lager strain CBS 1174 demonstrated the largest variation with a CV of 188, while the ale strain M2 had the smallest CV of 61, corresponding to a smaller degree of heterogeneity in neutral lipid content. There did not appear to be an obvious link between CV and strain type, with both lager and ale strains showing a range of values

within these extremes. This investigation provides evidence showing that the quantity of neutral lipid droplets does vary from cell-to-cell in yeast populations, and that the degree of heterogeneity significantly differs between the strains.

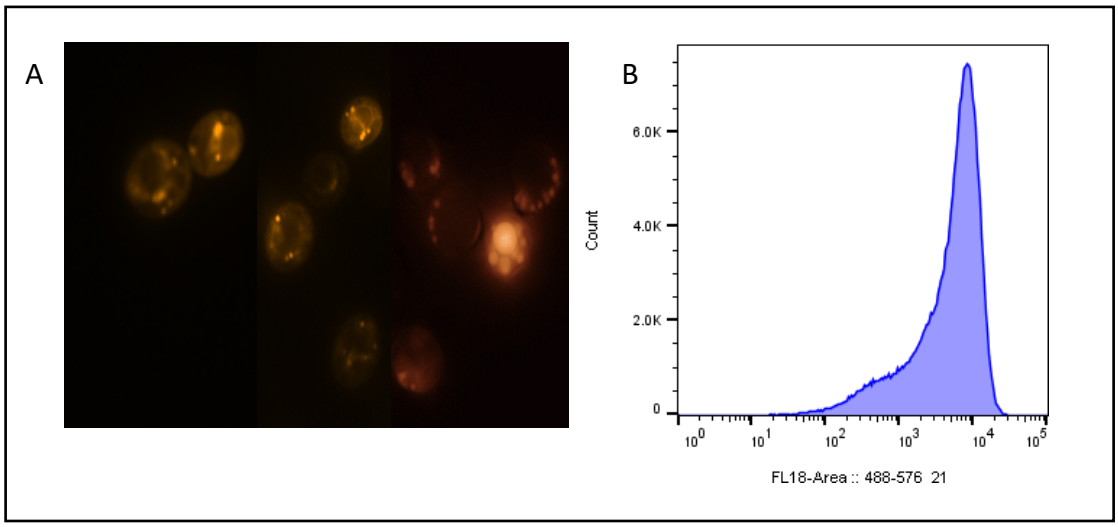

Figure 4.18. Assessment of neutral lipid content in stained W34/70 cells based on Nile red fluorescence. A: Stained W34/70 cells visualised using 400X magnification, excited using blue source light and yellow/orange fluorescence detected using a CYT emission filter. B, Fluorescence histogram peak for Nile red fluorescence among cells of W34/70. Histogram data represents the frequency (count) of cells based on NR fluorescence (FL18- Area) increasing along the x-axis.

Table 4.7. Population variance data for neutral lipid content based on Nile red fluorescence. Cell-to-cell variance was evaluated using the coefficient of variance for each of the studied strains.

Strain	**CV- NR**
W34/70 (Frohberg)	67
M2 (Ale)	61
NCYC 1332 (Ale)	97
CBS 1260 (Frohberg)	135
CBS 1174 (Saaz)	188
BA (Wild)	137
Kveik	102

4.3.4 Membrane sterol heterogeneity determination

Sterols are an important component of the yeast cell membrane, and the quantity and arrangement of these compounds can influence the ability for a yeast strain to respond to stress (Swan and Watson, 1999). To assess the heterogeneity in sterol content within populations, the fluorescent probe filipin was used. Initially, cell populations were stained with filipin and assessed by fluorescence microscopy. Using strain W34/70 as an example, it can be seen that sterols could be clearly visualised and that high concentrations were observed localised at the cell membrane, demonstrated by the presence of brighter blue spots on the outside of the cell (Figure 4.19a). Subsequently each of the other strains were analysed in the same way (data not shown) and heterogeneity in cellular sterol content was assessed using flow cytometry. As before, the singlet cell population for each strain was isolated and analysis of these cells using filipin was used to identify fluorescence intensity in individual cells, as shown in Figure 4.19b. From this data, insights into sterol heterogeneity could be gained by measuring the coefficient of variance across the population. The CV values for each strain are shown in Table 4.9 and from this data it appeared that heterogeneity was present in each strain, but to a relatively low degree, as indicated by the small CV values. The smallest CV obtained was for the lager strain W34/70 (CV: 37) while the largest CV was for the lager strain CBS 1174 (CV: 59). While differences in CV were present between strains, they were small, indicating that sterol content was mainly consistent under the defined conditions applied and likely not a major influential determinant on the heterogeneity observed in response to stress.

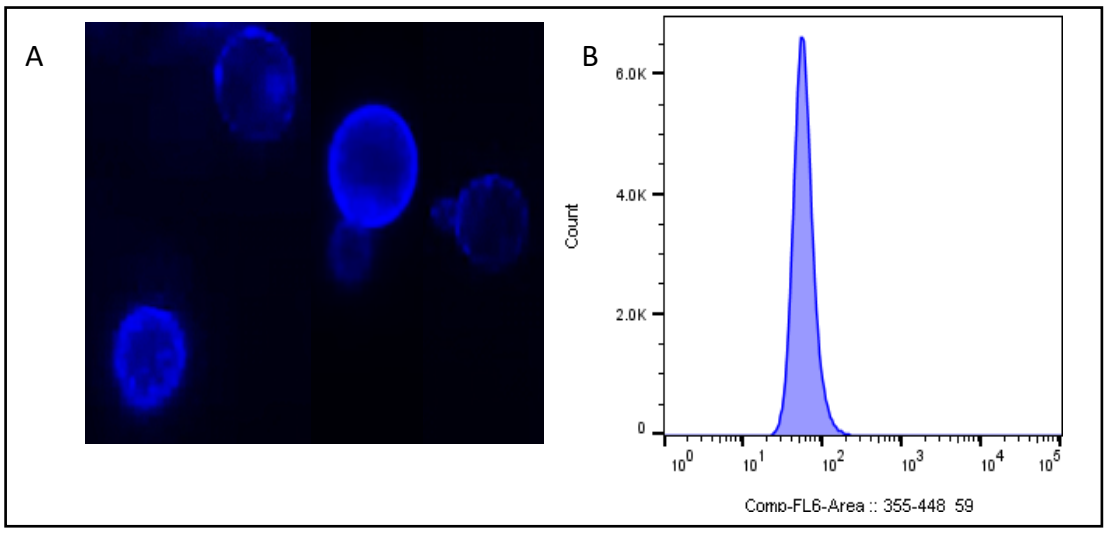

Figure 4.19. Assessment of sterol content in W34/70 cells stained using filipin. A: Fluorescence images of W34/70 cells stained with filipin. Stained cells visualised using 100X magnification, excited using UV source light and blue fluorescence detected using a DAPI emission filter. B: Fluorescence histogram peak for filipin fluorescence among cells of W34/70. Histogram data represents the frequency (count) of cells based on filipin fluorescence (FL6- Area) increasing along the x-axis.

Table 4.9. Population variance data for sterol content, based on filipin fluorescence. Cell-to-cell variance was evaluated using the coefficient of variance for each of the studied strains.

Strain	**CV- filipin**
W34/70 (Frohberg)	37
M2 (Ale)	43
NCYC 1332 (Ale)	49
CBS 1260 (Frohberg)	44
CBS 1174 (Saaz)	59
BA (Wild)	58
Kveik	47

4.3.5 Membrane fluidity heterogeneity determination

In addition to sterol content, membrane fluidity is also known to influence the capacity of a cell to tolerate brewing related stress factors. Cell membranes with a more liquid, disordered structure render themselves at risk to stressors such as ethanol (Ishmayana *et al*., 2017). Due to the close link between membrane fluidity and stress response it was hypothesised that variable membrane fluidity from cell-to-cell, could be a source of heterogeneity in stress response. To investigate this hypothesis, yeast cell populations were stained with the fluorescent probe Laurdan (6-Dodecanoyl-2-Dimethylaminonaphthalene). Initially, the performance of this stain was analysed using fluorescence microscopy to generate fluorescent images for the strain W34/70, as shown in Figure 4.20(a). This data indicated that the probe was able to successfully stain yeast cells, due to fluorescence localised at the cell membrane. Subsequently, analysis of populations was conducted to determine the cell-to-cell variation in membrane fluidity for each yeast strain. It should be noted that this technique is more complicated than with the previous cell probes; Laurdan is incorporated into the phospholipid bilayer and when excited with a 355nm laser, the fluorescence emission depends on the fluidity of the membrane, such that a more structured gel membrane emits fluorescence at ~440nm while in the liquid phase emits at 490nm (Learmonth and Gratton, 2002; Ishmayana *et al*., 2017). Therefore, fluorescence detection and variance were determined via flow cytometry using both the 448nm and 526nm channels in order to detect fluorescence in both states respectively. The single cell population was isolated as described previously and analysed for Laurdan fluorescence in each strain. Firstly, the fluorescence emitted from each cell within a population was assessed at 448nm emission (FL6-Area), which gives a measure of the ordered gel phase of the lipid bilayer. An example of this data using strain W34/70 can be seen in Figure 4.20(b). As before, this analysis was repeated for each strain and the collected data allowed a comparison of heterogeneity, where strains with a broader fluorescence peak were those with a greater degree of variation from cell-to-cell. To quantify the 448nm fluorescence peak width for each strain, the coefficient of variance was again used and the CVs for each strain can be seen in Table 4.10. From this data it was evident that heterogeneity was

present in each strain, however the degree of heterogeneity at this emission wavelength differed between each of the strains analysed. W34/70 exhibited the largest CV at 448nm emission (CV: 167), while CBS 1260 has presented the lowest CV (CV: 52), indicating a much smaller degree of heterogeneity. In addition to the heterogeneity data gained when analysing the fluorescence at 448nm, further insights were gathered from fluorescence emission at 526nm (FL7 Area). Measuring the emission at 526nm investigates the fluorescence produced by the Laurdan stain when situated in a more liquid, disordered membrane state. Again, the variation of fluorescence from cell-to-cell was measured for each strain and an example of this can be seen in Figure 4.10(c) for strain W34/70. Quantification of data for all of the yeast strains were used to calculate coefficient of variance values. CV data for 526nm emission is displayed in Table 4.10 and from this data it can be seen that large differences in variance were present between strains. In this instance, strain W34/70 exhibited the largest CV (CV: 114) while CBS 1260 had the lowest (CV: 44). However, when analysing the CVs from both emission channels, there was little evidence to suggest that there was a link between Laurdan stain emission heterogeneity and yeast species. However, there was a link between 448nm emission (FL6) and 526nm (FL7), with strains displaying a high CV in 448nm emission also giving a high CV in 526nm emission. While this indicates that heterogeneity in both membrane states is present in some strains to a higher degree than others, to determine the true heterogeneity in membrane fluidity the ratio of 448nm/526nm emission for each cell can be used. This value gives a ratio of ordered gel phase to liquid disorded phase for each cell, where a higher value corresponds to a cell with a more ordered membrane bilayer overall (Mazeres *et al*., 2017). The population mean for this metric of membrane fluidity is shown in Table 4.10. It can be seen that each strain displayed a similar mean 448nm/526nm ratio per cell, indicating similar membrane fluidity profiles across each strain population. However, of all of the strains, the wild BA yeast population exhibited a higher mean ratio, indicating a more ordered gel phase membrane within the culture. In order to assess the heterogeneity in membrane fluidity within each population, the 448nm/526nm emission ratio data was calculated on a cell-cell basis and the coefficient of variance determined. The resulting CV values for each strain were subsequently compared and differences in membrane fluidity heterogeneity

evaluated. From Table 4.10 it is apparent that the CV values for this measure of membrane fluidity were similar in all conventional brewing strains, ranging from 18.8-24.6. In contrast, the wild BA yeast strain and the Kveik yeast produced significantly higher CVs of 106 and 78.1 respectively, indicating that these strains have a greater degree of variance in membrane fluidity characteristics. This provides a potential rationale for the high degree of heterogeneity observed in the BA strain in response to stress, and perhaps also an indication as to why the Kveik yeast strain showed a high degree of tolerance to stress in general.

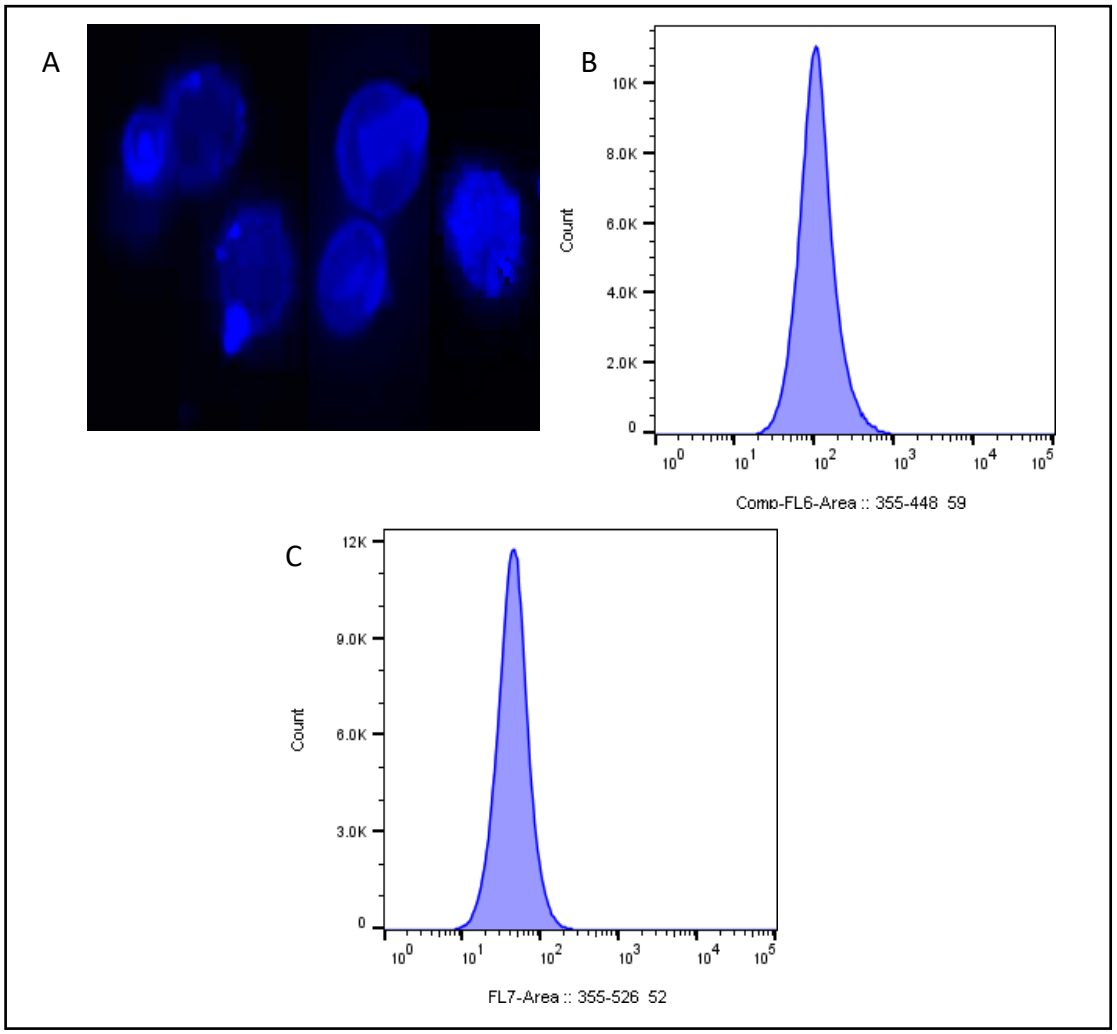

Figure 4.20. Assessment of membrane fluidity in W34/70 cells based on Laurdan fluorescence. A: Fluorescence images of W34/70 cells stained with Laurdan. Stained cells visualised using 400X magnification, excited using UV source light and blue fluorescence detected using a DAPI emission filter. B: Fluorescence histogram peak for Laurdan fluorescence among cells of W34/70. Data represents the frequency (count) of cells based on Laurdan fluorescence (FL6- Area) increasing along the x-axis. Fluorescence measured at 448nm (FL6) demonstrates the emission from an ordered gel phase membrane. C: Fluorescence histogram peak for Laurdan fluorescence among cells of W34/70. Data represents the frequency (count) of cells based on Laurdan fluorescence (FL7- Area) increasing along the x-axis. Fluorescence measured at 448nm (FL6) indicated a liquid disordered membrane.

Table 4.10. Population variance data for membrane fluidity, based on Laurdan fluorescence. Cell-to-cell variance was evaluated using the coefficient of variance for each of the studied strains.

Strain	CV- Laurdan FL 6	CV- Laurdan FL 7	448/536 Ratio Mean	CV- Ratio
W34/70 (Frohberg)	167	114	2.47	18.8
M2 (Ale)	81	64	2.87	21.4
NCYC 1332 (Ale)	92	61	2.12	24.6
CBS 1260 (Frohberg)	52	44	2.54	22.3
CBS 1174 (Saaz)	74	66	3.34	22.7
BA (Wild)	100	92	4.02	106
Kveik	72	51	2.45	78.1

4.4 Heterogeneity in cell size and age

Analysis of cell components indicated that there was significant variation within most yeast populations, irrespective of genus, species or strain type. However, while it is likely that this variation may manifest in terms of phenotypic and performance heterogeneity, the root causes of this variation remain unclear. Arguably the most basic factors that could impact variation within a population are related to cell size and age. Indeed, it is well documented that both cell size and age vary in brewing yeast populations. Under controlled laboratory conditions it is typical for cultures to consist of ~50% daughter cells but individuals showing a divisional age of 12 (divisions) have been observed (Barker and Smart, 1996). This wide distribution of cell age is likely to be linked to cell size since older cells are larger than newly produced daughter cells, and the distribution of cell size in a population closely matches that of cell age (Bartholomew and Mittwer, 1953; Barker and Smart, 1996; Powell *et al.*, 2000; Powell *et al.*, 2003). This variation has been implicated in changes to fermentation performance, demonstrated by links to flocculation efficiency and flavour production (Powell *et al.*, 2003). Consequently, determining the impact of size and age in yeast strains reflects an important starting point in further understanding the causes of heterogeneity in yeast strains. To investigate the presence of heterogeneity in cell size and age, each parameter was quantified using flow cytometry using light scatter for size in conjunction with fluorescent staining for age determination. It should be noted that brewing yeast cell-age nomenclature can vary depending on the process in question. Generation number can refer to how many cell divisions a cell has performed, or alternatively it can refer to the number of times that a yeast culture has been used for fermentation. For the purposes of this study, cell age will follow the format that daughter cells, referred to as generation 0, are those that have recently budded from a parent cell. Parent cells are therefore aged by the number of times they have budded to produce a new cell, with generation 1 having produced one cell, generation 2 having produced two cells etc.

4.4.1 Cell size heterogeneity

One of the most elementary forms of heterogeneity in microbial populations is cell size. It is common to observe a wide distribution of cell sizes, which can be accentuated when cells are exposed to stress and environmental conditions. For example, it is known that cell size can vary at different stages of fermentation (Speers *et al.*, 2006; Tibayrenc *et al.*, 2010). In order to determine the heterogeneity in cell size amongst various yeast strains, flow cytometry analysis was used. While flow cytometry does not give a specific measure of cell size without the use of reference beads, it can be used to obtain a comparative measure of cell size by analyzing the forward scatter light distribution produced from each measure event (cell). Furthermore, this technique facilitates the analysis of a large number of cells in a short space of time, making the process highly efficient as well as accurate.

Exponential phase yeast populations were prepared as described in Section 2.11.3 and the entire cell population was then analysed for size and granularity characteristics (FSC1-Area against SSC2-Area) as shown in Figure 4.21(a). The parameters FSC (forward scatter) and SSC (side scatter) refer to the way in which the light source scatters off the cell, with FSC giving a direct relation to the size of the cell, due to greater forward scatter being detected in larger cells. SSC corresponds to the cell granularity; cells that are more granular in topography scatter light to a greater extent, giving indications into large and more granular aggregates of cells. To gain an accurate snapshot of the population >100,000 events were analyzed for each strain. The main population of cells from each strain was gated to exclude large aggregates and debris (Figure 4.21a). In the example data shown, for the strain W34/70, this 'main population' consisted of 94% of the overall cells. Subsequently, this fraction was visualized based on the parameters FSC1-Width against FSC1-Height, allowing distinct sub-populations to be observed (Figure 4.21b). Fragments of cells, singlets, doublets and larger aggregates become visible using this analysis, as shown in the W34/70 data, and a further gate applied to isolate single cells, comprising 62% of the 'main population' defined above. At this point, only the singlet population was taken forward for further analysis; a histogram of these single cells was produced from the

flow cytometry data, displaying the range of cell sizes (FSC1-Area) within this population (Figure 4.21c). From this the horizontal breadth of the histogram peak can be used to measure cell size heterogeneity among each of the strains. Visually, a wider peak corresponds to a greater cell-to-cell variation in cell size and therefore higher degree of heterogeneity. Quantitatively, the degree of cell size variation within a population can be evaluated using the coefficient of variance (CV) for FSC1-Area. The coefficient of variance was obtained by dividing the standard deviation of cell sizes by the mean size in a population, resulting in a normalized measure of population heterogeneity. Using the example data obtained for strain W34/70, this evaluation produced a CV of 36 (Figure 4.21). This flow-path of analyses was performed on the remaining yeast strains and cell size heterogeneity evaluated using the CV values. The comparative CV values shown in Table 4.11 indicate that each strain exhibited differing degrees of variation in cell size. Strains CBS 1174 and BA (CV: 53 and 56 respectively) showed the highest variation, while strains M2 and CBS 1260 showed the smallest CV (CV: 30) and so possess lower heterogeneity in cell size. While this data was not unexpected for the wild BA yeast, which is known to be pleiomorphic in nature, it was interesting to note that each of the other strains had their own unique degree of cell size heterogeneity. Despite this, there was no apparent link between brewing strain type (lager/ale), indicating that species type did not impact this parameter *per se*. Furthermore, when comparing the cell size CV to the mean cell size (Table 4.11) there was an inverse relationship, such that strains with a higher CV generally exhibited a lower mean cell size and *vice versa*. While differences in variation were observed between strains, it is worth noting that these differences are relatively small and do not necessarily imply an impact on phenotype or physiological characteristics.

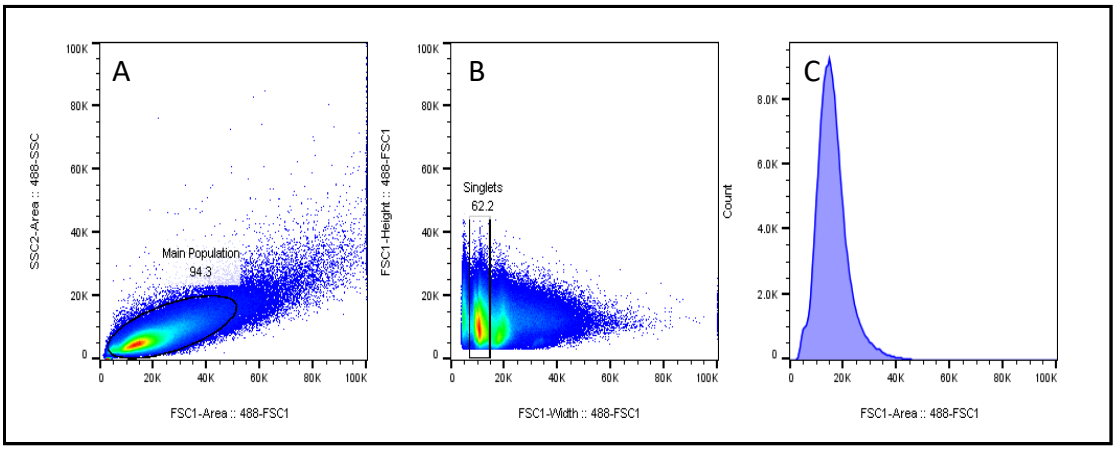

Figure 4.21. Cell size distribution in W34/70 populations after 48 hrs cultivation in YPD. Data was obtained using flow cytometry and coloured dots correspond to an event (cell). The heat map indicates the concentration of cells such that red zones have a greater concentration of cells, while blue indicates low density. A: side scatter area (SSC2-Area) vs forward scatter are a (FSC1-Area), analysis of cell granularity and size highlighting the 'Main Population' of cells (94.3%). B: forward scatter height (FSC1- Height) vs forward scatter width (FSC1- Width), cell discrimination highlighting the singlets (62.2%) from distinct fragment and doublet populations. C: Event count vs cell size (FSC1-Area), displaying the range and frequency of cell size among the singlet population.

Table 4.11 Yeast strain size variation and mean

Strain	CV- Size	Mean-Size (FSC1- Area)
W34/70 (Frohberg)	37	16171
M2 (Ale)	30	23013
NCYC 1332 (Ale)	48	21294
CBS 1260 (Frohberg)	30	17324
CBS 1174 (Saaz)	53	9967
BA (Wild)	56	9769
Kveik	33	20764

4.4.2 Cell age heterogeneity

Frequently related to cell size, cell age is also a potential source of heterogeneity within microbial populations. In fermentation environments, yeast cultures are known to comprise a wide distribution of cell ages and it is widely accepted that a cell age distribution follows the pattern of 50% daughter cells, 25% 1 cell division, 12.5% 2 cell divisions etc. (Powell *et al.*, 2003). As discussed above, this variation in cell age has been linked to changes in flocculation efficiency, sugar utilization and flavour production (Powell et al, 2003). Therefore, heterogeneity in age was determined for each strain using fluorescent stains in conjunction with flow cytometry. Yeast cell age was evaluated using the fluorescent stain calcofluor white which allows for the visualization of yeast bud scars. This stain was selected since it provides a good contrast between bud scars and other cellular material, and the longevity against photobleaching facilitates accurate cell age determination.

As with the cell size analysis in the previous section, each strain was first cultured to exponential phase and then cells were stained with calcofluor white and analysed using flow cytometry (Section 2.11.1 and Section 2.11.3). Firstly, each cell event was analysed for FSC1-Area against SSC2- Area to measure both cell size and cell granularity. As displayed in Figure 4.22(a), using strain W34/70 as an example, the 'main population' of yeast cells (excluding large aggregates and fragments) was gated, comprising 95% of cells. At this point, single cells within this fraction were identified and gated (Figure 4.22b), and individual cells were then measured for calcofluor white (bud scar) fluorescence. By evaluating the range and frequency of bud scar fluorescence intensity throughout the yeast population a measure of cell age heterogeneity was then determined. This was achieved by determining the calcofluor white fluorescence for each cell within the population (FL6- Area), measured and displayed as a frequency count. An example of this data for yeast strain W34/70 is shown in Figure 4.22(c). From these results, it can be seen that the width of the peak indicates the degree of variation in bud scar fluorescence from cell-to-cell. In order to quantify this variation, the coefficient of variance (CV) was again used, revealing a value for cell age heterogeneity for each yeast strain (Table 4.12). The CV analysis

showed that the strain with the lowest population variance in cell age was NCYC 1332 (CV: 60), while the largest was from the wild yeast strain BA (CV: 86). Interestingly the *S. cerevisiae* strains (NCYC 1332, M2 and Kveik) all had a lower CV than the lager strains, suggesting a greater heterogeneity in bud scar fluorescence and therefore a greater range in cell age in lager yeast strains. It should be noted that despite the affinity of the calcofluor white stain for yeast bud scars, this fluorescent probe does give some non-specific binding, therefore cell size may also have been an influential factor on the intensity of fluorescence emitted for each cell. To assess the impact of this, the age CV for each strain was compared with both the CV for cell size and the mean cell size for each strain (Table 4.12). From this it appeared that there was no strong evidence to suggest that strains with a higher CV in size also exhibited a high CV in age, indicating that the heterogeneity in calcofluor white staining was likely to be a result of bud scar quantity and not influenced by cell size. Irrespective, it is interesting to note that a previous, albeit limited, study has shown that ale strains show a reduced lifespan when compared to lager counterparts (Powell *et al*., 2000), which also somewhat corroborates this data. It is also worth noting, that while there are clear differences in CV between each of the strains in both cell size and age, when visually comparing the histogram peak widths between strains, the differences are relatively small. For example, this can be seen when comparing the data from W34/70 which has a high CV (Figure 4,22c), to NCYC 1332 which has the lowest CV for calcofluor white fluorescence (Figure 4.22d).

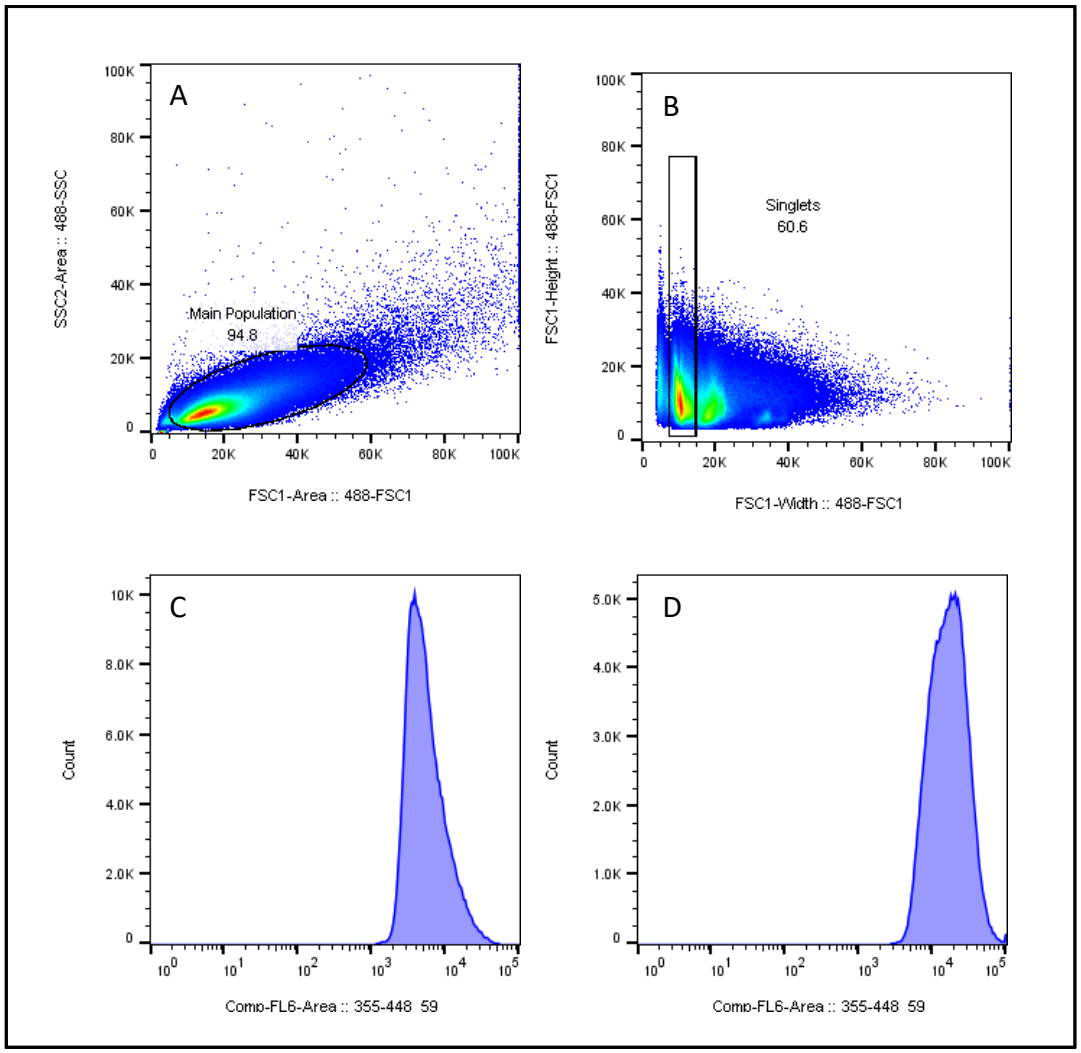

Figure 4.22. Cell age distribution of W34/70 cells after 48 hrs cultivation in YPD. Data was obtained using flow cytometry and coloured dots correspond to an event (cell). The heat map indicated the concentration of cells such that red zones have a greater concentration of cells and blue is low. A) W34/70 side scatter area (SSC2-Area) vs forward scatter are a (FSC1-Area), analysis of cell granularity and size highlighting the 'Main Population' of cells (94.8%). B) W34/70 forward scatter height (FSC1-Height) vs forward scatter width (FSC1- Width), cell discrimination highlighting the singlets (60.6%) from distinct fragment and doublet populations. C) W34/70 Event count vs calcofluor white fluorescence intensity (FL6-Area), displaying the range and frequency of bud scar fluorescence among the singlet population. D) NCYC 1332 Event count vs calcofluor white fluorescence intensity (FL6-Area), displaying the range and frequency of bud scar fluorescence among the singlet population.

Table 4.12 Yeast strain size and age variation

Strain	CV- Age	CV- Size	Mean-Size
W34/70 (Frohberg)	80	37	16171
M2 (Ale)	64	30	23013
NCYC 1332 (Ale)	60	48	21294
CBS 1260 (Frohberg)	75	30	17324
CBS 1174 (Saaz)	69	53	9967
BA (Wild)	86	56	9769
Kveik	65	33	20764

4.5 Conclusion

In this study, heterogeneity in the yeast stress response was investigated through the use of a cell cytotoxicity assay based on MTT staining. From this, data regarding population survival dynamics were obtained. This was initially expressed in the form of stress dose response curves, providing information ascertaining to the maximum tolerance limits of each stress and the degree of heterogeneity within each strain.

The data obtained here demonstrates that stress tolerance (IC50) varied between the studied strains and also between stress factors. Given the nature of stress response in yeast, it would be expected that the environmental stress response (ESR) would result in a holistic approach to stress, with some strains being more tolerant to all stresses and others having a lower tolerance to all stresses (Gasch and Werner-Washburne, 2002; Gasch, 2003). However, the data displayed here suggests that each stress is handled in a unique way and the response for each stress is controlled by a specific set of genes, with the ESR potentially providing a broad spectrum, basal level of stress tolerance. For example, strains with a high ethanol tolerance such as W34/70 and M2 did not necessarily have a high tolerance to all of the other stresses analysed. An exception to this was the Kveik yeast strain, which exhibited one of the highest IC50 (tolerance) values for all of the stress factors applied, indicating a greater adaptation to stress, likely a result of its genetic origin and industrial uses (Preiss *et al.*, 2018;

Garshol, 2020). Conversely, the wild strain BA routinely showed one of the lowest IC50 values for all of the stresses excluding copper, perhaps a reflection of the fact that this strain does not possess the same necessary adaptations to survive high concentrations of brewing related stress factors. However, this strain was able to survive at high concentrations of copper, a heavy metal more likely to be encountered in the wild than in controlled brewing environments (Holland *et al.*, 2014). When comparing *S. cerevisiae* strains to *S. pastorianus*, broadly the former showed a greater stress tolerance to all of the stress factors apart from copper stress, likely to be a result of heritage and the greater evolutionary period during which these strains have been able to adapt to industrial stresses, particularly those found in brewing environments (Gallone *et al.*, 2016). To clarify, ale strains (*S. cerevisiae*) have been present in brewing (and industry in general) for longer than lager strains, which are a more recently divergent hybrid yeast strain. However, these species are still closely related; *S. pastorianus* yeast contain genomic contributions from both the *S. cerevisiae* genome and a cold tolerant *S. eubayanus* yeast (Gallone *et al.*, 2016). This may have resulted in a reduced stress tolerance overall, albeit the cold tolerant characteristics make the latter ideal for lager production.

In this section it was also demonstrated that by exposing cell cultures to increasing concentrations of stress, cells of varying abilities to survive could be detected, indicating the presence of heterogeneity within yeast populations. The degree of heterogeneity was observed to vary between strain and was also dependent on individual stress factors. Interestingly, ethanol stress revealed the greatest disparity in degree of heterogeneity between the strains. In response to ethanol stress, strains M2 and NCYC 1332 displayed a very low degree of heterogeneity, while the wild yeast strain BA, the Kveik yeast and the lager strain CBS 1260 showed a very high degree of heterogeneity. These large differences in heterogeneity in response to ethanol stress suggest differences in survival strategy, indicating that some strains may be adopting a 'bet hedging' approach, choosing to expend energy preparing for unpredictable environments (Levy and Ziv, 2012). While this hypothesis is possible, it is worth noting the difficulty to accurately link observed heterogeneity with survival strategies such as bet hedging and division of labour. As such the reproducibility of the data displayed

here does demonstrate observable differences in stress response heterogeneity between the strains, the root cause of which may be an innate bet hedging strategy adopted by some strains more than others. In contrast, other strains adopted an alternative approach resulting in all of the cells within a population showing a similar tolerance to ethanol (low heterogeneity). This strategy is believed to be less 'energy intensive' but comes at a cost with regard to versatility and capacity to survive under changing conditions (Haaland *et al*., 2020).

Among the other brewing-related stress factors (osmotic and oxidative stress), all of the strains analysed appeared to have a similarly high degree of heterogeneity, excluding the Kveik yeast strain which showed a significantly lower degree of heterogeneity in response to oxidative stress. This finding suggests that, with the exception of the Kveik yeast, a bet hedging strategy is likely being applied to promote survival under adverse environmental conditions (Levy *et al*., 2012; Haaland *et al*., 2020).

This study of stress tolerance and heterogeneity determination demonstrates that there is no 'one size fits all' rule in tolerance to stress factors. High stress tolerance did not necessarily link itself to either low or high heterogeneity, and tolerance to one stress did not imply the same strategy for other stress factors. This demonstrates that yeast strains respond to stress factors in an individual manner, likely through altering expression of key genes for each individual stress (Gasch, 2003; Gasch *et al.*, 2017). As a result, the degree of heterogeneity within a population is likely to derive from differences in stress related gene expression, with some strains exhibiting greater stochasticity than others. Although this data provides evidence indicating heterogeneity in stress response, it does not give any indication as to the source of heterogeneity. Therefore, to fully understand the dynamics of heterogeneity individual cellular components and organelles known to be affected by yeast stress were investigated. It is known that cell components such as the mitochondria, neutral lipids, as well as internal membrane integrity, membrane fluidity and membrane sterol content are all involved in the yeast stress response (Alexandre *et al*., 1994; Bagnat *et al.*, 2000; Beney and Gervais, 2001; Pastor *et al.*, 2009; Müller and Reichert,

2011; Miyagawa *et al.*, 2014). Therefore, it was hypothesised that heterogeneity in these components may reflect the stress response. To study this, ethanol was selected as a key stress factor, since this is not only industrially relevant, but also revealed the greatest disparity in heterogeneity between strains. Through the use of fluorescence microscopy, the stains Nile red (lipid content), MitoTracker Green FM (mitochondrial mass), $DiOC_6$ (mitochondrial membrane potential), filipin (sterols) and Laurdan (cell membrane fluidity) were used to identify variation in the respective cell targets. Subsequently, by analysing the cell-to-cell variation (CV) in fluorescence intensity of these probes for each strain, differences could be observed. The data obtained using this method demonstrated that an element of heterogeneity was present in all of the cell targets across all of the yeast strains. However, the extent of heterogeneity varied between strains in each instance. One prime example was seen in the case of neutral lipid content, where CV values range between 61 and 188. Interestingly, the data also showed that strains which possessed a high population variation for neutral lipids, did not necessarily have the highest CV for other cell targets. Furthermore, yeast strains that displayed a high degree of heterogeneity in response to ethanol stress, did not necessarily have a high CV in all of the studied cell targets. This indicates that the heterogeneity observed in stress response may not derive from all stress related cell targets (such as those studied here) being heterogeneous in nature and may be due to an accumulation of various heterogeneous stress related factors unique to each strain. These stress factors are likely to comprise numerous cell targets, from which differences in observable heterogeneity are a result of variation on the epigenetic level. This was demonstrated clearly in the Kveik yeast strain, which displayed a low CV for neutral lipids, mitochondrial mass and sterols in comparison to the other strains, but a high CV for mitochondrial membrane potential and membrane fluidity. In this instance, however, it may indicate that the highly heterogeneous response to ethanol shown by the Kveik strain could be influenced by heterogeneity in mitochondrial membrane potential and membrane fluidity, and that in this strain these attributes are more important than mitochondrial number, neutral lipid content and membrane sterol content. Among the conventional brewing yeast strains, CBS 1260 and NCYC 1332 can be identified as adopting opposing strategies of survival in response to increasing concentrations of ethanol. CBS 1260 demonstrated a much

greater degree of heterogeneity in ethanol response than NCYC 1332 and, interestingly, when comparing the heterogeneity in cell stress targets between the two strains, CBS 1260 had more variation in neutral lipid content, mitochondrial mass and mitochondrial membrane potential, but less variation in sterol content and membrane fluidity. Therefore, the observed variation in neutral lipid content, mitochondria and internal membrane integrity are likely to be a more influential factors in ethanol stress response heterogeneity for this strain.

As a final determinant of potential causes of heterogeneity, yeast cell size and age were assessed through the use of flow cytometry and bud scar staining. The results of this analysis showed the existence of heterogeneity in all of the studied yeast strains, however the extent of variation differed. For age, this was not unexpected since it stands to reason that a population will always comprise different aged individuals (Powell *et al*., 2003). However, it was interesting to note that cell size was variable between different yeasts. For the non-brewing yeast strain BA, this was not unexpected as it is known that there can be considerable variation in size (pleiomorphy) within wild yeast populations. However, for the brewing strains, intra-population variability was low and this was also expected. Cell size is typically related to cell-cycle regulation and the requirement to achieve a critical size prior to passing through 'Start' within the cell cycle, a checkpoint that commits the cell to division (Hartwell, 1974; Johnston et al., 1977). This results in the majority of industrial strains typically having a similar size dispersion at the point of division. From a functional perspective this may also make sense as many cellular functions are dependent on cell size. For example, changes to cell volume can have a major impact on nutrient uptake, metabolic flux, and the capacity to biosynthesise important cellular compounds. Consequently, size variation may be restricted within industrial yeasts, since growth conditions are often controlled within tightly defined parameters, perhaps leading to a convergence in size distribution during the evolution of these strains.

Chapter 5. The relationship between cell age and stress response heterogeneity

5.1 Introduction

Yeast cells are capable of dividing using both sexual and asexual processes (Section 1.6.2). However, due to their complex genetic make-up, industrial yeasts tend only to divide asexually, theoretically generating a population of identical cells. Despite this, in the previous chapter it was demonstrated that heterogeneity within brewing yeast populations clearly exists (Chapter 4). Due to the nature of asexual division in yeast, it is both logical and typical to observe cultures that comprise ~50% daughter cells, ~25% first generation cells, ~12.5% second generation cells and so on (Powell *et al.*, 2003). Interestingly, cell age is known to be linked to some fundamental differences in yeast physiological characteristics, including size, which can in turn lead to differences in phenotypes such as the ability to assimilate and metabolise sugars (Powell *et al.*, 2003). Furthermore, the same authors also suggested that cell age may have an impact on flocculation efficiency, primarily because older cells are larger, more wrinkled and more hydrophobic, therefore making them more likely to form flocs (Jin *et al.*, 2001; Powell *et al.,* 2003; Rockenfeller and Madeo, 2008). Separate studies have also shown that internal pH may vary with age; older cells are typically more alkaline than newly formed daughters (Imai and Ohno, 1995). The precise reasons for this are unknown, but this may have important implications for yeast growth rate, metabolic efficiency and membrane transport systems, coincidentally all of which are believed to also vary with cell age (Serrano *et al*., 1986; Imai and Ohno, 1995). Finally, ageing in yeast has been linked to mitochondrial DNA stability and stress tolerance (Costa and Moradas-Ferreira, 2001; Laun *et al.*, 2001), which lends further support to the suggestion that age may be a significant cause of population heterogeneity. However, the fact that there are differences between the young and old cells within isogenic populations does not fully explain why strains display different degrees of heterogeneity *per se*. It does, however, raise the question as to whether heterogeneity is an inherited trait, or whether it is acquired over time based on external and/or environmental factors. Similarly, it is possible that the 'true'

heterogeneity of a yeast strain may be masked by differences between young and old cells.

Previous studies of ageing in yeast typically involving labour-intensive strategies to obtaining significant quantities of daughter and aged cells, including micromanipulation (Kennedy *et al.,* 1994; Park *et al.,* 2002), sucrose gradients (Powell, *et al*., 2003b), centrifugal elutriation (Woldringh *et al.,*1995), fixation of mother cells to membranes (Grzelak *et al.,* 2001), and separation of old cells using biotin-linked magnetic beads (Lam *et al.*, 2011). In this chapter we aim to demonstrate a technique for separating age fractions using fluorescence activated cell sorting (FACS) and to subsequently determine the relationship between cell age and population heterogeneity in key physiological attributes, including cell size, individual cell growth rate, and the response to stress factors. In addition, characteristics that can impact functionality were investigated including cellular mitochondrial content (biomass) and mitochondrial membrane potential, as well as overall cell membrane health and integrity and the neutral lipid content of individual cells. This was performed to ascertain differences between freshly formed daughter cells and to determine if the extent of these differences was the same as between older cells. Specifically, the ambition was to determine if heterogeneity is a trait which is acquired immediately at cell division and to highlight potential targets within the cell which could give rise to the sub-populations with variable properties.

5.2 Daughter cell isolation method development

In order to isolate daughter cell populations, two flow cytometric approaches were applied to separate and analyse cells. The first approach was based on cell size quantification, while the second used the presence/absence of bud scar material. For the latter, fluorescence activated cell sorting (FACS) was used in conjunction with the stains calcofluor white, FITC-wheat germ agglutinin (WGA), and congo red, all of which are known to have a high affinity to chitin, the major component of bud scars (Pringle, 1991; Chaudhari *et al.*, 2012; Linder, 2018). Initially preliminary analysis of the specificity of each stain to bud scars was conducted using fluorescence confocal microscopy (Section 2.11.2), and subsequently stained yeast populations were subjected to FACS for isolation of daughter cell sub-populations (Section 2.11.4). The resulting cells were further analysed by confocal microscopy to assess the purity of the population and the efficacy of each stain.

5.2.1 Daughter cell isolation based on cell size discrimination

Initially, the potential to isolate cell sub-populations based purely on cell size was investigated as a simple mechanism for obtaining daughter cells. Although this is not a recognised method for daughter cell isolation, it was tested here as a quick and simple technique that would negate the need for yeast cell staining (Park *et al.,* 2002). In order to assess this approach, a stationary phase mixed aged cell population of the strain W34/70 was analysed using flow cytometry (Section 2.11.3). This population was analysed using brightfield light scatter and cell size parameters (FSC- Height and FSC- Width) were used to allow the smallest ~10% of 'singlet' cells to be 'gated', as shown in Figure 5.1(a). These gated cells were sorted and re-analysed using the same technique. Figure 5.1(b) shows the positive events (suspected daughter cells) isolated from this round of sorting; it is evident that the percentage of cells within the gate 'small cells' had increased from 9.77% to 24.8%. However, the distribution of cell size among the entire population remained largely unchanged, suggesting a spill-over of cells from outside the gated area as a result of negative events being incorrectly sorted. The 24.8% of cells designated as 'small' via the initial gate were then targeted

for a second round of FACS to further purify this sub-population. The resulting isolated cells (Figure 5.1c), showed a small increase in the percentage of gated cells (27.3%), however significant spill-over was still observed. This indicated that cell size alone was not sufficient for accurate daughter cell isolation. It is likely that the resolution between positive and negative events using cell size parameters was not intricate enough to purify desired populations of cells. In addition, it has previously been found that older mother cells produce larger daughter cells that are sometimes indistinguishable from younger mother cells, based on size alone (Kennedy *et al*., 1994). Therefore, the use of bud scar stains was investigated in conjunction with cell size analysis for a more accurate isolation procedure.

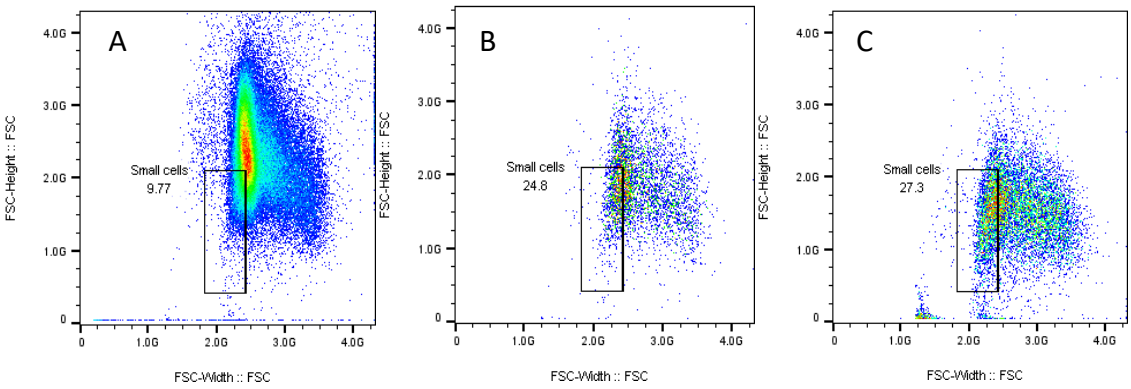

Figure 5.1. Flow cytometry data analysis for cell sorting based on size parameters. Each coloured dot represents a cell (W34/70) with red/yellow colour demonstrating a greater density of cells. (A) Singlet cells of the '*main population*' evaluated using the size parameters FSC-width and FSC-Height, with the smallest 9.77% of cells gated as suspected daughter cells. (B) Cells after the first round of cell sorting, displaying 24.8% of cells within the desired gate and suspected to be daughter cells. (C) Cells procured after a second round of sorting, demonstrating an increase in positive events falling within the designated gate and suspected to be daughter cells.

5.2.2 Bud scar stain assessment as a tool for daughter cell isolation

Given that sorting based on size alone was not sufficient to allow the accurate isolation of daughter cells (Section 5.2.1), yeast strains were analysed using fluorescent stains known to be specific to bud scar material that remains on the surface of mother cells following division. In order to visualise bud scars, cells were stained with either congo red, WGA-FITC or calcofluor white (Section 2.11.1), and the efficacy of staining was determined by visual analysis using confocal microscopy. Images were taken of single cells using both brightfield and fluorescence fields, and in both 2D and 3D using a series of Z stacks where 20-50 Z-planes were typically applied for each cell in order to produce a 3D image, depending on the size of the yeast cell. Cells stained with congo red were imaged using 488nm excitation and emission detected in the CY3 channel, WGA-FITC using 488nm excitation and detection in the GFP emission channel and calcofluor white using 405nm excitation with emission detected in the DAPI channel.

It can be seen from Figure 5.2 that all of the stains were efficient and allowed bud scars to be visualised. For WGA-FITC, the fluorescence and brightfield fields could be overlaid together, however for congo red and calcofluor white this was not possible as it obscured the fluorescence image clarity. Congo red was observed to have a relatively low specificity for the bud scars, with scar material appearing as dull spots on the cell surface. WGA-FITC and calcofluor white both exhibited comparatively improved bud scar staining. WGA-FITC had the advantage that bud scar specificity was clear with little staining around the remainder of the cell. However, during the course of confocal assessment, rapid photobleaching was observed. While the calcofluor white stain provided slightly less resolution between bud scar staining and the cell wall, this stain had improved photo-stability under the conditions applied. Given that the ultimate goal was to conduct several rounds of sorting and analysis, this meant that calcofluor white was selected for further studies.

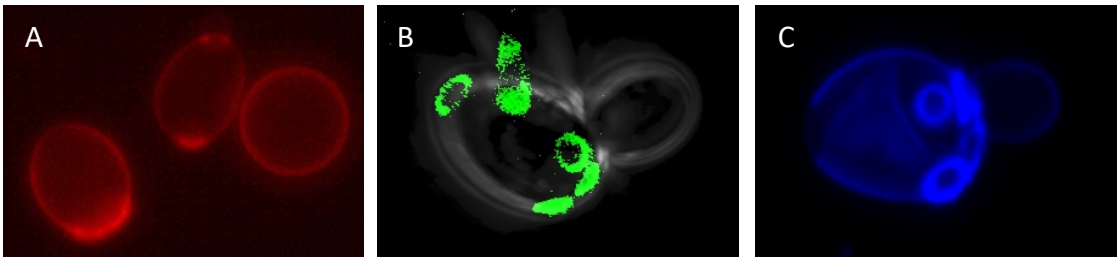

Figure 5.2. Yeast bud scar stain assessment. Fluorescence images for mixed aged W34/70 cells stained with congo red bud scar stain (A), WGA-FITC (B) and calcofluor white (C). images depict cell images taken in 3D using 60X-oil magnification and 488nm excitation energy (A and B), and 405nm excitation (C). For A-C, emission was detected in the CY3 channel, GFP channel and DAPI channel respectively. Bright rings at the cell poles demonstrate the presence of successful bud scar staining.

The staining efficiency of each bud scar stain on yeast populations was further evaluated by the isolation of daughter cells using FACS. Initially mixed aged yeast populations were analysed using flow cytometry, firstly examining the brightfield source light scatter of each cell based on cell size (FSC1 Area) and cell granularity (SSC Area). This allowed identification of the main population of yeast, discounting large aggregates. An example of this is displayed in Figure 5.3(a) for mixed aged W34/70 cells, although the same analysis was performed for each strain (data not shown). Initially a 'main population' was identified based on standard size/granularity, comprising 83.3% of all cell events. Subsequently, the cells in this selected population were analysed based on cell height (FSC- height) and width (FSC- width), allowing for the discrimination between cell fragments/debris, singlets, doublets, triplets and larger aggregates. As displayed in Figure 5.3(b), the population of yeast gated as 'singlets' represented the key population of interest, comprising 83.9% of the 'main population'. A further distinct population, identified to the right on the x-axis, corresponded to doublets and triplets, while events to the left reflect cell debris; both of these were discarded from further analysis. Once single cells derived from the main population had been identified for each population, these singlets were then examined for fluorescence using congo red, WGA-FITC and calcofluor white. Events were recorded by tracking individual cells by size (FSC- Area) against fluorescence intensity (bud scar matter). An example of this using calcofluor white can be seen in Figure 5.3(c), where cell size (y-axis) and bud scar stain fluorescence (x-axis) for each

cell were recorded. Note that this was repeated for each yeast strain/fluorescent stain combination (data not shown). From this data, the smallest and least fluorescent ~10-15% cells were gated for sorting using FACS, such that any cell detected with FSC-Area (size) and fluorescence values falling within this gate were deemed to be positive events and were isolated. This small fraction of cells was chosen due to the expected reduced size of daughter cells and the reduced bud scar stain fluorescence intensity emitted, as a result of the absence of brightly fluorescent bud scars. This process of sorting was performed twice, with the first round of FACS used in 'enrich mode', where recovery quantity is the main priority (Figure 5.3d-f). In this mode all cell events that are in the desired gated population were sorted, however this can result in some negative events also being sorted, due to their close proximity to positive events. A second round of sorting was then performed on these enriched cells using the same gating parameters, however this was conducted in 'purify mode' (Figure 5.3g-i). This mode functions to ensure a high purity of the desired cells is achieved with only positive events adjacent to other positive events being sorted, while all other events are discarded.

From this sorting procedure it was evident that a specific sub-population of cells could be selected during the first round of sorting, with an increase of cells falling with the gate designated as daughter cells. However, sorting using WGA-FITC (Figure 5.3e and h) was not as effective as for the other stains analysed. This trend was also seen after the second round of sorting, although the fraction of cells within the gate (i.e. suspected to be daughter cells) increased for congo red and WGA-FITC stained cells. For calcofluor stained cells, the fraction of events remained at around 50% positive, indicating that spillover had shifted significantly on the y-plane and that cells were isolated more accurately based on fluorescence rather than size, corroborating the data obtained shown in Section 5.2.1.

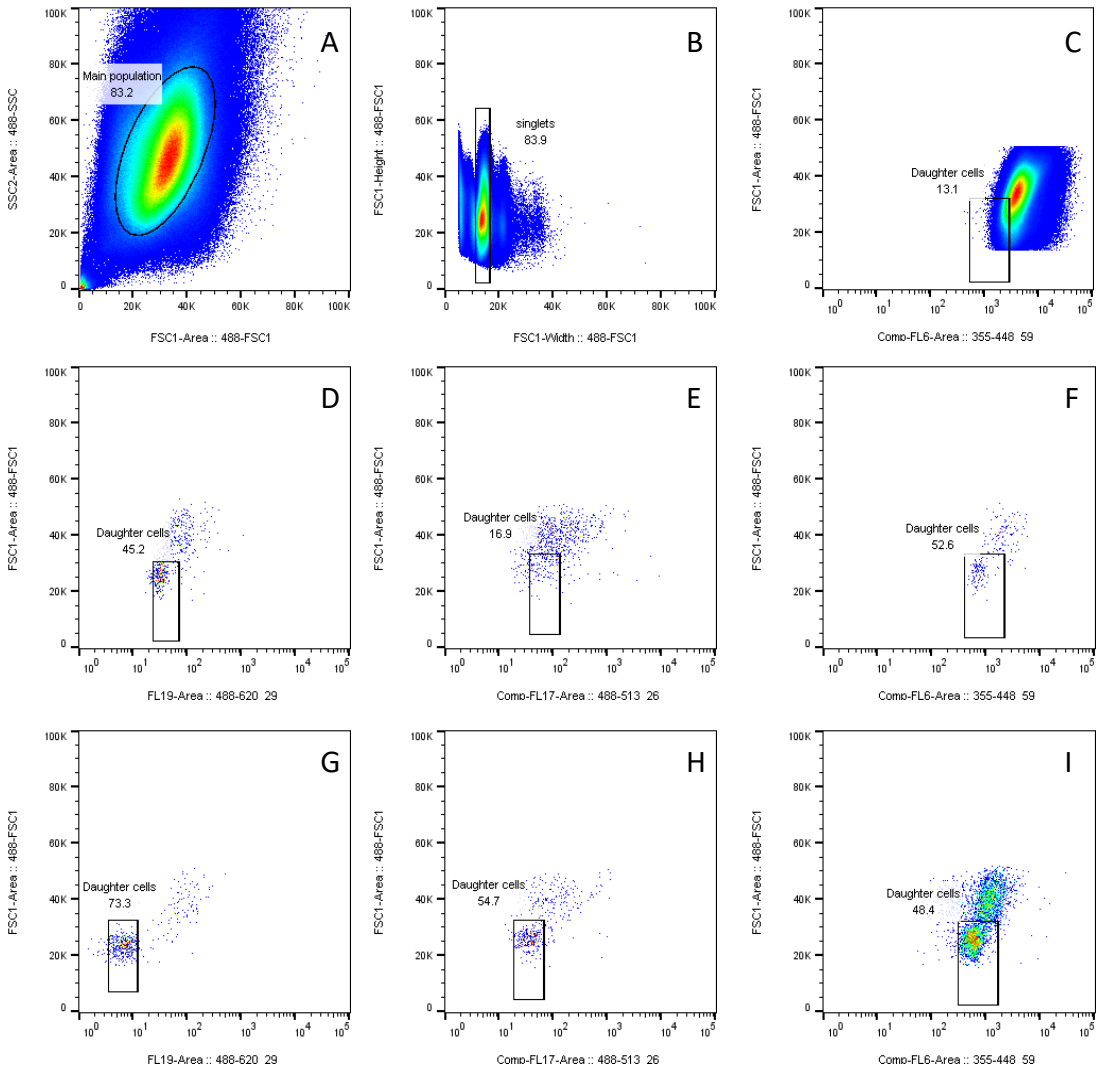

Figure 5.3. The assessment of bud scar stains for isolation of daughter cells using FACS. Flow cytometry data for the first and second rounds of FACS using stained, mixed aged W34/70 cell populations as a reference strain. A: W34/70 cell population plotted based on the forward scatter (FSC1- Area) and side scatter (SSC2- Area) detected from each cell, with the '*Main Population*' gated. B: *Main Population* analysed by the cell size parameters (FSC1-Width) and (FSC1- Height) in order to identify sub-groups of cells, highlighting the singlet population. C: *Singlet* population visualised based on cell size (FSC1- Area) against bud scar stain fluorescence intensity (FL6- Area) with suspected daughter cells gated as positive events. D, E, F represent suspected daughter cell populations isolated from the first round of FACS using congo red (D), WGA-FITC (E) and calcofluor white (F). G, H, I suspected daughter cell populations isolated from a second round of FACS using congo red (G), WGA-FITC (H) and calcofluor white (I).

The cells isolated through two rounds of FACS were subsequently analysed using confocal microscopy to assess the purity of daughter cells. The images shown in Figure 5.4(a-c) show an example of the subset of W34/70 selected based on each fluorescent stain. It can be seen that cells stained using congo red (a) were less fluorescent than before pre-sorting, which suggested photobleaching had occurred. Furthermore, when the images were brightened post acquisition many of the cells still appeared to possess brighter areas on the cell surface, indicating the presence of scar material, likely to be birth scars. A similar trend was observed for WGA-FITC where cells displayed very little staining (b), again indicating successful sorting. There was some WGA-FITC fluorescence identified at the poles of sorted cells, but as the fluorescence intensity was low, it is again believed likely to be birth scar staining. The calcofluor white stained cells (c) showed good visualisation with minimal bleaching and no scar material at all. Given that the images showed a lack of distinct bud scars, cells were subsequently visualised in both 2D and 3D to confirm the absence of bud scars. A total of 50 cells were imaged for each stain and all had similar characteristics. The isolated cells showed an absence of calcofluor white bud scar fluorescence, demonstrating that a high purity of daughter cells could be achieved using this staining protocol in conjunction with FACS. As a result, calcofluor white staining with 2 rounds of FACS was adopted as the mechanism for obtaining a high purity of daughter cells.

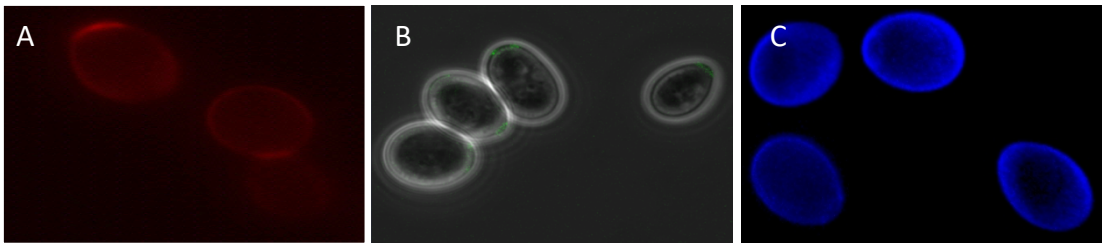

Figure 5.4 3D Fluorescence image for cells obtained from two rounds of FACS. Suspected daughter cells isolated based on congo red staining (A), WGA-FITC (B) and calcofluor white (C).

Once the protocol above had been established using W34/70, the same methodology was performed using strains CBS 1260 and NCYC 1332, selected for their opposing heterogeneous survival strategies in response to ethanol stress and cold shock

(Section 4.2.1). As a reminder, CBS1260 previously displayed a high degree of heterogeneity and the NCYC 1332 a low degree of heterogeneity to these stress factors. Following two rounds of cell sorting, newly isolated daughter cell populations from CBS 1260 and NCYC 1332 were assessed for purity in order to confirm the accuracy of the FACS, using confocal microscopy. However, in this instance a direct comparison to mixed aged cells was also performed for each strain respectively (Figure 5.5). It can be seen that mixed aged populations (Figure 5.5a and c) for each yeast strain allowed clear identification of bud scars. When comparing these images with those of suspected daughter cell populations acquired by two rounds of FACS (Figure 5.5b and d), it was evident that the purified fractions comprised individuals with a distinct lack of bud scars, clearly indicating that they were daughter cells. It should be noted that these cell images represent a small subset of cells analysed from a total of ~50 cells imaged. Therefore, while these results indicate a successful sorting, the possibility of human error resulting in some cells with bud scars being overlooked during the confocal microscopy remained. As such, the purity of the resulting suspected daughter cells was further quantified using imaging flow cytometry.

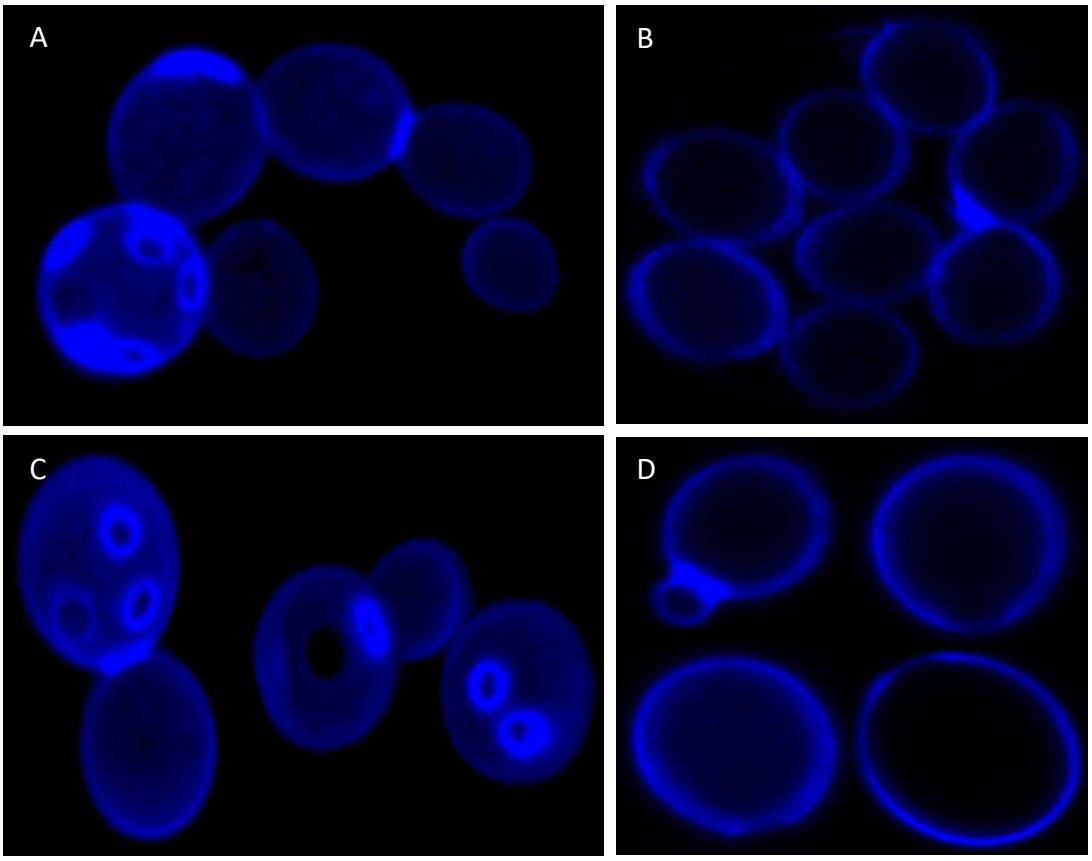

Figure 5.5. Bud scar stain assessment for CBS 1260 and NCYC 1332. 3D Fluorescence images of cells stained with the bud scar stain calcofluor white. 405nm excitation and detection in the DAPI channel. A, mixed aged CBS 1260 cells displaying clearly visible fluorescent bud scar rings. B, CBS 1260 cells isolated from two rounds of FACS suspected to be purified daughter cells. C, mixed aged NCYC 1332 cells displaying clearly visible fluorescent bud scar rings. B, NCYC 1332 cells isolated from two rounds of FACS suspected to be purified daughter cells

5.2.3 Quantitative assessment of daughter cell purity

Imaging flow cytometry combines the principles of single cell fluorescence microscopy and flow cytometry. The micro-fluidics section works in similar way to conventional flow cytometry, such that single cells are separated into a constant flow of cells past selected laser light sources (Barteneva *et al.*, 2012). In addition, cells also pass through a brightfield light source and in total, the brightfield, scatter and fluorescence signal for each cell are detected and used to provide separate brightfield and fluorescence images for each cell (Basiji *et al.*, 2007). This analysis was utilised to both visualise and

quantify bud scar fluorescence on brewing yeast cells, a novel method of yeast age determination and population assessment, while also being used to evaluate the purity of the suspected daughter cell population obtained in the previous section. Examples of brightfield and calcofluor white fluorescence images for strains CBS1260 and NCYC 1332 can be seen in Figure 5.6.

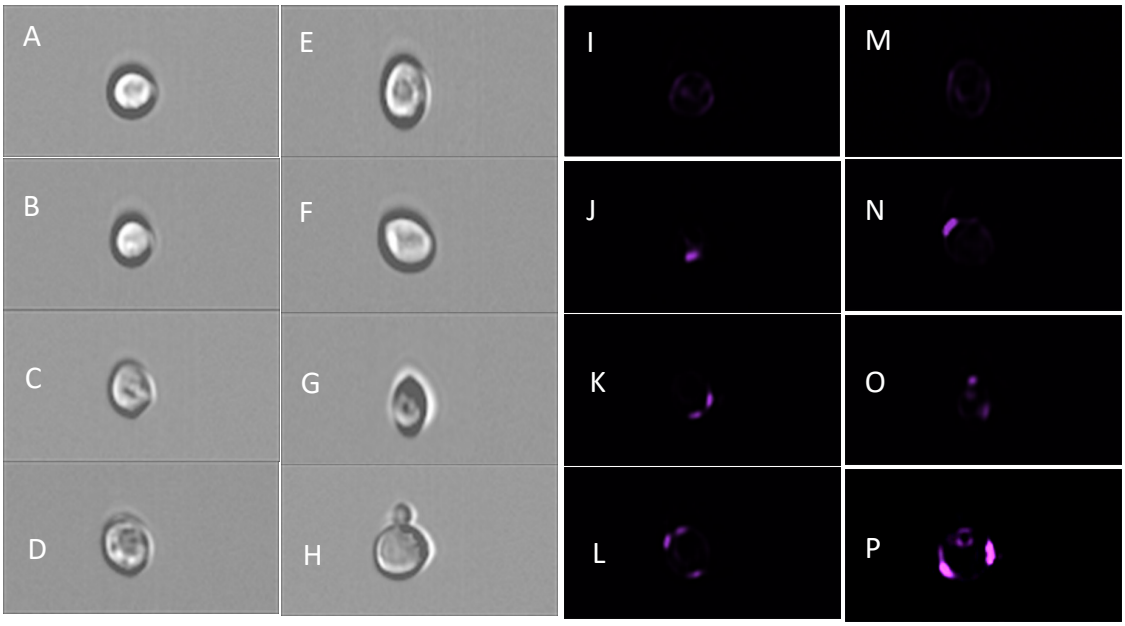

Figure 5.6 Cell age assessment using imaging flow cytometry. Brightfield and fluorescence images of mixed aged CBS 1260 and NCYC 1332 cells stained with calcofluor white and procured using Imagestream flow cytometry. A, B, C, D: CBS 1260 Brightfield images obtained using 40X magnification. E, F, G, H: NCYC 1332 brightfield images. I, J, K, L: CBS 1260 Fluorescence images of the same cells obtained using excitation light of 405nm and emission detected in Channel 7. M, N, O, P: NCYC 1332 fluorescence images of the corresponding cells. Cells in I, J, K, L and M, N, O, P, represent cells with 0, 1, 2, 3 bud scars respectively, counted and binned using the Spot Count feature.

Analysis of mixed aged yeast cells in this way demonstrated the clarity of the brightfield images, but also showed the successful visualisation and ability to overlay data from calcofluor white bud scar staining. It was anticipated that the clearly visible bud scars identified using this technique could also be utilised as a method of cell age determination, established by counting the fluorescent scars on each cell. In order to achieve this the stained cell populations were analysed using conventional flow cytometry parameters; each cell event detected was visualised by aspect ratio

(AspectRatio_M01) against area (Area_M01) to identify the singlet population of each strain (Figure 5.7a-b). Once identified, this population was used for further analysis.

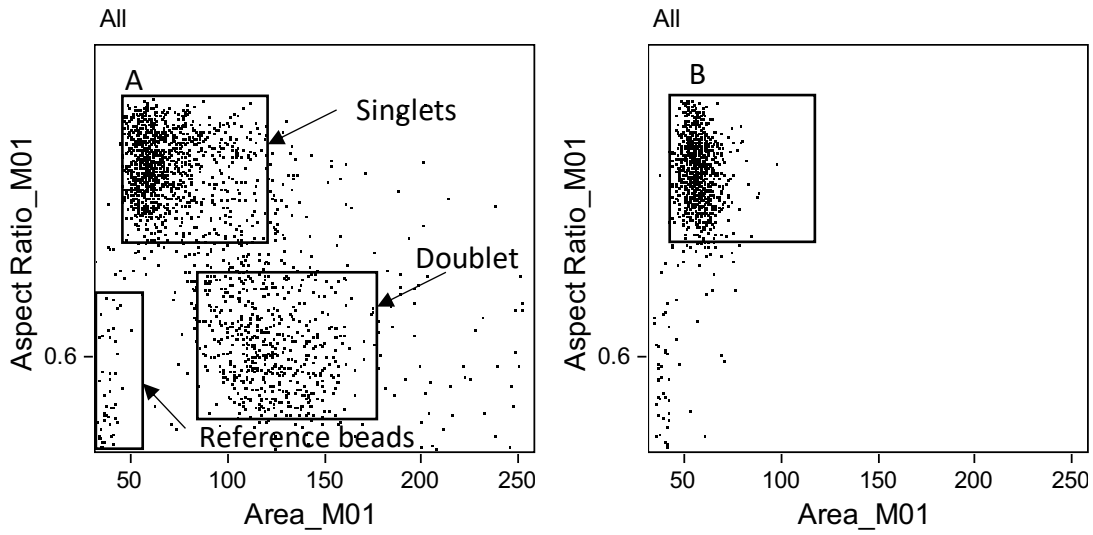

Figure 5.7 CBS 1260 cell distribution before and after daughter cell isolation. Imagestream flow cytometry data for strain CBS 1260. A, Mixed aged cells analysed using size parameters Area and Aspect Ratio, clearly identifying singlets and doublets from which only the singlet population was analysed further. B, demonstrated the same cell size discrimination performed on CBS 1260 daughter cells obtained through two rounds of FACS. The light scatter from the reference beads are also identified in graph A, these were used in order to synchronise the sample flow and maintain accurate focus doing the run

Utilising the 'spot count' feature within the IDEAS data analysis software (Section 2.11.5), fluorescence images for each singlet cell were obtained and bud scars were counted for each individual cell. By setting the peak calcofluor white fluorescence to a specific threshold (17.5- an arbitrary value assigned to specific fluorescence intensity), only areas of fluorescence intensity above this peak were counted as bud scars. As displayed in the procured images (Figure 5.6), brightly fluorescent bud scars were clearly distinguishable from the rest of the cell, therefore the peak fluorescence was set to detect these distinct bud scars and count them. Several cell images of each fraction (0 bud scars, 1 bud scar, 2 bud scar etc) were reviewed to ensure that no cells had been placed in the wrong category as a result of the peak threshold being

incorrect (images I-P; Figure 5.6). Once the correct peak fluorescence was determined, the number of bud scars for each cell was enumerated and cells were 'binned' into different age groups, giving a novel method of age determination. Bud scar enumeration and age determination was performed using mixed aged cells from populations of CBS 1260 and NCYC 1332; the fraction of single cells within each age group was evaluated and results are displayed in Figure 5.8. The data for both strains demonstrate a wide distribution of bud scar number, however, the age distribution for mixed aged populations of yeast is similar to that which would be expected. For example, populations consisted of 54% and 41% daughter cells (0 bud scars) for the strains CBS 1260 and NCYC 1332 respectively, with older age fractions decreasing in quantity by approximately half each time, a trait expected in light of current literature (Powell *et al*., 2003).

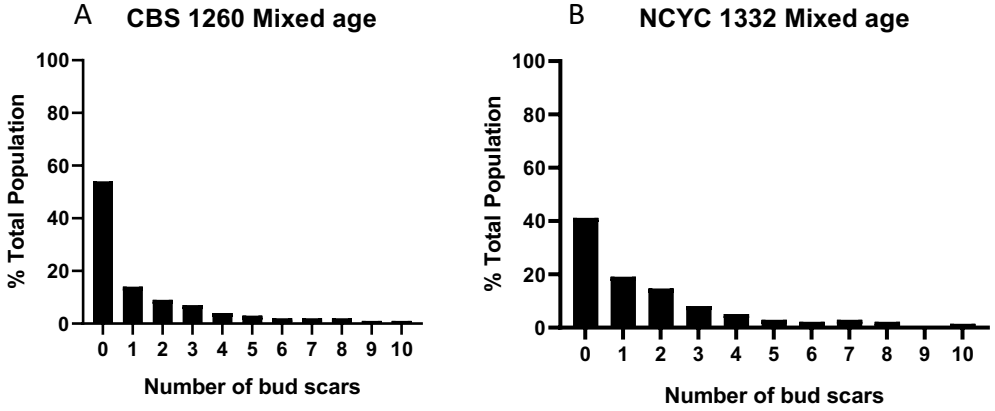

Figure 5.8 Bud scar number variation present in mixed aged cells of strains CBS 1260 (A) and NCYC 1332 (B).

This method of cell age determination was then utilised to assess the purity of daughter cell populations isolated previously (Section 5.2.2). It can be seen that daughter cells were successfully isolated using cell sorting based on visualisation of cells using brightfield and fluorescence analysis (calcofluor white) (Figure 5.9), presenting the distinct absence of bud scars. Confirmation of this can be seen in the

analytical data (Figure 5.10), which demonstrates that these sorted populations comprised 96% cells with 0 bud scar for both CBS 1260 and NCYC 1332 populations.

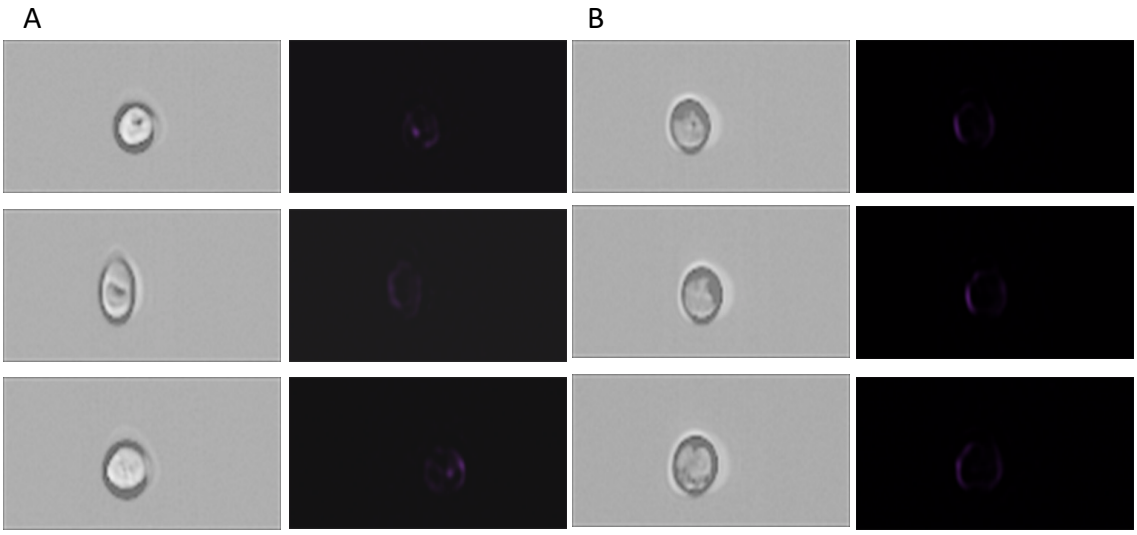

Figure 5.9 Visual assessment of daughter cell purity. Brightfield and fluorescence images of A, CBS 1260 daughter cells and B, NCYC 1332 daughter cells stained with calcofluor white and procured using imagestream flow cytometry. Brightfield images obtained using 40X magnification. Fluorescence images obtained using excitation light of 405nm and emission detected in Channel 7. Calcofluor white fluorescence is clearly still present in both strains, however distinct bud scar fluorescence is not detected.

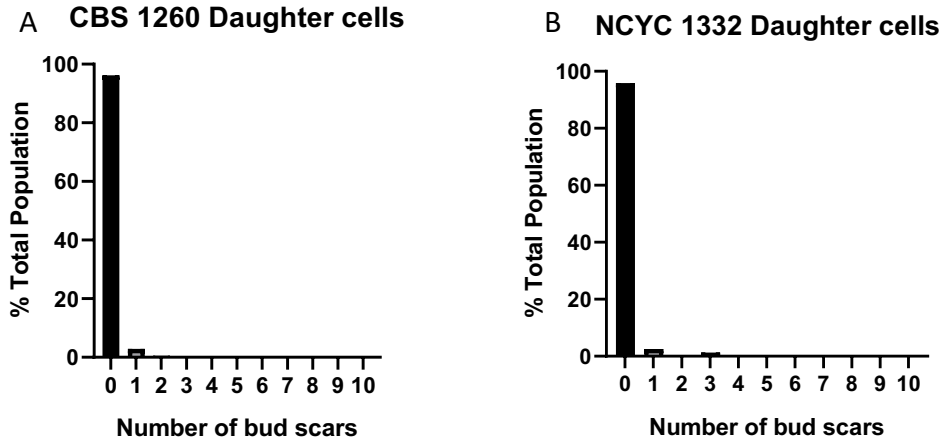

Figure 5.10 Quantitative assessment of daughter cell purity. Bud scar number variation present in suspected daughter cell populations of strains CBS 1260 (A) and NCYC 1332 (B).

5.2.4 Calcofluor white- yeast cell growth impact

It has been reported that when yeast cells are grown in high concentrations of calcofluor white, damage can occur to the yeast cell wall, impacting compositional integrity, cell division and eliciting a stress response (Ram and Klis, 2006). However, this is only of practical concern when yeast cultures are grown in the presence of calcofluor white for a significant period of time. In our current study, cell aliquots were stained for 20 minutes before excess stain was washed off and consequently the impact of calcofluor on the integrity of the yeast cells is likely to be minimal. However, to assess the impact of the staining protocol on yeast cell growth and yeast stress, mixed aged yeast cells were stained with calcofluor white as above. Following this, stained cell populations of the stains CBS 1260 and NCYC 1332 were seeded into wells of a 96 well plate (10^6 cell/ml) and cultured in conditions of increasing stress 0-10% ethanol (v/v) with YPD to a total volume of 200µl. This range of ethanol was chosen to allow cells to be stressed, while maintaining a high degree of viability based on previous data (Section 3.6.1). The cultures were then grown for 2 days at 25ºC until stationary phase had been reached and growth was measured using a Tecan plate reader to determine cell density at OD 600nm every 15 mins (Section 2.11.6). The resulting growth curves were subsequently compared to those produced by un-stained populations of CBS 1260 and NCYC 1332. It can be seen from Figure 5.11(a) (CBS1260) and Figure 5.11(b) (NCYC 13320) that the differences in growth and stress survival between stained and unstained yeast populations were negligible. As a result, the purified daughter cells obtained in the previous section could be used with confidence in future experiments, such that stress response characteristics would reflect the imposed stress and not the calcofluor white staining procedure.

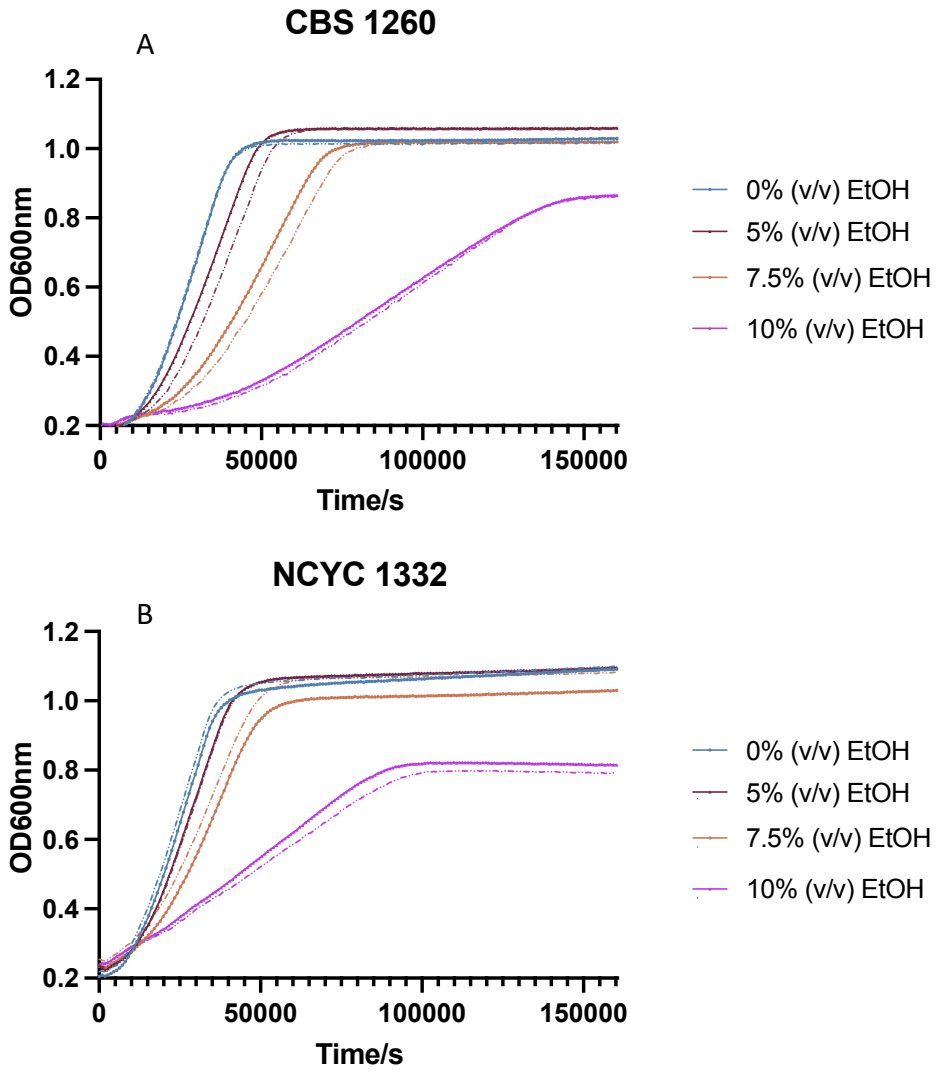

Figure 5.11. The impact of calcofluor white stain on yeast growth and stress response. Growth curves for cell populations both stained with calcofluor white and unstained, grown in 0-10% (v/v) ethanol stress conditions. A, CBS 1260. B, NCYC 1332 Solid lines: Unstained cell populations, Hashed lines: Calcofluor white stained populations.

5.3 Stress response heterogeneity in daughter cell populations.

In order to assess the innate (pre-programmed) stress response of yeast cell populations, isolated daughter cells were obtained (Section 5.2.2) and analysed for heterogeneity using the MTT assay developed previously (Chapter 4; Section 2.9.2). The FACS protocol described in the Section 5.2 was used to isolate individual daughter cells, with minor adjustments to the cell sorting parameters. Following a primary enrichment to select the least fluorescent and smallest ~10% of cells (as before), the sorting mode was then switched to 'Single' instead of 'Purify'. It should be noted that the purification parameters in 'Single' mode are identical to 'Purify' but ensure only one event per drop as a priority. This certifies that only strictly positive events are counted and selected for with a higher degree of accuracy, while also allowing individual cells to be counted. Using this system, exactly 5000 positive events (daughter cells) were sorted directly into wells of a 96 well plate, containing YPD supplemented with increasing concentrations of a designated stressor (ethanol, sorbitol, hydrogen peroxide, copper sulphate or zinc sulphate) to a total volume of 200µl, as described in Section 2.9.1.

To study the extent of phenotypic heterogeneity in these daughter cell populations, the MTT cell cytotoxicity assay developed previously was implemented as before (Chapter 4; Section 2.9.2). The percentage viability was determined by comparing the surviving population under stress conditions in reference to an un-stressed control for each strain. This data was subsequently formatted to produce sigmoidal dose response curves from which insights into survival dynamics, tolerance and heterogeneity were obtained. To assess the innate heterogeneity in yeast stress response, the two stains CBS 1260 and NCYC 1332 were selected, primarily due to their significantly opposing response to ethanol stress. Previous data (Chapter 4) showed that CBS 1260 exhibited a highly heterogeneous stress response, while NCYC 1332 produced a low degree of heterogeneity. However, initially, in order to ensure accuracy of method development and to benchmarks strains in general, daughter cells of the remaining 'conventional' brewing yeasts (W34/70, M2 and CBS 1174) were also

tested for their survival dynamics to ethanol stress. It should be noted that while the Kveik strain analysed in Chapter 4 presented interesting stress response data, it was not carried forward for further analysis as it exhibited a rapid growth pattern which did not facilitate daughter cell selection. Similarly, due to the morphology and cell division properties of *Brettanomyces* yeasts, strain BA could not be investigated here as obtaining large quantities of single daughter cells was not possible.

5.3.1 Ethanol stress heterogeneity in daughter cell populations

Initially, daughter cell populations (5000 cells) for each strain were exposed to increasing concentrations of ethanol ranging from 0-25% (v/v) and stress response dynamics determined using the MTT assay (Section 2.9.1). After 72 hours of growth, surviving cells were stained using MTT, from which the purple colour was evaluated at OD 570nm (Section 2.9.2). The data obtained and the resultant sigmoidal dose response curves can be seen in Figure 5.12, reflecting the survival dynamics of the initial seed of 5000 daughter cells. When the dose response curves were compared it became evident that each strain followed the same general sigmoidal shape, however the horizontal positioning and slope gradient vary significantly. It is clear from Figure 5.12 that CBS 1260 daughter cells exhibit the most gradual curvature, while daughter cells derived from strains M2 and NCYC 1332 show the steepest dose response, indicating differences in survival dynamics. Furthermore, the horizontal positioning of the M2 and NCYC 1332 dose response curves, shifted to the right on the x-axis, suggests these strains have a higher tolerance to ethanol. Conversely, strains CBS 1174 and CBS 1260 show a shift to the left suggesting lower tolerance to ethanol overall (Figure 5.12).

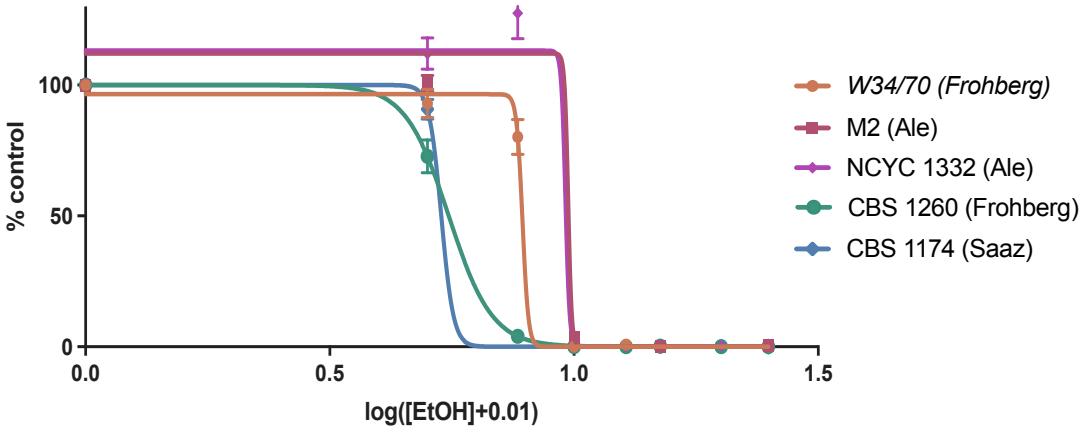

Figure 5.12. Dose response curves in response to ethanol for daughter cell populations of each strain, obtained from the MTT cell cytotoxicity assay. Yeast strains were exposed to increasing concentrations of ethanol (0-25% v/v) and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

In order to determine the tolerance limits of daughter cell populations more precisely, the IC50 values were evaluated as with previous mixed-aged experiments (Chapter 4; Section 2.9.2). This was achieved by measuring the stress dose concentration at which 50% viability was reached, and is directly related to the horizontal position of the maximum and minimum asymptotes for each strain. This data, referred to as IC50, for daughter cell populations is displayed in Figure 5.13. Among the conventional brewing strains studied, daughter cells of the *S. cerevisiae* strains M2 and NCYC 1332 demonstrated the highest IC50 of 9.6% ethanol (v/v), closely related to the IC50 of their mixed aged counterparts (9.7%; Section 4.2.1). In addition, CBS 1260 and CBS 1174 displayed the lowest IC50 of 5.5% and 5.3% respectively, similar to that seen in mixed aged populations (Section 4.2.1). However, in this instance the IC50 values were lower in daughter cell than mixed aged cell populations, with IC50s of 6.1% and 7.3% respectively. Furthermore, while W34/70 was again the most tolerant lager strain, the IC50 data for daughter cells showed a reduction in tolerance at 7.8% ethanol (v/v) when compared to mixed aged cells (9.6%). Despite these differences between daughter cell and mixed aged populations, the general pattern of tolerance was closely related. The reduction in tolerance (IC50) in some of the daughter cell populations may directly reflect their reduced stress tolerance, a trait which has

previously been observed, although the effect may be more pronounced in some strains than others (Costa and Moradas-Ferreira, 2001; Laun *et al.*, 2001; Powell *et al*., 2003a).

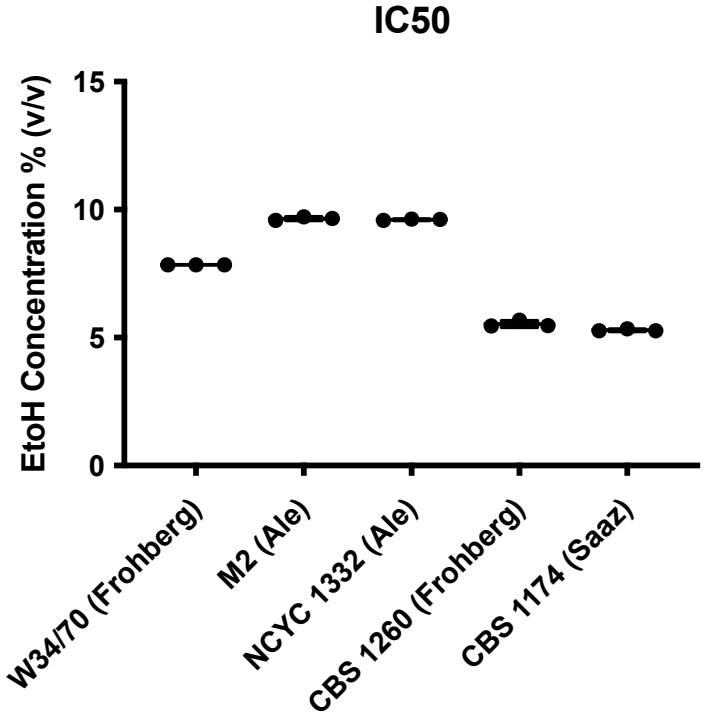

Figure 5.13. Daughter cell IC50 values generated from the ethanol dose response curves. The data displayed represents the ethanol concentration at which 50% viability is achieved for daughter cell populations of each strain. Values are a mean of triplicate data with error illustrating the standard deviation.

In addition to allowing IC50 data to be calculated, the dose response curves in Figure 5.12 also present insight into the degree of heterogeneity within each daughter cell population. To investigate this, the gradient of the dose response curves evaluated at the point of sigmoidal curve inflection was determined for each strain (Section 2.9.4) to create hillslope gradient data as shown in Figure 5.14. It can be seen that significant differences in hillslope gradient were observed; data showing large negative values correspond to dose response curves with a greater gradient as a result of a more

sudden decline in population viability (low heterogeneity) and data with small negative values represent curves with a more gradual slope gradient (higher heterogeneity). Daughter cells derived from strain CBS 1260 yielded a hillslope gradient of -9.9, representing a gradual decline in viability as ethanol concentration increased, likely reflective of heterogeneous sub-populations. Conversely daughter cells belonging to strain NCYC 1332 produced larger values (-108), indicating a lower degree of heterogeneity within the population where the majority of cells showed similar tolerance dynamics. When comparing the hillslope gradients of the daughter cell populations (Figure 5.14) with their mixed aged counterparts (Section 4.2.1; repeated here as Figure 5.15) it is interesting to note that the degree of heterogeneity was unchanged in all of the strains investigated. This observation was corroborated using Tukeys multiple comparison test, which did not show any significant differences between data sets. Due to the similarity in heterogeneity between mixed age and daughter populations, it can be concluded that the observed degree of heterogeneity in response to increasing concentrations of ethanol is an innate feature of yeast cells, gained immediately upon budding from a parent cell and not a trait that is acquired over time, for example due to a gradual adaptation to the environmental or fermentation conditions.

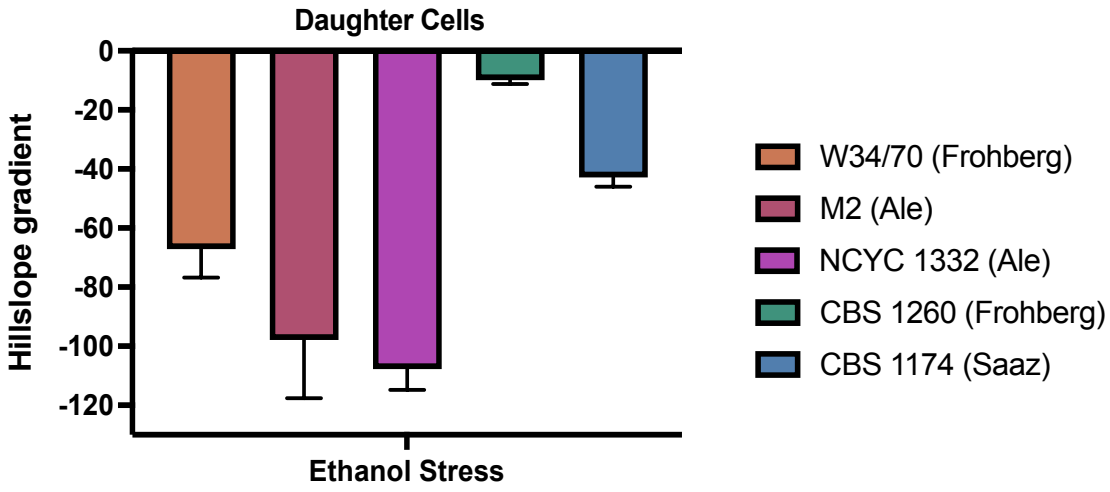

Figure 5.14. Daughter cell ethanol stress response heterogeneity assessment. Hillslope gradient data obtained from dose response curves generated by exposing daughter cell populations to increasing concentrations of ethanol.

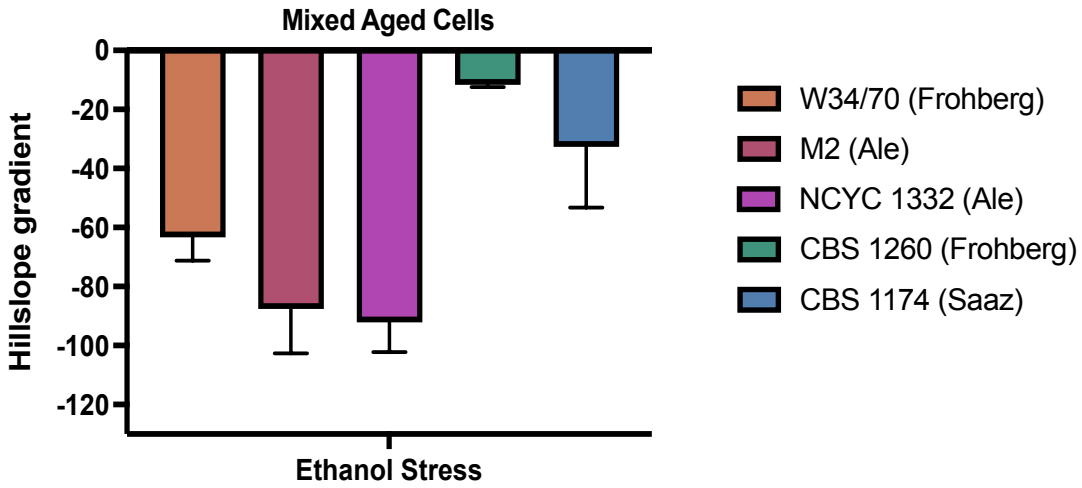

Figure 5.15 Mixed aged cell ethanol stress response heterogeneity assessment. Hillslope gradient data obtained from dose response curves generated by exposing mixed aged yeast populations to increasing concentrations of ethanol.

5.4 Stress response heterogeneity in daughter cell populations of CBS 1260 and NCYC 1332

Due to the data obtained previously, daughter cells belonging to the strains CBS 1260 and NCYC 1332 were further tested for their response to additional stresses. These two strains were primarily chosen due to their opposing stress response dynamics, where CBS 1260 showed a high degree of heterogeneity and NCYC 1332 a low degree of heterogeneity in response to ethanol.

5.4.1 Osmotic stress response heterogeneity in daughter cells

As with their mixed aged counterparts (Section 4.2.2), daughter cell populations for the strains NCYC 1332 and CBS 1260 were exposed to sorbitol ranging from 0-60% (w/v) in order to induce osmotic stress. The stress response dynamics were subsequently determined using the MTT assay and dose response curves obtained (Figure 5.16a). The obtained IC50 data gave values of 36.6% (NCYC 1332) and 36.9% (CBS 1260) sorbitol (w/v) (Figure 5.16b). This pattern was similar to that observed in mixed aged populations, however for the latter, the IC50 values were greater (44.4%

and 43.3% sorbitol w/v) indicating a reduction in osmotic stress tolerance in newly produced daughter cells. Figure 5.17(a) displays the hillslope gradients of daughter cell populations for the two studied strains, both of which presented similar values of -8.4 (NCYC 1332) and -10.1 (CBS1260). This indicated a high degree of osmotic stress response heterogeneity within these cell populations. When comparing these values to the mixed aged cell populations (Figure 5.17b), there was no significant difference, with the latter showing hillslope gradients measuring -9.1 (NCYC 1332) and -8.2 (CBS 1260). This data therefore further emphasises that observed heterogeneity is indeed a feature innate to the yeast cell immediately after it is produced and not a characteristic acquired over time.

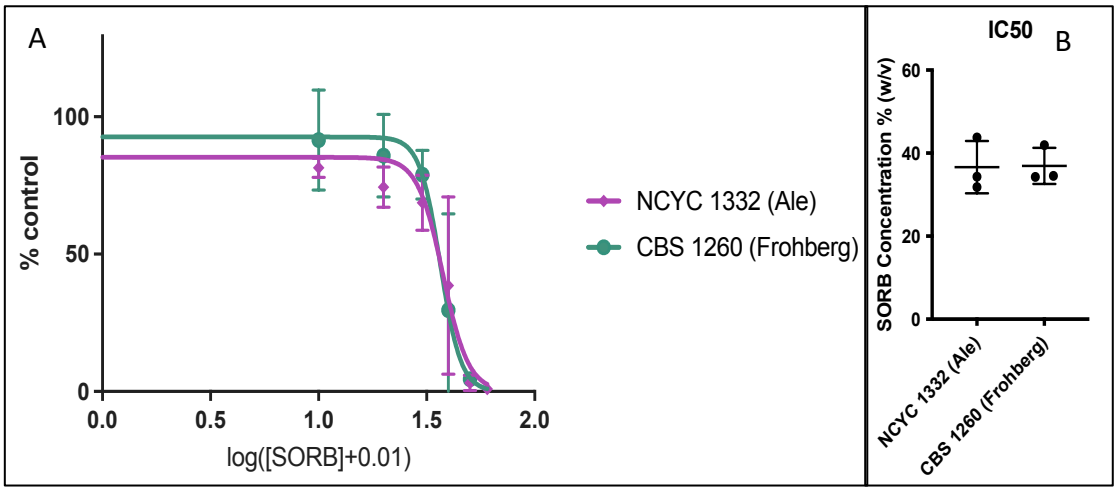

Figure 5.16 Daughter cell growth assessment in response to osmotic stress. A, Dose response curves in response to osmotic stress for daughter cell populations of each strain, obtained from the MTT cell cytotoxicity assay. B, IC50 values generated for the sorbitol concentration at which 50% viability is achieved. Yeast strains were exposed to increasing concentrations of sorbitol (0-60% w/v) and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

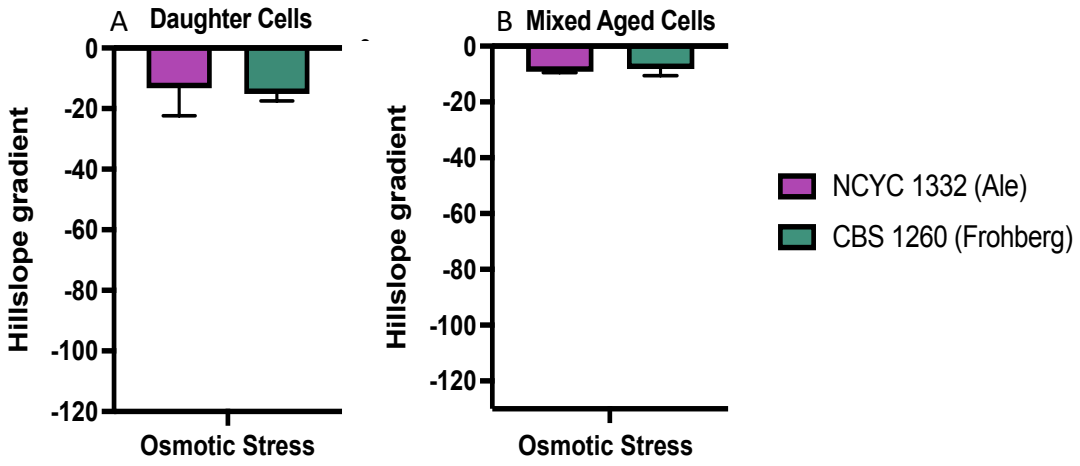

Figure 5.17. Osmotic stress response heterogeneity comparison between daughter and mixed aged cells. Hillslope gradient data obtained from dose response curves generated by exposing daughter cell populations to increasing concentrations of sorbitol (A). Hillslope gradient data for mixed aged NCYC 1332 and CBS 1260 cell populations (B).

5.4.2 Oxidative stress response heterogeneity in daughter cells

Daughter cell populations derived from the strains NCYC 1332 and CBS 1260 were also exposed to oxidative stress by culturing in increasing concentrations of hydrogen peroxide (0-6mM). The stress dose response curves obtained and shown in Figure 5.18(a) displayed significant differences between strains, derived from the fact that strain NCYC 1332 reached ~75% growth (compared to the unstressed control) faster than CBS 1260. The dose response curves were used to calculate IC50 values for each strain (Figure 5.18b). It can be seen that daughter cells from both strains displayed a similar IC50 of 2.9mM H_2O_2 for NCYC 1332 and 3.3mM H_2O_2 for CBS 1260. This similarity in IC50 was somewhat surprising, given that for their mixed aged counterparts the IC50 values measured 4.1mM (NCYC 1332) and 3.1mM (CBS 1260), showing a significant reduction in IC50 and therefore poorer oxidative stress tolerance for strain NCYC 1332. However, the hillslope gradients shown in Figure 5.19(a) indicate that both strains exhibited similar patterns of heterogeneity (-11.4 NCYC 1332 and -13.7 CBS 1260). When comparing these results to the mixed aged cell populations (Figure 5.19b) these data were not statistically significant, and a high degree of

heterogeneity was observed in both age populations, further demonstrating that heterogeneity is an innate property to yeast daughter cells.

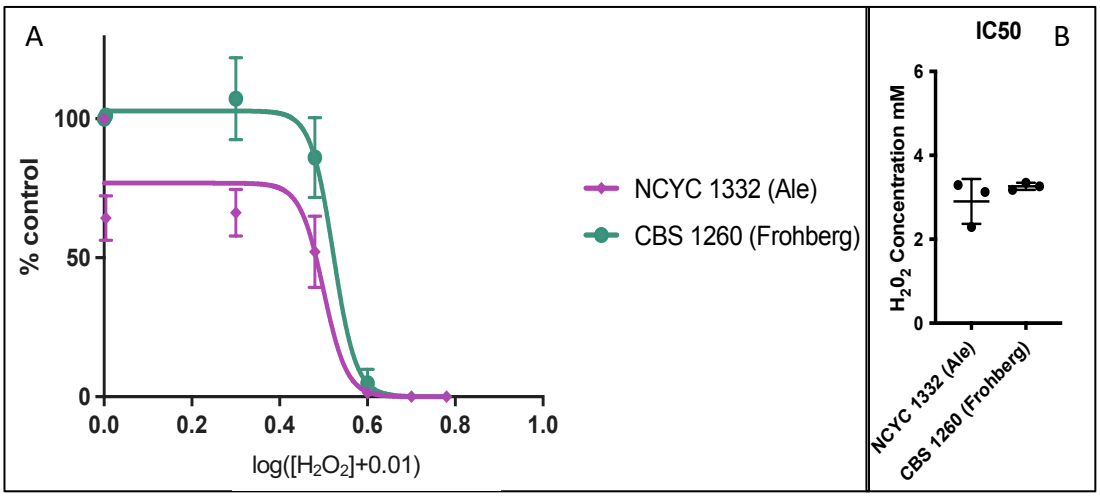

Figure 5.18. Daughter cell growth assessment in response to oxidative stress. A, Dose response curves in response to oxidative stress for daughter cell populations of each strain, obtained from the MTT cell cytotoxicity assay. B, IC50 values generated for the hydrogen peroxide concentration at which 50% viability is achieved. Yeast strains were exposed to increasing concentrations of hydrogen peroxide and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

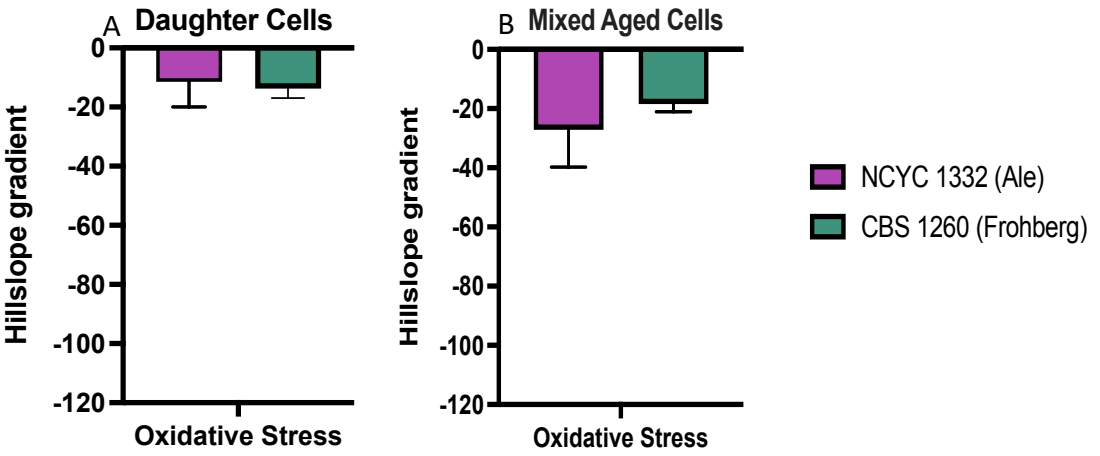

Figure 5.19. Oxidative stress response heterogeneity comparison between daughter and mixed aged cells. Hillslope gradient data obtained from dose response curves generated by exposing daughter cell populations to increasing concentrations of hydrogen peroxide (A). Hillslope gradient data for mixed aged NCYC 1332 and CBS 1260 cell populations (B).

5.4.3 Copper stress response heterogeneity in daughter cells

Isolated daughter cells were tested for their stress response dynamics after exposure to increasing concentrations of copper sulphate pentahydrate (0-8mM). Figure 5.20(a)displays the sigmoidal dose response curves obtained when the strains CBS 1260 and NCYC 1332 were exposed increasing concentrations of copper. Analysing the IC50 in response to increasing concentrations of copper revealed differences in tolerance levels between the two strains. Daughter cells from NCYC 1332 had an IC50 of 2.1mM $CuSO_4H_2O$ while CBS 1260 measured 3.4mM $CuSO_4H_2O$ (Figure 5.20b). These values were significantly higher than the IC50 data calculated from mixed aged yeast populations, which were 1.1mM and 1.3mM $CuSO_4H_2O$ respectively. This increase in tolerance by daughter cell populations is surprising and contrary to the previous stressors. This may be a result of an innate bet hedging strategy utilised by brewing yeast, whereby upon budding, newly produced daughter cells show enhanced resistance to heavy metals. It is possible that, due to the fact that brewing yeasts rarely come into contact with toxic concentrations of copper, this trait is rapidly lost in older cells in favour for other more useful cell attributes. In addition, this may be a result of reduced gene expression in genes involved in heavy metal stress response due to a lack of inducing chemical signal (copper ions) (Estruch, 2000).

The hillslope gradient data obtained from the point of curve inflection for daughter cells of the two studied strains showed similar values. Strain NCYC 1332 had a gradient of -1.5 while CBS 1260 measured -4.7 (Figure 5.21a), indicating that both daughter cell populations exhibited a high degree of heterogeneity in response to copper. When comparing this measure of heterogeneity with their mixed aged counterparts (Figure 5.21b), no significant differences were found with similar hillslope gradients of -2 and -2.4 (NCYC 1332 and CBS 1260 respectively), again indicating that heterogeneity is an acquired trait.

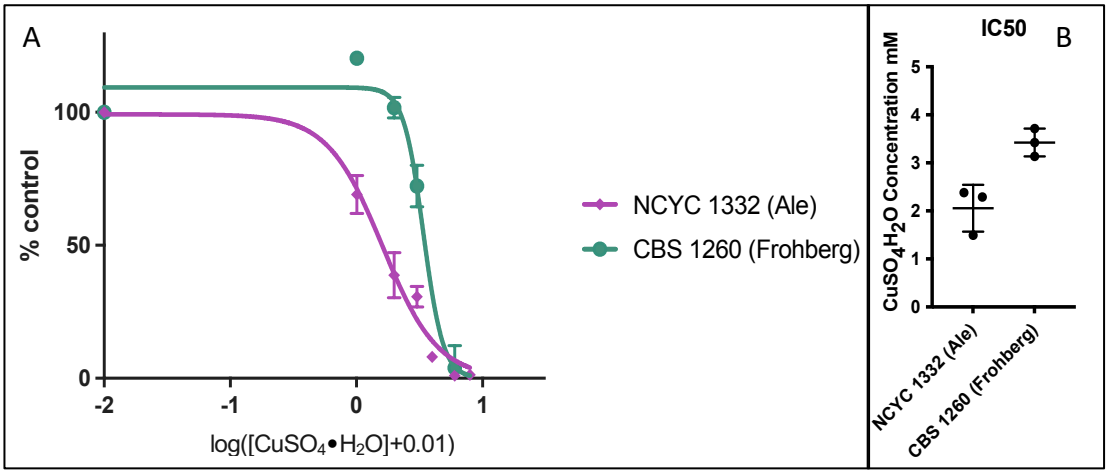

Figure 5.20. Daughter cell growth assessment in response to copper ion toxicity A, Dose response curves in response to copper stress for daughter cell populations of each strain, obtained from the MTT cell cytotoxicity assay. B, IC50 values generated for the copper sulphate concentration at which 50% viability is achieved. Yeast strains were exposed to increasing concentrations of copper sulphate and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

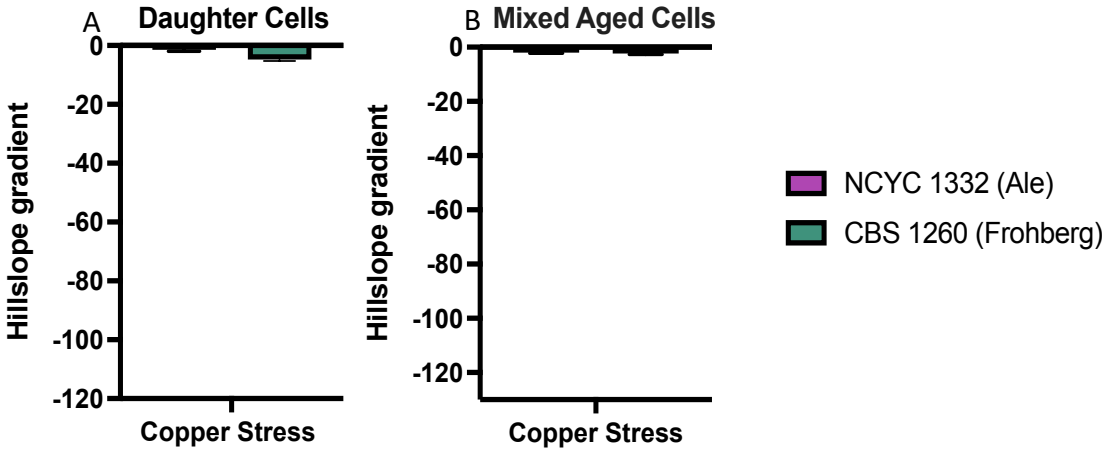

Figure 5.21. Copper stress response heterogeneity comparison between daughter and mixed aged cells. Hillslope gradient data obtained from dose response curves generated by exposing daughter cell populations to increasing concentrations of copper sulphate(A). Hillslope gradient data for mixed aged NCYC 1332 and CBS 1260 cell populations (B).

5.4.4 Zinc stress response heterogeneity in daughter cells

By exposing daughter cell populations of strains CBS 1260 and NCYC 1332 to 0-5mM $ZnSO_4 \bullet 7H_2O$ tolerance, heterogeneity data was obtained as with the previous stressors. The sigmoidal dose response curves gathered from the MTT assay are shown in Figure 5.22(a). The IC50 data was extracted from these curves as a measure of tolerance and shown in Figure 5.22(b). The IC50 value for daughter cells of the strain NCYC 1332 measured 2.8mM $ZnSO_4 \bullet 7H_2O$, while for CBS 1260 it was 2.5mM $ZnSO_4 \bullet 7H_2O$. This similarity in tolerance was also observed in their mixed aged counterparts which measured 2.6 for NCYC 1332 and 2.8 for CBS 1260, demonstrating no significant differences. The gradient of the dose response curves were subsequently used to gain insight into the degree of heterogeneity present in daughter cell populations in response to zinc. The hillslope gradients taken at the point of curve inflection are displayed in Figure 5.23(a). From these data it is evident that both strains showed a similar gradient, and therefore similar degree of heterogeneity. Daughter cells of NCYC 1332 produced a gradient of -40, while CBS 1260 was -34. When comparing this data to mixed aged cells, again no significant differences were determined (Figure 5.23b). As before, this provides further evidence that heterogeneity is an acquired characteristic in the yeast strains analysed.

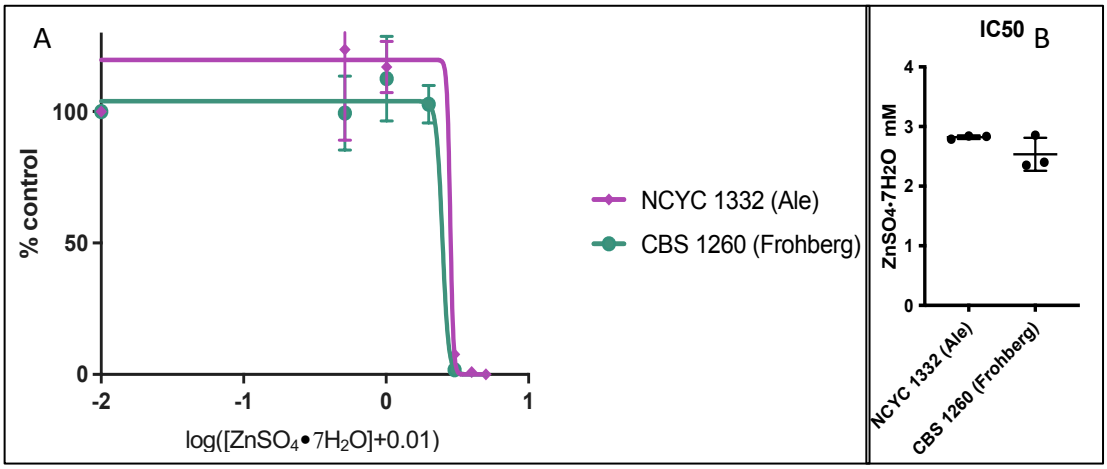

Figure 5.22. Daughter cell growth assessment in response to zinc ion toxicity A, Dose response curves in response to zinc stress for daughter cell populations of each strain, obtained from the MTT cell cytotoxicity assay. B, IC50 values generated for the zinc sulphate concentration at which 50% viability is achieved. Yeast strains were exposed to increasing concentrations of zinc sulphate and data obtained in triplicate. Error bars represent the standard deviation of the collated data for each strain.

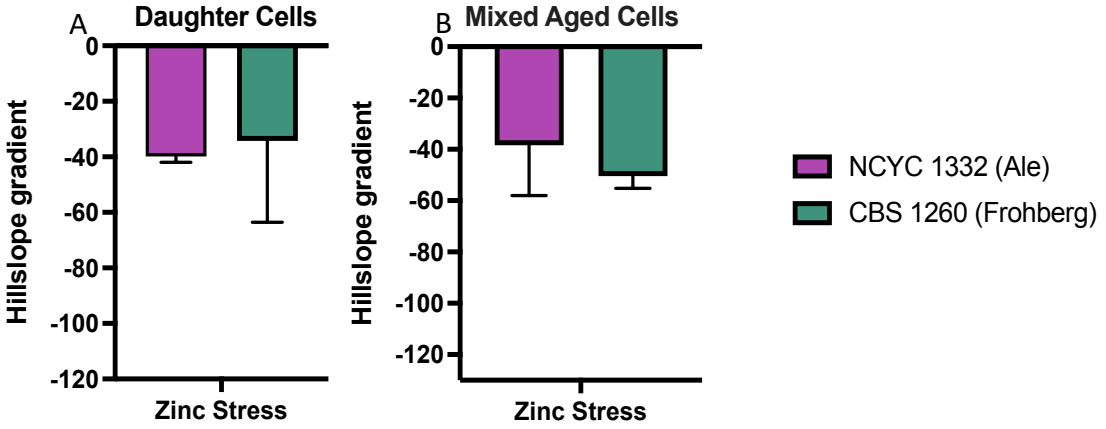

Figure 5.23. Zinc stress response heterogeneity comparison between daughter and mixed aged cells. Hillslope gradient data obtained from dose response curves generated by exposing daughter cell populations to increasing concentrations of zinc sulphate(A). Hillslope gradient data for mixed aged NCYC 1332 and CBS 1260 cell populations (B).

5.5 Heterogeneity in growth under standard and cold conditions

Chemical stressors such as those focussed on in the previous section are not the only source of stress for yeast populations in industrial environments. For example, it is common in modern day breweries to reuse yeast cultures in a process known as serial re-pitching. This practice involves the periodic storage of yeast cultures under refrigerated conditions (Somani *et al.*, 2012) before being recycled. It is know that cold shock can induce significant changes to yeast cells, resulting in up/down regulation of a large number of genes, ultimately leading to changes in membrane structure, storage compounds and potentially also impacting on viability (Rodriguez-Vargas *et al*., 2002). The changes brought about by the expression of cold shock related genes may also affect the recovery of yeast cells when placed in more favourable conditions. Furthermore cell-to-cell variation in cold shock recovery within a cell population, may have significant impacts on fermentation consistency and predictability (Phadtare *et al*., 1999; Al-Fageeh and Smales, 2006). In order to determine the susceptibility and variation within the cold shock response in brewing yeast, single daughter cell growth dynamics were measured under standard optimum conditions and following a period of cold shock.

To obtain daughter cells, the same isolation procedure was performed as above, using a 2 stage FACS isolation process (Section 2.11.4; Section 5.2.2) allowing individual daughter cells to be sorted directly into individual wells within a 96 well plate. These individual single daughter cells were grown in 200µl YPD at 25ºC and population growth was monitored until stationary phase was achieved using an automated plate reader measuring optical density at OD 600nm every 15 minutes (Section 2.4). Based on this analysis, the population growth per well can be considered to be a direct reflection of the performance of the initial single daughter cell seed, and therefore differences in population growth dynamics between theoretically identical daughter cells could be observed.

In total, 97% and 93% of the single isolated daughter cells for CBS 1260 and NCYC 1332 (respectively) produced viable growth (from a total of 96 cells in each instance). Subsequently, the growth curves derived from each daughter cell were overlaid, allowing percentage growth to be visualised against time, as shown in Figure 5.24. From these graphs it is evident that all cultures produced a typical yeast growth profile displaying the expected lag, exponential, and stationary phases. From a purely visual analysis of data, it can be seen there was variation in growth kinetics between cultures derived from single daughter cells in both strains (Figure 5.24). However, by measuring the time taken for each single cell culture to reach 50% growth, comparisons between individual curves could be made. In order to achieve this, the coefficient of variance (CV) for the time taken for each culture to achieve 50% growth was used as a quantitative measure of heterogeneity. The CV for daughter cells derived from the strains CBS 1260 and NCYC 1332 under standard growth conditions were 3.1 and 2.6 respectively. These variance values are low, indicating that heterogeneity in growth dynamics was relatively small for both strains. However, it is interesting to note that daughter cells from strain CBS 1260 (which showed a higher degree of heterogeneity in response to ethanol stress), also displayed a slightly higher variance in growth kinetics compared to NCYC 1332.

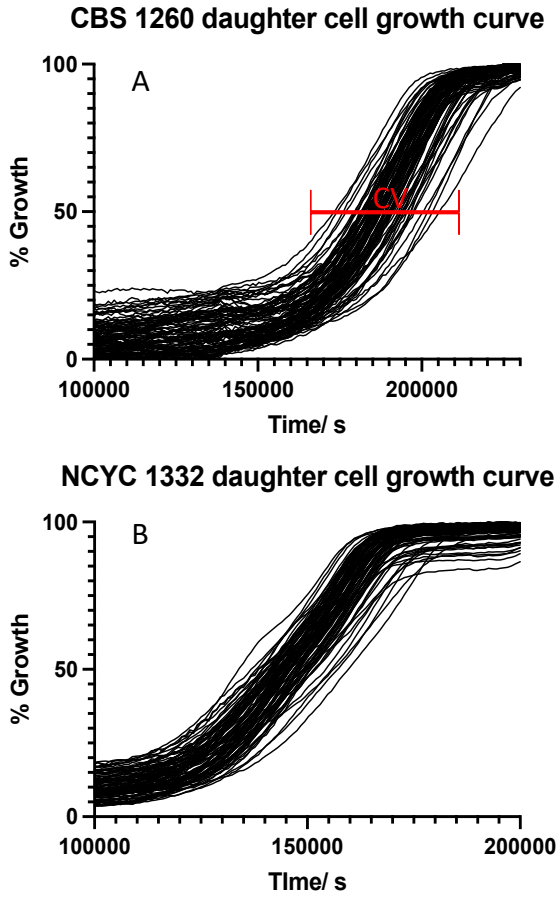

Figure 5.24. Assessment of daughter cell growth curves derived from single cells. A, Daughter cells of CBS 1260 and B, daughter cell of NCYC 1332 grown at 25°C. Marked on graph A is a representation of the variance measured the CV of time taken to achieve 50% growth.

In order to determine the effect of cold shock on growth dynamics, the same experimental conditions were applied to individual daughter cells placed in individual wells within a 96 well plate and immediately exposed to cold shock at 4°C for 12 hours (Section 2.12). These conditions are representative of industrial storage practices, and also allow time for gene expression profiles to tailor themselves in response to sub-optimal temperature (Homma *et al.*, 2003; Murata *et al.*, 2006). Following cold shock, daughter cells from both strains were incubated at 25ºC as before, and the resulting growth was monitored using a plate reader, with growth measured every 15 mins based on absorbance at 600nm. Each daughter cell was used to seed a population and grown to stationary phase, at which point the growth curves were extracted and

overlaid on a single graph for each strain as previously described. Greater than 95% of single cell seeds survived to produce growth curves. A visual examination of the data suggested that there was evidence that CBS 1260 daughter cells exhibited greater variation in the time taken to reach 50% growth than NCYC 1332 as displayed in Figure 5.25. To quantify this and to gain a measure of growth heterogeneity the CV was again used as before. Interestingly, the CV for both strain populations increased compared to non-stressed samples (11.6 and 8.5 for CBS 1260 and NCYC 1332) respectively (Table 5.1). The general increase in CV after cold shock provides evidence suggesting some yeast cells may be superior in their recovery from cold shock than others, potentially confirming the presence of sub-populations with varying capacities to withstand cold shock, perhaps related to key gene expression profiles. Furthermore, the fact that strain CBS 1260 exhibited greater variation in cold shock response than NCYC 1332, again suggests a greater degree of cell-to-cell heterogeneity, matching the previous heterogeneity profiles exhibited in response to ethanol stress and providing further evidence that heterogeneity is both an innate and strain specific phenomenon.

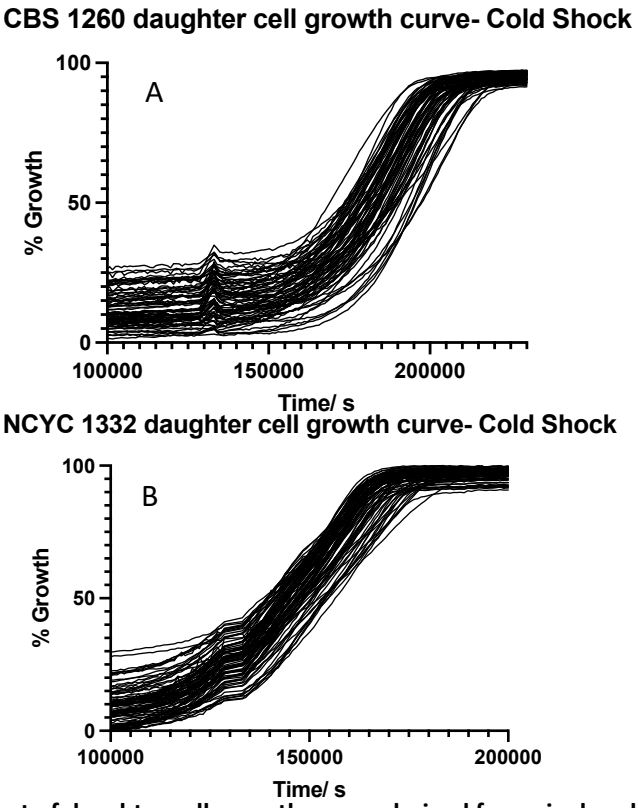

Figure 5.25. Assessment of daughter cell growth curves derived from single cells exposed to cold shock. A, Daughter cells of CBS 1260 and B, daughter cell of NCYC 1332 grown at 25°C after cold shock.

Table 5.1. Coefficient of variance values for the time taken to reach 50% growth in daughter cell derived cultures in normal conditions and after the induction of cold shock.

Strain	CV (normal)	CV (cold shock)
CBS 1260	3.1	11.6
NCYC 1332	2.6	8.5

5.6 Heterogeneity in stress related cell targets.

In the previous sections, the isolation of newly produced daughter cells and the subsequent heterogeneity determination for stress response provided novel insights into the true innate heterogeneity observed in brewing yeast populations. Daughter cells of strains CBS 1260 and NCYC 1332 exhibited opposing strategies of survival in response to ethanol stress and cold shock, with CBS 1260 displaying a high degree of innate heterogeneity compared to NCYC 1332 (Sections 5.3.1 and 5.5). Once this had been established, potential sources of cell-to-cell variation were then the focus of attention. In Chapter 4, differences in heterogeneity were observed in cell attributes such as mitochondria, membrane integrity, neutral lipid content, membrane fluidity and membrane sterols. It is important to note that this was seen in mixed aged yeast cells, such that variation in cell size and cell age could influence the observed heterogeneity (Powell *et al*., 2003b; Powell and Smart, 2004). Therefore, as in the previous sections, to ensure that the true, innate, cell-to-cell variation was assessed, uniform daughter cell populations were investigated for potential sources of heterogeneity.

To achieve this, targeted cell staining was used in conjunction with imaging flow cytometry (Section 2.13). The cell stains used were Mitotracker Green to target mitochondria, $DiOC_6$ targeting internal membranes and mitochondrial membrane potential (MMP), and finally Nile red (NR) for measuring neutral lipid droplets (Section 2.13). These stains were chosen due to their targets being linked with the yeast ethanol stress response (Cox *et al*., 1997; Stanley *et al.*, 2010; Miyagawa *et al.*, 2014; Sommer, 2020) and based on previous data which showed they were particularly useful for heterogeneity studies (Chapter 4). To investigate heterogeneity in cell populations of different age the fluorescent stain calcofluor white was again used to enumerate cell bud scars and determine the age of each cell (Section 5.2.3). However, a result of using calcofluor was that the stains Filipin and Laurdan previously used for sterols and membrane fluidity, respectively, could not be included in this study due to an overlap in fluorescence emission. Unfortunately, both stains emit blue

fluorescence at the same wavelength as calcofluor white, an issue which could not be resolved.

To measure heterogeneity in these stress related cell targets populations of the strains CBS 1260 and NCYC 1332 (mixed age and daughter aged fractions) were cultured in YPD for 48 hours and subsequently prepared for cell staining and heterogeneity determination (Section 2.3). In parallel, identical cell cultures were prepared that had been grown for 48hrs in YPD supplemented with 5% (v/v) ethanol. This was performed to consider whether the degree of heterogeneity observed in these cell targets was affected by sub-lethal stress. A concentration of 5% (v/v) ethanol was selected as an efficient stressor, sufficient to stress brewing yeast with minimal impact to population viability (Piper *et al.*, 1994; Piper, 1995). Following this, cultures were co-stained with calcofluor white and either Mitotracker Green, DIOC or Nile red as described in Section 2.13.1. The cell cultures were then analysed for stain heterogeneity in different age sub-populations using imaging flow cytometry in conjunction with bud scar enumeration as before.

5.6.1 Stain compensation assessment

Due to the nature of co-staining, the fluorescence observed in one channel of detection from a stain can spill over into other channels if the emission spectra overlap. This is not a concern when only one fluorescent stain is applied, however if spill-over is detected in the channel of a second co-stain, the results may lose accuracy and resolution. To prevent this, compensation is usually required, whereby the compensation settings are altered until no fluorescence is detected in unwanted channels. To assess the need for compensation, initially, mixed aged cells stained with the individual stains calcofluor white, NR, Mitotracker Green and $DiOC_6$ were analysed (Section 2.13.1). In each stained cell population, the singlet population was identified by analysing each cell by aspect ratio (AspectRatio_M01) against area (Area_M01). From the singlet population the fluorescence detected in each emission channel was then determined (2.13.2). Figure 5.26(a) shows an example of CBS 1260 cells stained with calcofluor white; the fluorescence is detected with the best resolution in Channel

7, where the bud scars are clearly visible. However, there did appear to be spill-over into detection channel 6 (and a small amount into channel 4), indicating that compensation was required. Cells stained with NR were also assessed for their emission characteristics (Figure 5.26b). NR is most accurately detected in channel 4, however spill-over was detected in channels 2, 3 and 6. Mitotracker Green and $DiOC_6$ are both detected in channel 2 and, as shown in Figures 5.26(c) and 5.26(d), spill over was detected in channels 3 and 5. While overlaps in emission spectra did cause spill over to be detected in other channels, there were no instances where the spill over from any of the targeted cell stains (NR, Mitotracker Green or DIOC) were detected in channel 7 (calcofluor white). Therefore most of the co-stains did not interfere with the bud scar stain, and as a result only a very small amount of compensation was required due to a low fluorescence signal detected in channel 4 (NR emission) from cells stained with calcofluor white.

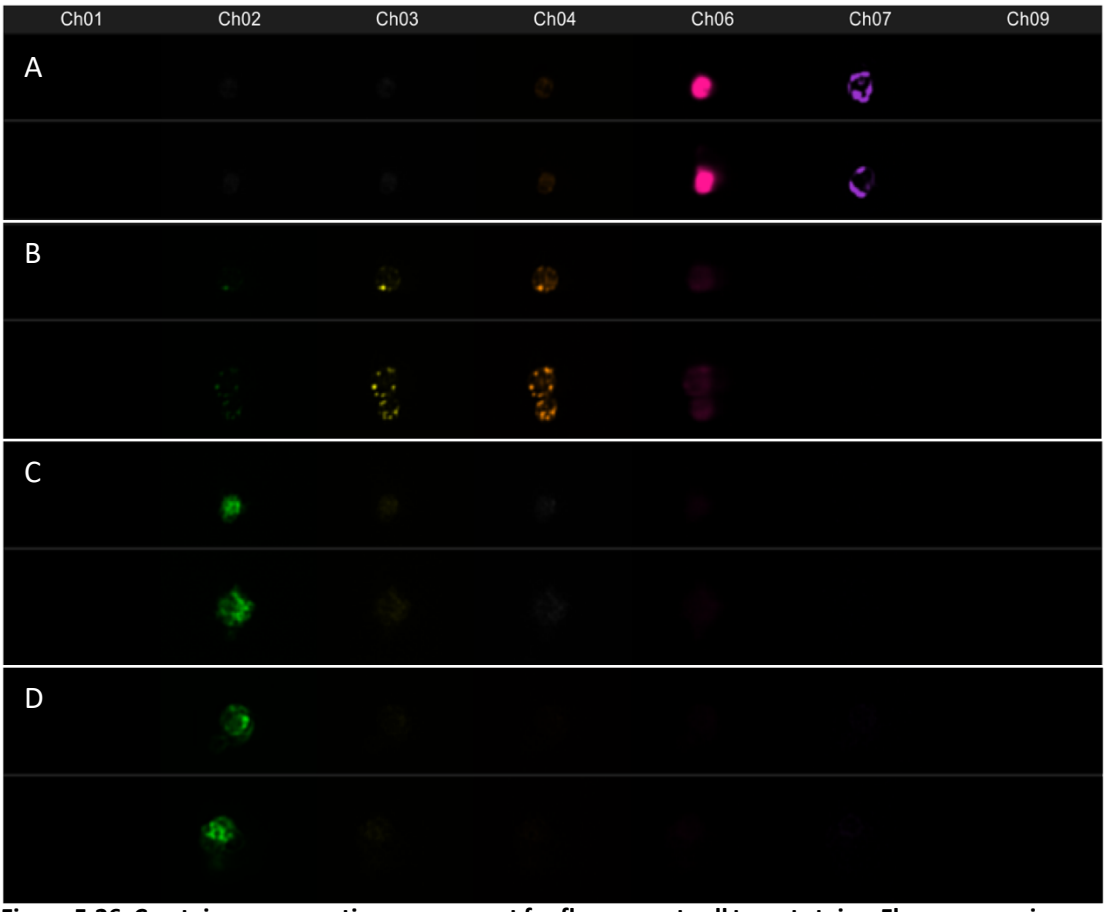

Figure 5.26. Co-stain compensation assessment for fluorescent cell target stains. Fluorescence images obtained for single CBS 1260 cells stained with calcofluor white, using an excitation energy of 405nm the emitted fluorescence detected in channels 1-9 are displayed (A). Nile red using an excitation energy of 461nm (B). Mitotracker green using an excitation energy of 488nm (C). $DIOC_6$ using an excitation energy of 488nm (D).

Once compensation had been factored into the analysis model of both strains, co-stained mixed aged samples of the strains CBS1260 and NCYC 1332 were investigated for cell-to-cell stain intensity variation.

5.6.2 The assessment of neutral lipid heterogeneity among different aged cell populations

In order to examine neutral lipid content, images of cells from CBS 1260 and NCYC 1332 populations were obtained using brightfield and fluorescence analysis using 400X magnification. For NR/calcofluor white co-staining, excitation lasers of 561nm (NR) and 405nm (calcofluor white) were used with detection in channels 4 (NR) and 7 (calcofluor white) (Section 2.13). An example of the images procured for CBS 1260 cells can be seen in Figure 5.27, displaying Brightfield (A), calcofluor white (B), NR (C) and an overlay of all three (D). These images show evidence that the co-staining was successfully, in this instance highlighting bud scar fluorescence while also identifying and localising neutral lipids.

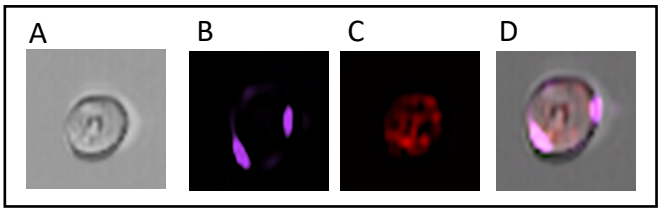

Figure 5.27. Neutral lipid and bud scar co-stain assessment. Brightfield and fluorescence images for single cells of CBS 1260 in 40X magnification. A, brightfield. B, calcofluor white florescence. C, Nile red fluorescence. D, overlap of brightfield and fluorescence.

Subsequently, by analysing each cell for its aspect ratio vs cell area the single cell population could be identified as before, excluding fragments, doublets and triplets (Figure 5.28). The singlet population was taken forward for further analysis. As in Section 5.2.3, bud scar enumeration was performed using the spot count feature, allowing florescent intensities of calcofluor white over a threshold (i.e. bud scars) to be enumerated and providing age determination of each individual cell. For example, in Figure 5.27(b) it is clear that the cell analysed has two bud scars and was therefore designated a cell age of 2. The distinction between different cell ages allowed each cell to be pooled according to age, with subsequent analysis performed on daughter cells (0-bud scars), cells with 1 bud scar and cells with 2 bud scars for both yeast strains.

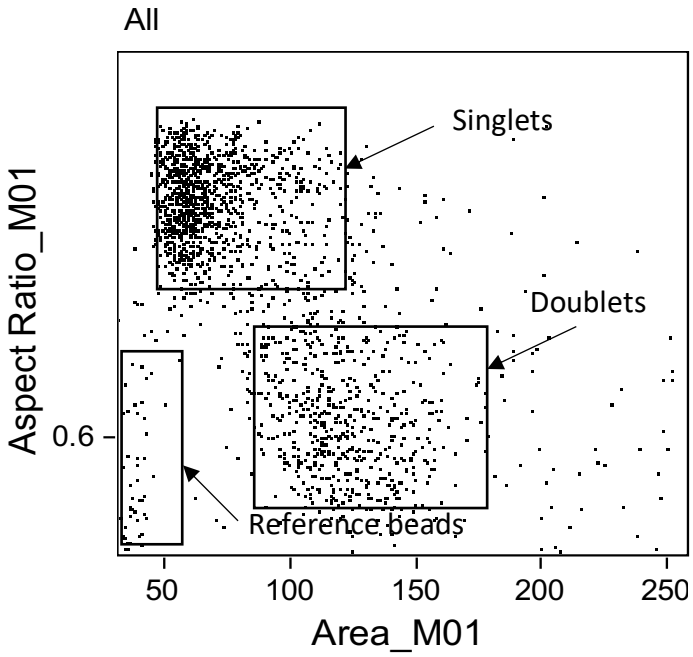

Figure 5.28. Cell population distribution determined using Imagestream flow cytometry. Example data displayed for strain CBS 1260. Mixed aged cells analysed using size parameters Area and Aspect Ratio, clearly identifying singlets and doublets from which only the singlet population was analysed further. The light scatter from the reference beads are also identified, these were used in order to synchronise the sample flow and maintain accurate focus during the run.

The cells within each age fraction were investigated for their emitted fluorescence intensity for the NR stain (Section 2.13.2). This was visualised by plotting NR fluorescence intensity (Intensity_M04_Ch04) against frequency, as shown in Figure 5.29 which displays data for cells of each strain with 0 bud scars. The histogram shown presents an insight into NR staining variation, where the wider the histogram peak the greater variation in neutral lipid throughout the population. To quantify this, the coefficient of variance (CV) was used. This process of variation detection was subsequently performed on both strains for unstressed and stressed samples, ultimately providing CV values for 0, 1 and 2 bud scar cell populations for both of the strains studied. Daughter cells (0 buds scars) of unstressed cell populations revealed a CV in neutral lipid content of 98 for CBS 1260 and 60 for NCYC 1332. These CV values demonstrate that variation is present in both populations, but to a much greater extent in the daughter cells of CBS 1260, indicating a higher degree of neutral lipid

heterogeneity. The comparison of all CV values between the two studied strains is presented in Figure 5.30 , with raw data in Table 5.2. From these data it is evident that under unstressed conditions, CBS 1260 cell populations possess a higher degree of heterogeneity in neutral lipid content compared to NCYC 1332. Interestingly this difference was greater in the daughter cell populations, which we have previously suggested represents the 'true' innate heterogeneity as it is observed immediately after budding and before any influence from external factors.

In cell populations stressed using 5% (v/v) ethanol, the pattern of greater heterogeneity was similar, with CBS 1260 being more heterogeneous compared to NCYC 1332, and the difference was again greater within the daughter cell population compared to mixed aged. However, the CV values for all stressed cell populations was reduced compared to unstressed conditions. Furthermore, CBS 1260 demonstrated a greater reduction in CV within stressed cells, although this finding may be a direct result of the initial heterogeneity present in unstressed yeast cells. Upon addition of ethanol stress, it is possible that underperforming sub-populations may be selected out, leaving only cells that have the necessary adaptations to survive. This can be further corroborated by the fact that variation in NCYC 1332 populations changed to a much lesser extent, perhaps due to the non-existence of underperforming heterogeneous sub-populations meaning that a higher population tolerance was ascertained as reported previously in Chapter 4.

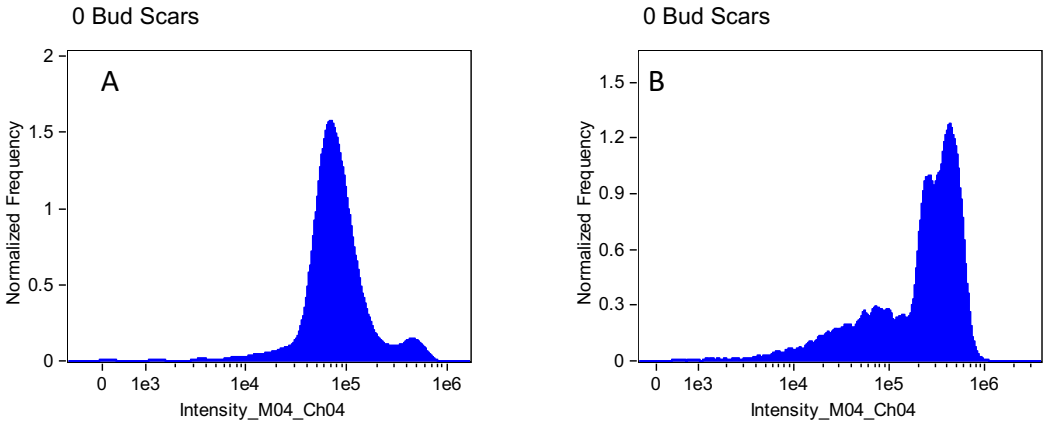

Figure 5.29. Fluorescence intensity variation for Nile red staining. Histogram data displays the fluorescence data for single daughter cells (0 bud scars) of the strain CBS 1260 (A) and NCYC 1332 (B).

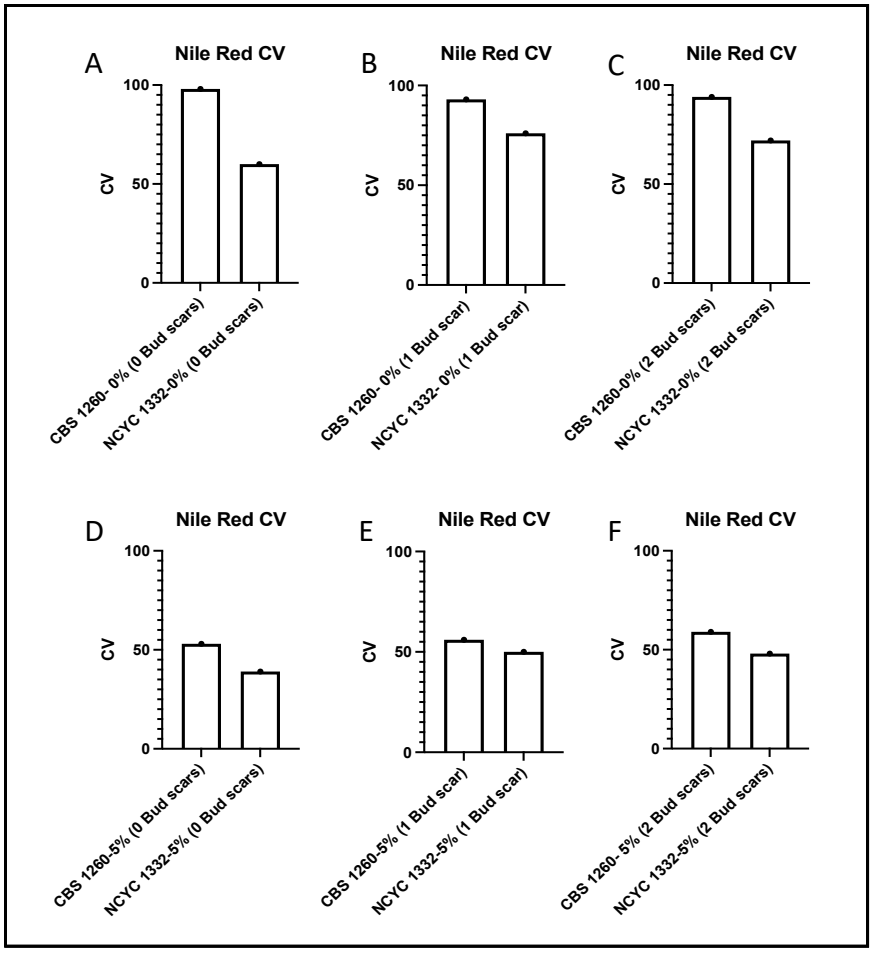

Figure 5.30. Assessment of neutral lipid variation among different CBS 1260 and NCYC 1332 cell populations. CVs for unstressed cell populations with 0 bud scars (A), 1 bud scar (B) and 2 bud scars (C). CVs for stressed (5% (v/v) ethanol) cell populations with 0 bud scars (D), 1 bud scar (E) and 2 bud scars (F).

5.6.3 The assessment of mitochondrial mass heterogeneity among different aged cell populations

In order to analyse mitochondrial mass (content), Mitotracker Green fluorescence was applied to different cell aged populations using co-staining with calcofluor white in conjunction with imaging flow cytometer analysis as described previously (Section 2.13.1). Once the single cell population had been identified, brightfield and fluorescence images were taken of each cell using 488nm excitation for Mitotracker Green and 405nm for calcofluor white, with fluorescence detection in channel 2 (MTG) and channel 7 (calcofluor white)(Section 2.13.1). An example of the procured images for CBS 1260 cells can be seen in Figure 5.31, which visibly show the staining of bud scars and the efficient staining and localisation of mitochondria within the cell.

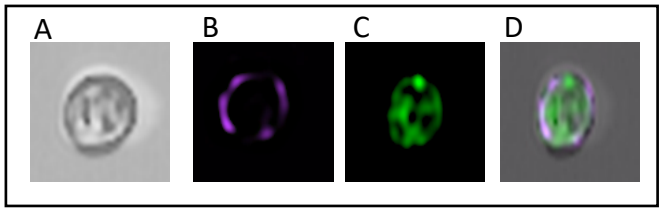

Figure 5.31. Mitochondrial biomass and bud scar co-stain assessment. Brightfield and fluorescence images for single cells of CBS 1260 in 40X magnification. A, brightfield. B, calcofluor white florescence. C, Mitotracker green fluorescence. D, overlay of brightfield and fluorescence.

Mitochondrial mass for each cell age population was investigated by plotting fluorescence intensity (M02_CH02) against normalised frequency. An example of this analysis for daughter cell populations derived from both strains in unstressed conditions can be seen in Figure 5.32. The variation in mitochondrial mass was again quantified using the CV of the Mitotracker green fluorescence intensity for each cell within a population. This method of population variation assessment was performed on all aged fractions for both stressed and unstressed samples of CBS 1260 and NCYC 1332. Figure 5.33 demonstrates the CV comparisons for each of these conditions with raw data in Table 5.2. In unstressed cell populations, CBS 1260 cells of all ages showed a higher degree of variation than NCYC 1332. As with neutral lipid assessment above,

the difference in CV appeared greater in the daughter cell populations when present in unstressed conditions. The addition of stress (5% (v/v) ethanol) resulted in similar CV profiles between the two strains; CBS 1260 again had a greater degree of fluorescence variation among each of the differed aged populations. However, in the stressed daughter cell sample the CV for CBS 1260 reduced compared to the unstressed counter parts. This again could indicate the eradication of underperforming sub-populations from a population as discussed above.

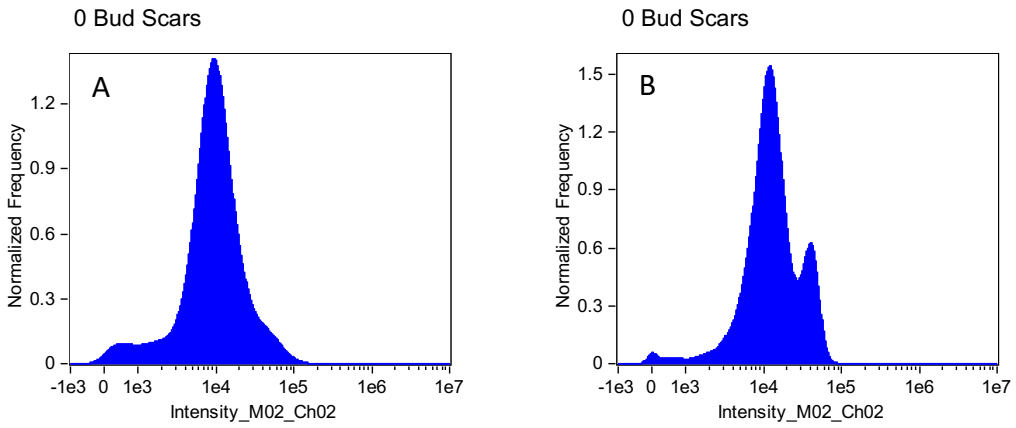

Figure 5.32. Fluorescence intensity variation for Mitotracker green staining. Data displays the fluorescence data for single daughter cells (0 bud scars) of the strain CBS 1260 (A) and NCYC 1332 (B).

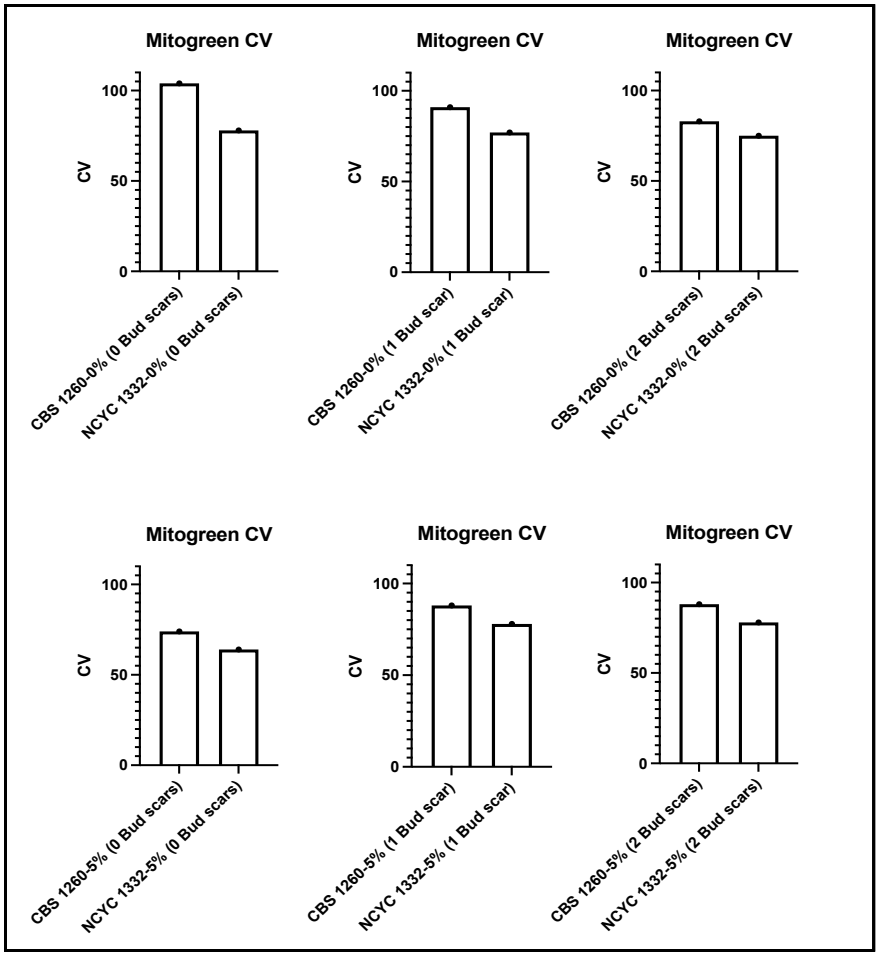

Figure 5.33. Assessment of Mitochondrial biomass variation among different CBS 1260 and NCYC 1332 cell populations. Graphs display Mitotracker green variance data. CVs for unstressed cell populations with 0 bud scars (A), 1 bud scar (B) and 2 bud scars (C). CVs for stressed (5% (v/v) ethanol) cell populations with 0 bud scars (D), 1 bud scar (E) and 2 bud scars (F).

5.6.4 Assessment of internal membrane integrity among different aged cell populations

Variation in internal membrane integrity and mitochondrial membrane potential (MMP) was analysed in a similar fashion to described previously, using $DiOC_6$ in conjunction with calcofluor white for age determination (Section 2.13.1). Brightfield and fluorescence images were procured using the 488nm excitation laser for $DIOC_6$ and 405nm laser for calcofluor white, with detection in channels 2 ($DIOC_6$) and channel 7 (calcofluor white). Figure 5.34 presents examples of the brightfield and fluorescence images obtained for the strain CBS 1260. The images indicate the accurate staining of bud scars using calcofluor white (B) and the successful staining of the $DIOC_6$ target structures (C).

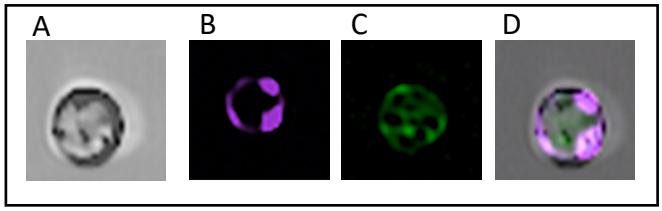

Figure 5.34. . MMP and internal membrane integrity coupled with bud scar co-stain assessment. Brightfield and fluorescence images for single cells of CBS 1260 in 40X magnification. A, brightfield. B, calcofluor white florescence. C, $DiOC_6$ fluorescence. D, overlay of brightfield and fluorescence.

Cell-to-cell variation in internal membrane integrity for each populations was determined by analysing the fluorescence emission intensity in Channel 2 (480-560nm) resulting from 488nm excitation (Section 2.13.2). An example of the results is displayed in Figure 5.35, which shows the fluorescence intensity (Intensity_M02_Ch02) against normalised frequency for daughter cells of CBS 1260(a) and NCYC 1332(b). Using the example of the daughter cell population from unstressed CBS 1260 cells (Figure 5.35), the CV of this fluorescence peak could be calculated to be 107. When comparing this to the same cell population in unstressed daughter cells of the same strain a CV value of 57 was obtained. It is therefore apparent that there was a large difference in degree of variation, indicating the presence of greater cell-to-cell heterogeneity in internal membrane integrity and MMP for the strain CBS 1260.

A similar analysis was performed for all cell age populations in both stressed and unstressed samples for each strain, and the comparisons of the resulting CVs are shown in Figure 5.36 with raw data in Table 5.2. In unstressed cell populations, the comparisons in CV give evidence suggesting CBS 1260 had a much greater degree of variation for internal membrane integrity from cell-to-cell compared to populations of NCYC 1332. Interestingly the biggest difference in CV was again observed in the unstressed daughter cell populations. The 5% **(v/v)** ethanol stressed cell populations also showed similar patterns of variation, with CBS 1260 displaying higher variation than NCYC 1332 populations in general. However, in stressed daughter cell populations, CBS 1260 showed a CV of ~100, a value that was double that observed for NCYC 1332 (~ 50). Interestingly a similar ratio was also seen in all three of the aged populations investigated. This suggests, unlike the previous cell targets, that heterogeneity in internal membrane integrity and MMP is maintained in the event that ethanol stress is imposed.

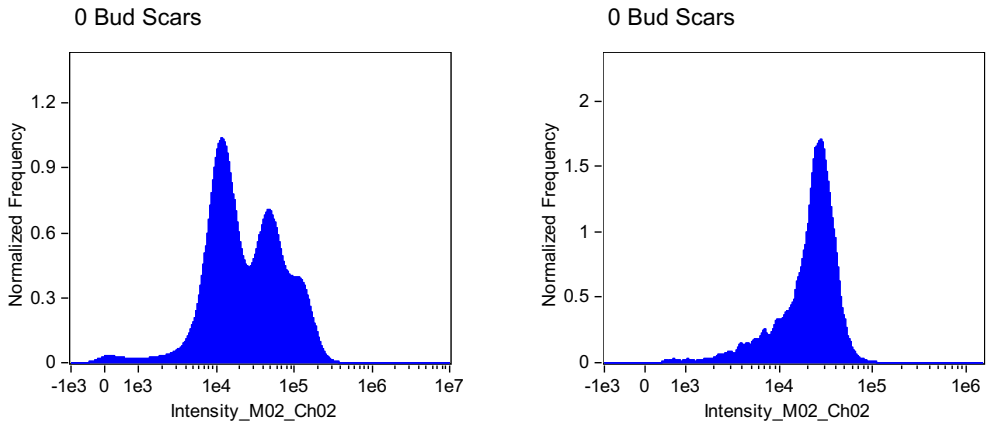

Figure 5.35. Fluorescence intensity variation for $DiOC_6$ staining. Data displays the fluorescence data for single daughter cells (0 bud scars) of the strain CBS 1260 (A) and NCYC 1332 (B).

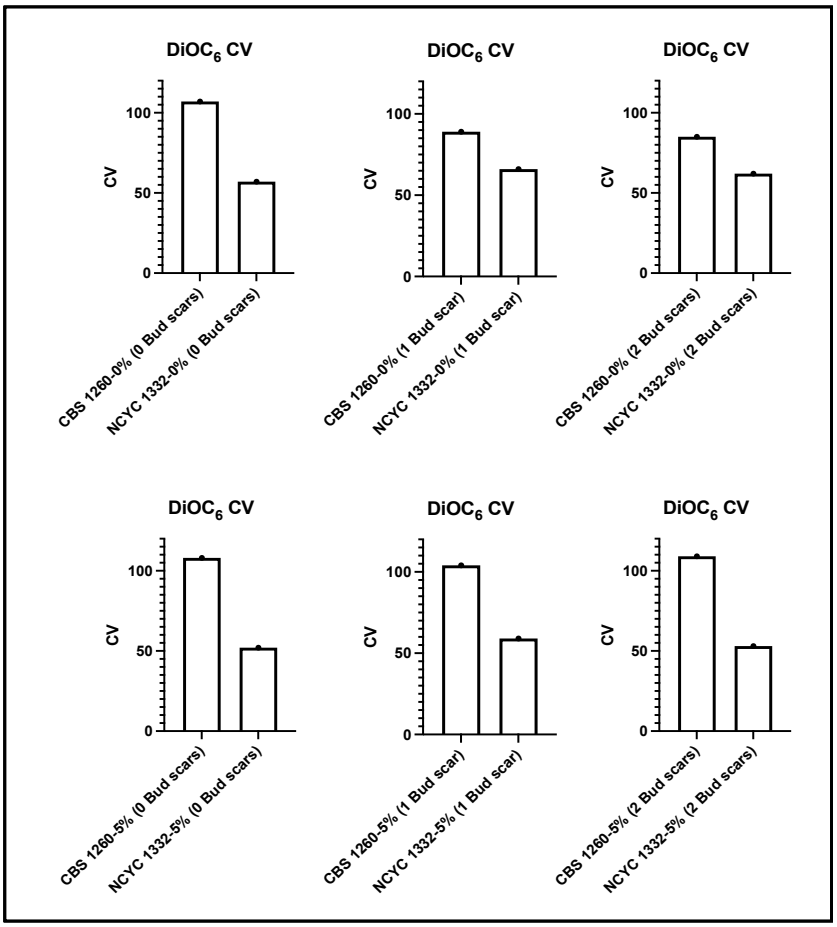

Figure 5.36. Assessment of MMP and internal membrane integrity variation among different CBS 1260 and NCYC 1332 cell populations. Graphs display the $DIOC_6$ variance data. CVs for unstressed cell populations with 0 bud scars (A), 1 bud scar (B) and 2 bud scars (C). CVs for stressed (5% (v/v) ethanol) cell populations with 0 bud scars (D), 1 bud scar (E) and 2 bud scars (F).

Table 5.2. Raw CV data for targeted organelle stains in stressed and unstressed cells. Data represents the fluorescence intensity CV obtained from the strains CBS 1260 and NCYC 1332 in both unstressed and stressed conditions. Data for cell populations with 0 bud scars (daughter cells), 1 bud scar and 2 bud scars are presented.

Strain and condition	Bud scar no.	MitoTracker Green (Mitochondria biomass)- CV	$DIOC_6$ (MMP and internal membrane integrity) -CV	Nile red (Neutral lipid content)- CV
CBS 1260-0% (v/v) Ethanol	0	104	107	98
	1	91	89	93
	2	83	85	94
CBS 1260-5% (v/v) Ethanol	0	74	108	53
	1	88	104	56
	2	88	109	59
NCYC 1332-0% (v/v) Ethanol	0	78	57	60
	1	77	66	76
	2	75	62	72
NCYC 1332-5% (v/v) Ethanol	0	64	52	39
	1	78	59	50
	2	78	53	48

5.6.5 The impact of cell size variation on organelle heterogeneity

In addition to cell age, cell size could also be a source of heterogeneity among yeast cell populations (Powell *et al*., 2003b). To some extent this is controlled within this study by separating cells based on cell size and fluorescence by flow cytometry. However, within each age group it is likely that there may be some level of cell size variation. In order to assess the impact that cell size had on the obtained data, the variation in cell area was measured for each of the cell age populations in both strains for unstressed and stressed samples (Section 2.13.2). This was achieved by measuring the coefficient of variance for the area of each cell, measured using imaging flow cytometry. The results for this analysis are displayed in Table 5.3. The variation in cell size CV among all populations was observed to differ, from 13.6-23.8, however this difference was deemed to be relatively small. Furthermore, there was no evidence to suggest a link between cell size CV and fluorescence intensity CV for any of the studied stain. Therefore, this suggests that cell size variation does not impact the data presented in this section.

Table 5.3. CV data for cell size of the studied cell populations.

Strain and condition	Bud scar no.	MitoTracker Green (Mitochondria biomass)- CV	$DIOC_6$ (MMP and internal membrane integrity) CV	Nile red (Neutral lipid content) CV
CBS 1260-0% (v/v) Ethanol	0	15.55	16.92	16.15
	1	17.08	19.4	18.77
	2	19.88	20.29	19.26
CBS 1260-5% (v/v) Ethanol	0	13.59	15.03	13.66
	1	21.1	22.83	21.8
	2	23.77	23.37	22.28
NCYC 1332-0% (v/v) Ethanol	0	20.39	21	21.34
	1	21.89	21.18	22.63
	2	22.7	22.32	23.46
NCYC 1332-5% (v/v) Ethanol	0	17.76	17.89	17.38
	1	19.06	19.22	19.22
	2	18.68	18.55	19.09

5.7 Conclusion

In Chapter 4, the presence of heterogeneity in stress response was evaluated and data was obtained showing that each yeast strain varied in terms of the extent of heterogeneity, which was also variable from stress to stress. While this data provided a high throughout method of assessing population heterogeneity in response to environmental challenges, the results were obtained for mixed aged cell populations alone. As discussed previously, age heterogeneity itself can be a source of significant variation in other cell aspects (Costa and Moradas-Ferreira, 2001; Laun *et al.*, 2001; Powell *et al*., 2003a; Knorre *et al.*, 2018). As a result, to ensure a true reflection of yeast strain heterogeneity was being obtained, similar heterogeneity studies were performed on populations of daughter yeast cells. This allowed a uniform aged population to be investigated and the 'innate' heterogeneity of cells immediately after budding to be assessed.

In order to obtain purified daughter cells from a mixed aged population of yeast, fluorescence activated cell sorting, confocal microscopy and single cell imaging flow cytometry were all utilised. It was demonstrated that the bud scar stain calcofluor white displayed good bud scar resolution and was amenable to FACS to allow isolation of purified daughter cell populations (Pringle, 1991). While WGA-FITC provided the best bud scar resolution as observed in other studies (Powell, *et al*., 2003a), this stain lost efficacy when FACS and co-staining of other cell structures was performed. Similarly congo red was also deemed an inefficient method of bud scar fluorescence. As a result, the stain calcofluor white was subsequently used to isolate daughter cells of the strains CBS 1260 and NCYC 1332. These strains were selected due to their opposing response to ethanol stress, where CBS 1260 displayed a high degree of heterogeneity and NCYC 1332 a much lower degree of heterogeneity.

In this Chapter, imaging flow cytometry was used to develop a novel method of cell age determination, resulting in a quantitative assessment of daughter cell purity of greater than 95%. These cells were analysed using the MTT assay and it was shown

that the degree of heterogeneity was similar to their mixed aged counterparts. In response to ethanol stress, daughter cells of the strain CBS 1260 exhibited significantly greater population variation than daughter cells of NCYC 1332. This similarity in heterogeneity between daughter cells and mixed aged cell populations was also seen for the 4 other studied stresses. Interestingly while the degree of heterogeneity remained un-changed, the MTT assay did provide data demonstrating differences between mixed aged and daughter cell populations. The IC50 data relating to the maximum stress tolerance of each strain was lower for both ethanol and osmotic stress response in many daughter cell populations. This was a trait also seen in the oxidative stress response, but only for daughter cells of NCYC 1332 (CBS 1260 displayed a similar IC50 to the mixed aged population). These differences in maximum tolerance are likely a result of the adaptation of older yeast cells, which are able to procure a more developed stress response likely due to metabolic changes over time (Knorre *et al.*, 2018). However, in response to copper stress, daughter cells had a higher IC50 than mixed aged cells. This contrasting mechanism is perhaps surprising, considering the response to conventional brewing stresses. Although, it may be explained by that fact that copper is rarely present in brewing fermentations at toxic concentrations (Pagenstecher *et al.*, 2021). As a result, yeast daughter cells may use an innate, pre-determined copper stress response which is activated in a bet hedging strategy of survival immediately after budding (Grimbergen *et al.*, 2015). In contrast, older cells which may have never been exposed to toxic concentrations of copper may lose some of their tolerance in favour of more pertinent metabolic pathways. Therefore, when these older cells are exposed to high concentrations of copper they have a reduced basal tolerance in comparison to daughter cells. This innate bet hedging, heterogenous response, has previously been observed in response to other heavy metals (Holland *et al.*, 2014). While differences in maximum tolerance between mixed aged cells and daughter cells were observed, the degree of heterogeneity remained the same. This indicates that stress response heterogeneity is an innate property of yeast cells and a factor that is present in daughter cells immediately upon budding and not acquired over time due to environmental factors. In addition to chemical stressors, analysis of both growth rate and the cold shock response provided

further evidence that heterogeneity is present to a greater extent in daughter cells of the strain CBS 1260 than in NCYC 1332.

Once the true 'innate' heterogeneity of the yeast stress response had been discovered, the source of this heterogeneity was subsequently investigated. By targeting stress related cell attributes such as mitochondria, internal membrane structure, mitochondrial membrane potential and neutral lipid content, potential sources and causes of heterogeneity were assessed. This was achieved by employing a novel method of yeast cell age determination using co-staining, spot counting and imaging flow cytometry. In this way, heterogeneity was discovered in all of these cell targets and across different ages of cells, primarily focussed on daughter cells (0 bud scars) and different fractions of aged cells (1 bud scar and 2 bud scar). The data presented further demonstrates that the strain CBS 1260 is more heterogeneous, having greater cell-to-cell variation in mitochondrial number, mitochondrial membrane potential and internal membrane integrity, and neutral lipid content. In unstressed yeast populations the difference in heterogeneity between the two strains CBS 1260 and NCYC 1332 was also observed in the daughter cell populations, supporting the previous hypothesis that observed heterogeneity is an innate attribute of yeast cells. Further to this, the degree of heterogeneity found in CBS 1260 cell populations appeared to reduce slightly under stressed conditions, in particular for neutral lipid content and mitochondrial mass. This is likely a result of underperforming sub-populations within the CBS 1260 cell populations being selected out as reported previously (Holland *et al.*, 2014). This was not seen in the strain NCYC 1332 perhaps as a result of the absence of underperforming sub-populations due to improved stress tolerance (Section 4.2.1) and a lack of heterogenous sub-population within this strain. This pattern was not seen for $DIOC_6$ staining of internal membrane structures, where the difference in heterogeneity between both daughter cells vs older cells and between stressed vs non-stressed was largely the same in all conditions. Interestingly, in this instance the difference in CV between the two strains did increase when stress was induced, indicating that the CBS 1260 populations had become more heterogeneous for MMP and internal membrane integrity and NCYC 1332 had become less heterogeneous. This may be explained by CBS 1260 cells retaining their

innate bet hedging and generalist nature meaning that cells with reduced membrane structures were not selected out when stress was applied, a trait that has been previously reported (Beaumont *et al.*, 2009; Haaland *et al*., 2020).

The results in this section give evidence showing that heterogeneity is an innate property of yeast cells and is not something acquired over time. Interestingly it does appear that in response to some stresses, such as ethanol, maximum tolerance can be acquired and increase over time in aged cells, although the actual degree of heterogeneity from cell-to-cell remains the same (Watson and Cavicchioli, 1983; Costa *et al.*, 1993). In addition, stress related cell targets such as mitochondria, neutral lipid content, MMP and internal membrane integrity all appear to have more cell-to-cell variation in the strain CBS 1260 regardless of cell age and stress state. As a result, it is therefore likely that other stress related metabolites may vary more in CBS 1260 than in NCYC 1332. Further to this, the source of heterogeneity is likely caused by differences in gene expression from one cell to another. These two additional aspects of heterogeneity will be investigated further in Chapter 6.

Chapter 6. Investigation into the source of heterogeneity using metabolomics and single cell RNA sequencing

6.1 Introduction

In Chapter 4 it was shown that the yeast stress response was heterogeneous within brewing yeast populations. Further to this, in Chapter 5, heterogeneity was also identified in key cellular structural components, including mitochondrial mass, neutral lipid content, sterols and membrane fluidity, suggesting potential sources of phenotypic variation. However, the data indicated that both the observed heterogeneity and the source of heterogeneity were complex, with significant variation observed between the studied strains. In addition, the data presented in Chapter 4 and 5 demonstrated that a high degree of heterogeneity in stress response (for example in response to ethanol) did not necessarily mean a high degree of heterogeneity in all of the studied key stress related targets. This suggests that the observed stress response heterogeneity is likely a result of the accumulation of different stress related cell aspects, each contributing to the overall degree of heterogeneity. Furthermore, the cell targets selected for analysis are themselves impacted by many cell processes. For example, neutral lipid content relies on lipid biosynthesis, lipid metabolism, cell membrane lipid re-organisation, lipid transport etc. (Müllner and Daum, 2004; Czabany *et al*., 2007; Daum *et al.*, 2007). Each of these processes and the metabolites involved in production are controlled by specific sets of genes encoding a series of proteins and enzymes, all of which are likely to alter their expression levels in response to exogeneous signals. As a result, it is likely that both the source of heterogeneity and the physiological manifestation of stress response heterogeneity are related to differences in the gene expression of stress related genes between individual cells of a population.

Therefore, to further investigate the root causes of phenotypic heterogeneity, in this Chapter a yeast metabolomic approach was employed to identify compounds up-regulated in yeast cell populations when stressed with sub-lethal concentrations of

ethanol (5% (v/v) ethanol). The compounds identified in this section were then used to narrow down potential gene targets to be investigated using single cell RNA sequencing. This approach was taken since previous studies have shown that gene expression at the single cell level allows cell-to-cell variation to be characterised, thus providing a mechanism for strain heterogeneity comparison (Gasch *et al.*, 2017). It was anticipated that the presence of divergent sub-populations, and variations in gene expression, for certain target stress related genes would allow both the genetic sources of this variation and potential key products to be identified. To conduct this analysis, two brewing yeast strains were chosen, one with high ethanol stress response heterogeneity (CBS 1260) and one with a lower degree of heterogeneity (M2). Consequently, metabolomic screening was performed using liquid chromatography mass spectrometry to identify compounds of interest, followed by single cell RNA sequencing.

6.2 Selection of yeast strains

It should be noted that the yeast used as a representative 'low heterogeneity' strain (NCYC 1332) in the previous sections, was not taken forward for gene expression or metabolomic analysis here. This is because NCYC 1332 exhibited a propensity to form doublets, which were seen to comprise a significant fraction of the population. As a result, yeast cells from this strain caused blockages in the single cell microfluidics stage of RNA sequencing cell preparation, even with attempts to decouple cells using sonication. Hence it was advised to discontinue its use. As a result, NCYC 1332 was replaced with strain M2 as the most comparative strain of the same species (*Saccharomyces cerevisiae*). These two strains showed similar degrees of stress heterogeneity and maximum tolerance limits (Chapter 4). For example, in response to ethanol stress the two strains showed identical IC-50 (9.7%) in mixed aged populations and similar stress dose response curves. In addition, the strain M2 had also been investigated for heterogeneity in key cell targets, including mitochondria, internal membrane integrity and MMP, and neutral lipid content, as described in Chapter 5. Using the same analysis to measure cell staining in different aged cell populations the

coefficient of variance for each stain for M2, along with the previously obtained data for CBS 1260 and NCYC 1332 can be seen (Table 6.1). From this data it was evident that the strain M2 presents a significantly lower degree of heterogeneity compared to CBS 1260 in all cell populations. Furthermore, M2 appears to have a lower degree of heterogeneity for cell targets than the previous yeast NYCC 1332, making it a highly suitable alternative. The fact that strain M2 was also more preferential for the production of discrete single cells, compared to the doublets observed in NCYC 1332 populations, made this strain the preferred choice for single cell RNA sequencing.

Table 6.1. Raw CV data for variation in key organelle structures, including mitochondrial mass, internal membrane integrity and neutral lipids in stressed and unstressed cells of different divisional ages. Data represents the CV of fluorescence intensity obtained from the strains CBS 1260, NCYC 1332 and M2 and data for cell populations with 0 bud scars (daughter cells), 1 bud scar and 2 bud scars are presented.

Strain and condition	Bud scar no.	MitoTracker Green (Mitochondria biomass) - CV	$DIOC_6$ (MMP and internal membrane integrity) - CV	Nile red (Neutral lipid content) - CV
CBS 1260-0% (v/v) Ethanol	0	104	107	98
	1	91	89	93
	2	83	85	94
CBS 1260-5% (v/v) Ethanol	0	74	108	53
	1	88	104	56
	2	88	109	59
NCYC 1332-0% (v/v) Ethanol	0	78	57	60
	1	77	66	76
	2	75	62	72
NCYC 1332-5% (v/v) Ethanol	0	64	52	39
	1	78	59	50
	2	78	53	48
M2-0% (v/v) Ethanol	0	51	40	32
	1	52	35	36
	2	41	33	35
M2-5% (v/v) Ethanol	0	48	37	40
	1	44	42	46
	2	41	40	41

6.3 Yeast metabolomics analysis using Liquid Chromatography- Mass Spectrometry

To identify compounds that were up-regulated and down-regulated in stressed yeast cell populations, LC-MS was utilised in both reverse phase (positive and negative mode), and via hydrophilic interaction chromatography (HILIC) according to Section 2.14. These techniques were applied to reveal compounds of interest that were altered in yeast cells in response to sub-lethal ethanol stress (5% v/v). The analytes obtained using LC-MS were identified using SIRIUS LC-MS/MS data analysis platform as described in Section 2.14.4 and only analytes with high SIRIUS scores and high percentage accuracy were taken forward, due to the respective compound being reliably identified. These compounds were used to produce heat maps based on the chromatography phase in each of the following sections. For each analysis, the Quality Control (QC) mix data was included in the heat map, used to confirm the chromatography column performance and, as a result, should indicate a high abundance of each of the identified compounds. The strains CBS 1260 (high heterogeneity) and both strains with low heterogeneity (NCYC 1332 and M2) were assessed in this study, even though only M2 was used subsequently for single cell gene expression analysis.

6.3.1 Analysis of compounds up-regulated in response to stress using reverse phase chromatography - positive mode

Reverse phase chromatography utilises alkyl chains covalently attached to the stationary phase (chromatography column), this develops a hydrophobic, non-polar stationary phase and is used in conjunction with a polar mobile phase (water mixed with acetonitrile) (Atkinson *et al.*, 2011). This functions to separate compounds that are less polar in nature, since these have a high retention time on the column. In the positive mode, protonated analytes are preferentially observed, while in the negative mode the analytes correspond to de-protonated molecules. This was used to identify compounds that were significantly up-regulated in stressed cell populations. The heat map shown in Figure 6.1 presents a combined list of compounds that were identified

to be up-regulated under stressed conditions in all 3 strains, when analysed in positive mode. Specifically, the data shows analytes that were of highest abundance in stressed cell populations, and those that had a greater than 15x fold change in stressed cell populations compared to unstressed. From these heat maps it was evident that many of the analytes are up-regulated to different degrees, depending on the strain. This was determined by the colour of the segment allocated to that analyte, with darker red representing a higher abundance through to dark blue which represents a very small abundance.

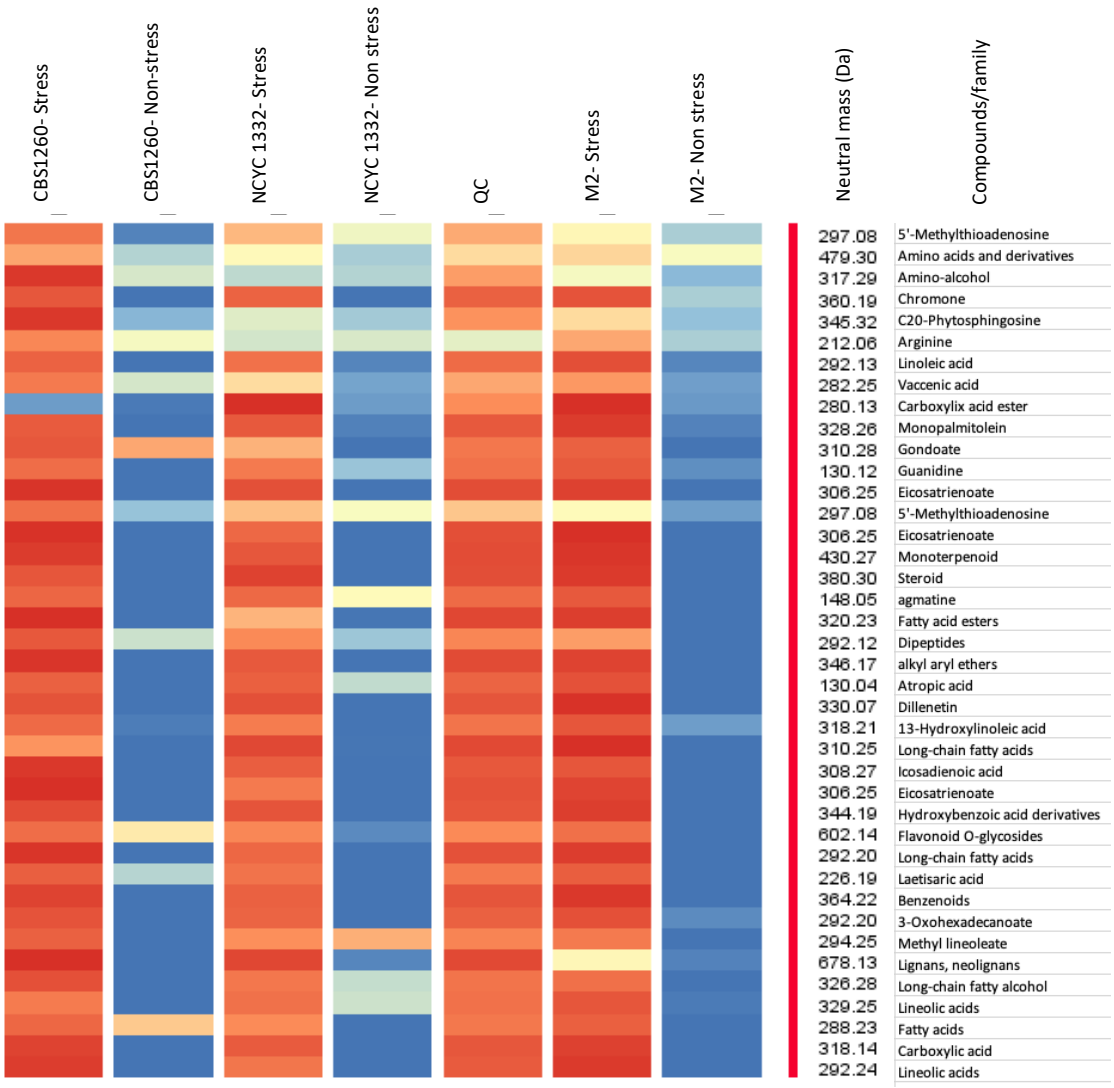

Figure 6.1. Analyte abundance as detected using reverse phase chromatography in positive mode. The heat map presents both the most abundant analytes detected in stressed cell populations and

those positively identified with a fold change of greater than 15x in stressed cell populations. Neutral mass of each analyte is presented in Da and the QC control sample included.

Many of the analytes detected displayed a high normalized abundance under stressed conditions and a small abundance in unstressed conditions in all three strains. Examples include compounds with a neutral mass of 306, 430 and 380 (Da), which were identified as being linoleic acid, a lipid and an undefined steroid respectively. There were, however, compounds in this list that were up-regulated differentially between strains. An example includes the analyte with a neutral mass of 280.13Da, corresponding to a carboxylic acid ester. This analyte was up-regulated in all stressed cell populations but only to a small extent in the stressed CBS 1260 cell population. Another example of this variation can be observed for the analyte with a neutral mass of 678.13Da, which corresponds to a lignan. This molecule was up-regulated to a greater extent in the stressed cell populations for the strains CBS 1260 and NCYC 1332, than for M2. Interestingly the compounds of greatest fold change (>15x) present on this list almost entirely consisted of linoleic acids, such as methyl linoleate and eicosatrienoate, a group of polyunsaturated fatty acids and long chain fatty acids including myristoleate and stearic acid. Furthermore, the high abundance analytes found to be up-regulated under stressed conditions mostly consisted of amino acids, including arginine which has previously been implicated as a stress protectant (Cheng *et al.*, 2016), dipeptides such as adenylthiomethylpentose, and fatty acids such as gondoate. The up-regulated analytes discovered largely correspond to those that have previously been linked to the yeast stress response, including an increase in cellular lipid content in response to ethanol stress. It is known that these lipids function by altering the membrane composition in order to counteract changes in membrane fluidity imposed by ethanol (Šajbidor *et al*., 1995; You, *et al*., 2003).

6.3.2 Analysis of compounds up-regulated in response to stress using reverse phase chromatography - negative mode

As with the previous section, this mode of chromatography utilises a non-polar, hydrophobic stationary phase in conjunction with a polar mobile phase. In negative mode this method allows for the detection of de-protonated analytes presenting a whole new range of identifiable compounds. The analytes presented in the heatmap in Figure 6.2 represent a combination of those up-regulated in stressed cell populations with a large fold change (>15x) and those of high abundance in all stressed cell populations. From this heatmap it is again evident that many of these analytes are up-regulated to a similar extent in stressed populations compared to unstressed for all of the strains studied. Examples include analytes with neutral masses of 330.07, 182.07 and 666.22 (Da), corresponding to dillenetin, sorbitol and glycogen. However, there were also analytes that varied in their degree of upregulation from strain to strain, such as those with natural masses of 297.08, 342.11 and 300.26 (Da) corresponding to 5'-methylthioadenosine, trehalose and 2-hydroxystearate respectively. The two latter compounds were slightly up-regulated in non-stressed populations of the strain NCYC 1332, while being significantly up-regulated in stressed populations of CBS 1260 and M2. This was an unusual finding, particularly with regard to the disaccharide trehalose, a well-known ethanol stress protectant and key reserve carbohydrate source, known to be produced in times of stress (Bandara *et al.*, 2009). However, due to its recognised importance, it was included in the heatmap for NCYC 1332, especially since it was one of most abundant identified compounds in all of the stressed yeast populations. Interestingly the list of most abundant and most up-regulated compounds by fold change (>15x), comprised many sugars, including sorbitol, glycogen, trehalose, and UDP-glucose, an activated form of glucose essential in the production of glycogen (Lomako *et al.*, 1993). All of these sugars are well documented as either stress protectants or as reserve carbon sources (Bleoanca *et al.*, 2013). In addition, many of the other analytes up-regulated in this analysis were long chain fatty acids such as 2,4-dioxo-tridecanoic acid and 2-hydroxystearate, among other members of the fatty acid class. Long chain fatty acids are crucial for lipid production, ensuring the structural integrity of the membrane is maintained and for

storing as a reserve carbohydrate to be utilised in stressful conditions (Bauer and Pretorius, 2000; Black and DiRusso, 2003).

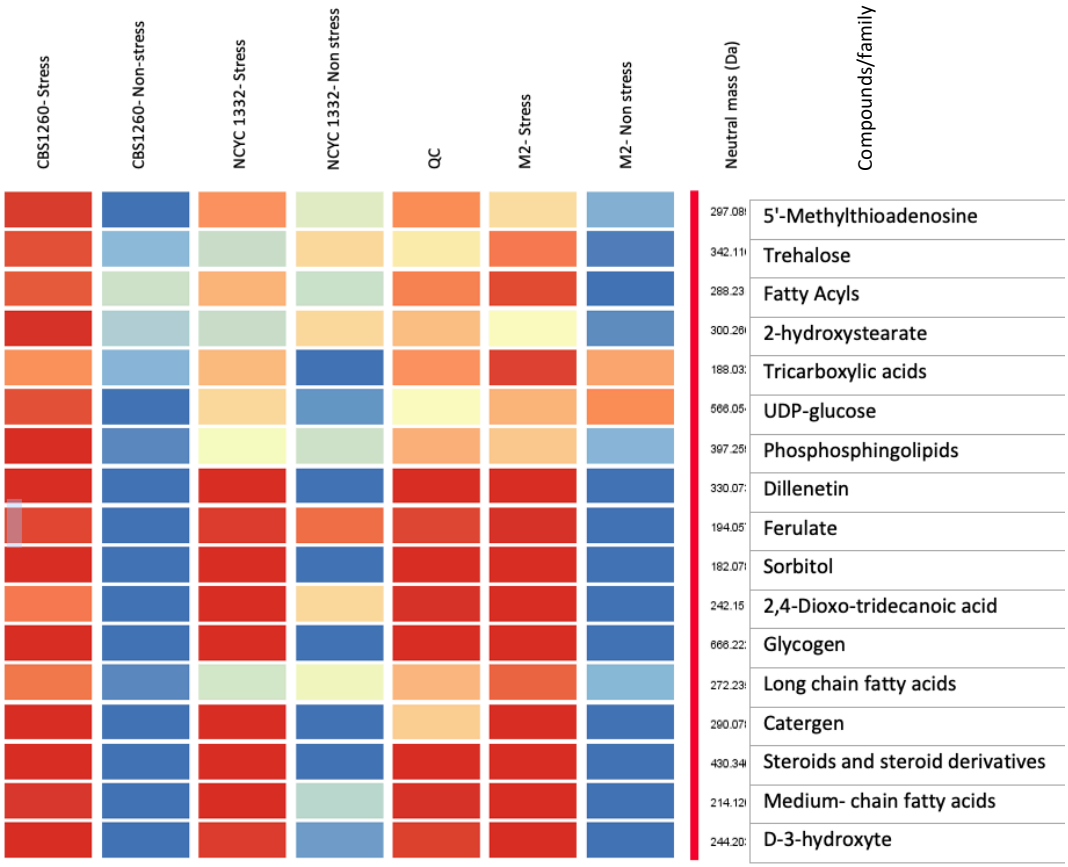

Figure 6.2. Analyte abundance as detected using reverse phase chromatography in negative mode. The heat map presents both the most abundant analytes detected in stressed cell populations and those positively identified with a fold change of greater than 15x in stressed cell populations. Neutral mass of each analyte is presented in Da and the QC control sample included.

6.3.3 Analysis of compounds up-regulated in response to stress using Hydrophilic Interaction Chromatography

Hydrophilic interaction chromatography (HILIC) was also utilised to identify compounds that were up-regulated and down regulated in each yeast population. This method of liquid chromatography utilises a polar stationary phase with mobile phase consisting of water and low polar solvent (acetonitrile); an effective method of isolating smaller polar compounds (Buszewski and Noga, 2012). The heatmap in Figure 6.3 presents analytes identified with a large, up-regulated fold change (>15x) in abundance for stressed cell populations, and analytes identified to be of greatest abundance in stressed cell populations for all strains combined. Analytes that were significantly up-regulated in stressed populations for all three strains include those with the neutral masses 356.16, 330.18, 172.11, 232.05 and 159.089 (Da). These correspond to a long chain fatty acid, dillenetin, 9-oxononanoate, a dipeptide and 3-dehydrocarnitine respectively. However, there were also many other analytes presented in this heatmap that had varying degrees of upregulation in stressed cell populations for the studied strains. For example, the compounds with neutral mass of 297.09, 282.26, 161.07 and 200.05 (Da) all displayed varying degrees of upregulation between the strains. These analytes correspond to the compounds: 5'-methylthioadenosine, 10-hydroxystearic acid, an unidentified amino acid, and 3,4,5-trihydroxy-2-methoxypentanal. Interestingly there were a few analytes that were found in high abundance in stressed cell populations but were further up-regulated in unstressed NCYC 1332 cells. These analytes were 188.08, 298.25, 272.24 and 316.26 (Da) which were all long chain fatty acids. The analytes presented in this heat map almost entirely consist of long chain fatty acids and amino acid derivatives (such as 3-dehydrocarnitine and 5'-methylthioadenosine), indicating that many of the compounds found to increase in abundance in stressed yeast cells were those involved in lipid storage, and membrane structure and re-organisation. Further to this, an increase in abundance of amino acid derivatives was observed, possibly linked to the increase in production and degradation of proteins through the 'unfolded protein response' (UPR), as a result of ethanol induced protein damage (Harding *et al.*, 2003).

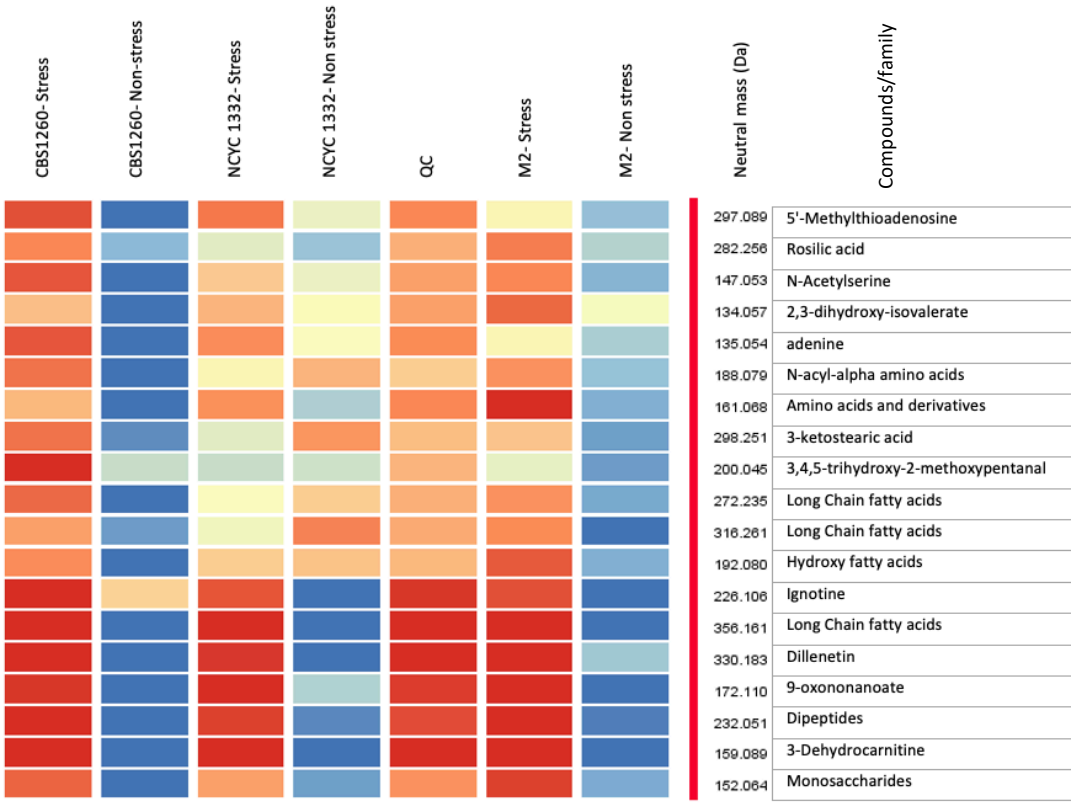

Figure 6.3. Analyte abundance as determined using HILIC chromatography. The heat map presents both the most abundant analytes detected in stressed cell populations and those positively identified with a fold change of greater than 15x in stressed cell populations. Neutral mass of each analyte is presented in Da and the QC control sample included.

6.3.4 Analysis of compounds down-regulated in response to stress using reverse phase chromatography - positive mode

As with the up-regulated compounds identified in the previous sections, the analytes down-regulated in stressed cell populations were also identified in each phase of chromatography. Using reverse phase chromatography in positive mode, a heat map was produced as with the previous sections (Figure 6.4). From this there is evidence that several analytes were down-regulated in stressed populations to a similar extent in all strains. For example, analytes of neutral mass at 162.10, 488.10, 170.10 and 269.23 (Da) were identified, corresponding to the compounds hydroxylysinate, cytidine 5'-diphosphocoline, N-alpha-acetyl-L-lysine and an unidentified alpha-amino

acid. There were also compounds identified, including lysophosphatidylethanolamine (425.25 Da) and saccharopine (314.08 Da), that were more abundant in non-stressed cell populations of each strain, however the difference in abundance between stress states was relatively small, demonstrated by the small change in colouration on the heat map (Figure 6.4). Similarly, there were analytes identified that were abundant in unstressed conditions, however down-regulated in some yeast strains under stressed conditions but up-regulated in other strains. These include the analytes of neutral mass at 161.10, 137.04 and 314.08 Da, corresponding to carnitine, 4-aminobenzoic acid and saccharopine, which were all down-regulated in the stressed cell populations of NCYC 1332 and M2, but up-regulated in stressed CBS 1260 cells.

Interestingly, almost all of the compounds down-regulated in stressed cell populations consisted of amino acids and amino acid derivatives, obtained from leucine, valine, lysine, isoleucine and phenylalanine. It should be noted that these were a different set of amino acids/derivatives than those observed to be up-regulated in stressed cell populations. This trait has previously been observed in similar studies, including the gene expression analysis performed by Chandler *et al.*,(2004), which demonstrated that after an induced ethanol stress of 5% there was both upregulation and down regulation of different amino acid metabolism and biosynthesis genes. It has been suggested that the downregulation of such attributes in stressed cell populations is likely a result of cells entering stress induced cell cycle arrest (Gasch *et al.*, 2000; Chandler *et al.*, 2004).

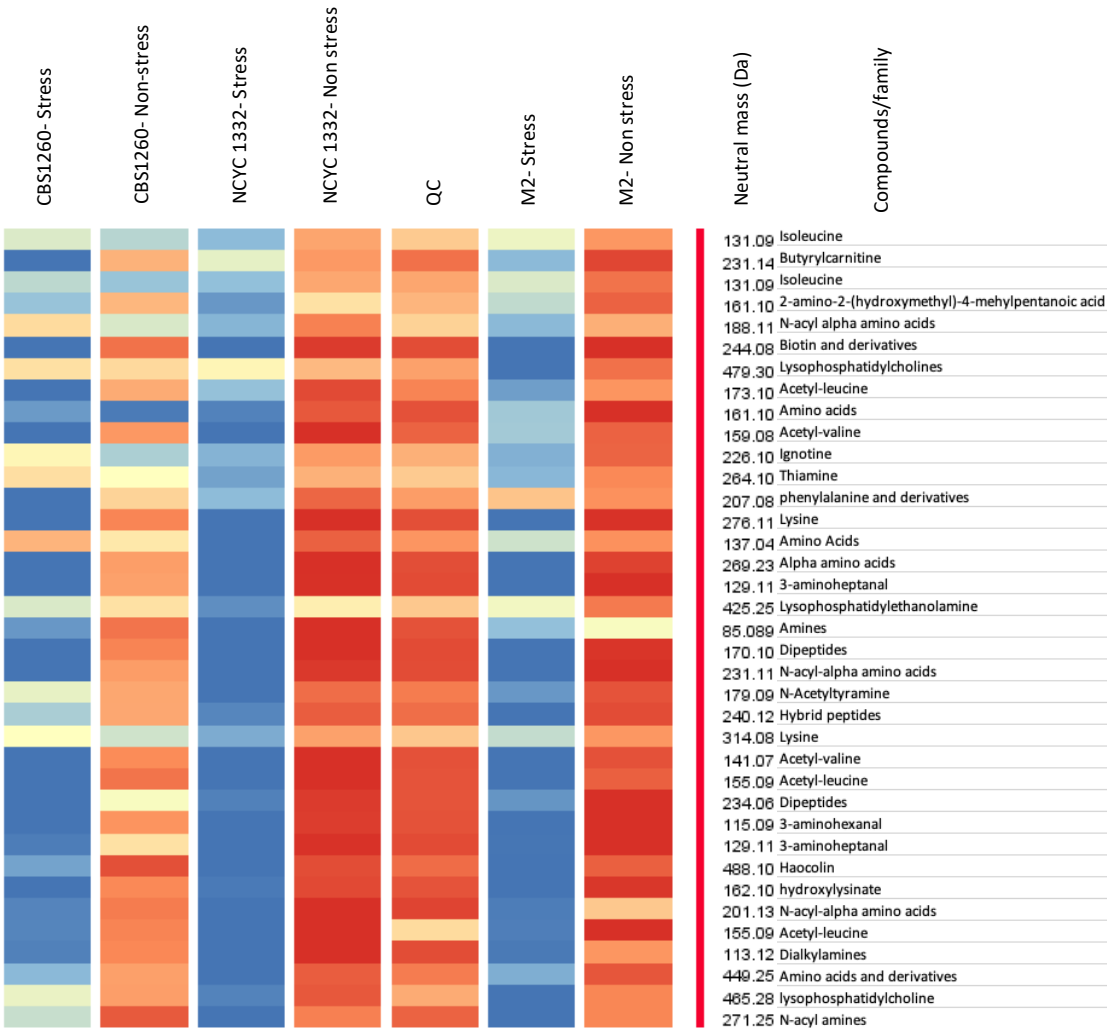

Figure 6.4. Analyte abundance as determined using reverse phase chromatography in positive mode. The heat map presents both the most abundant analytes detected in non-stressed cell populations and those positively identified with a fold change of greater than 15x in non-stressed cell populations. Neutral mass of each analyte is presented in Da and the QC control sample included.

6.3.5 Analysis of compounds down-regulated in response to stress using reverse phase chromatography - negative mode

Analytes of a large fold change (>15x) and of high abundance in all unstressed cell populations, as such downregulated in stressed populations were also isolated using reverse phase chromatography in negative mode and used to generate a heatmap of abundance (Figure 6.5). From this data it was evident that many of these analytes corresponded to compounds that were significantly down-regulated to a similar extent in stressed populations from all three strains. These analytes included those that had a neutral mass of 175.08, 187.12, 189.10 and 254.18 (Da), corresponding to an unidentified alpha-amino acid, 8-amino-7-oxononanoate, an unidentified amino acid and an unidentified linoleic acid. However, the top half of the heatmap demonstrated much greater variation in the abundance of each analyte between each cell population. One example is the analyte with a mass of 173.10 Da, which corresponded to the compound acetyl-DL-leucine, down-regulated in all stressed cell populations, albeit the degree of downregulation varied greatly between strains. Additionally, the analyte with mass 176.10 Da (a methyl branched fatty acid) was a highly abundant compound in all of the unstressed cell populations and down-regulated in stressed cell populations of NCYC 1332 and M2. However, in this instance the normalised abundance was relatively low with no difference in fold-change between the unstressed and stressed population for strain CBS 1260. The data presented in this heat map does suggest that compounds that appear to be present at high concentration in unstressed populations of NCYC 1332 and M2 (signified by the darker red colour) appeared to vary much more than in strain CBS 1260, with the degree of fold change not being uniformly high.

The compounds displayed in the heat map in Figure 6.5, which were down-regulated to a similar degree in stressed conditions in all strains, consisted mainly of amino acids and their derivatives corresponding to the analytes of neutral mass 173.11, 175.08, 189.10, 223.08, 240.12, 376.13, and 404 (Da). This suggests that protein production may be arrested in stressed cell populations, likely as a result of cell entering programmed cell death as suggested previously (Gasch *et al.*, 2000; Chandler *et al.*,

2004), and/or that the stressed cell populations preferentially shifted their metabolism in favour of producing lipids and storage compounds, and for improving cell wall integrity and stress response survival, rather than synthesising or assimilating free amino acids (Pham and Wright, 2008). An alternative theory is that during initial exposure to ethanol stress, many enzymes and proteins involved in the stress response could have been over-expressed, resulting in available free amino acids being utilised immediately (Majara *et al.*, 1996; Chandler *et al.*, 2004). Finally, it is possible that due to changes in membrane integrity as a result of ethanol stress, amino acids could be leaking out of the cell (Salgueiro *et al.*, 1988; Piper, 1995), although further analysis would be required to determine if this was indeed the case.

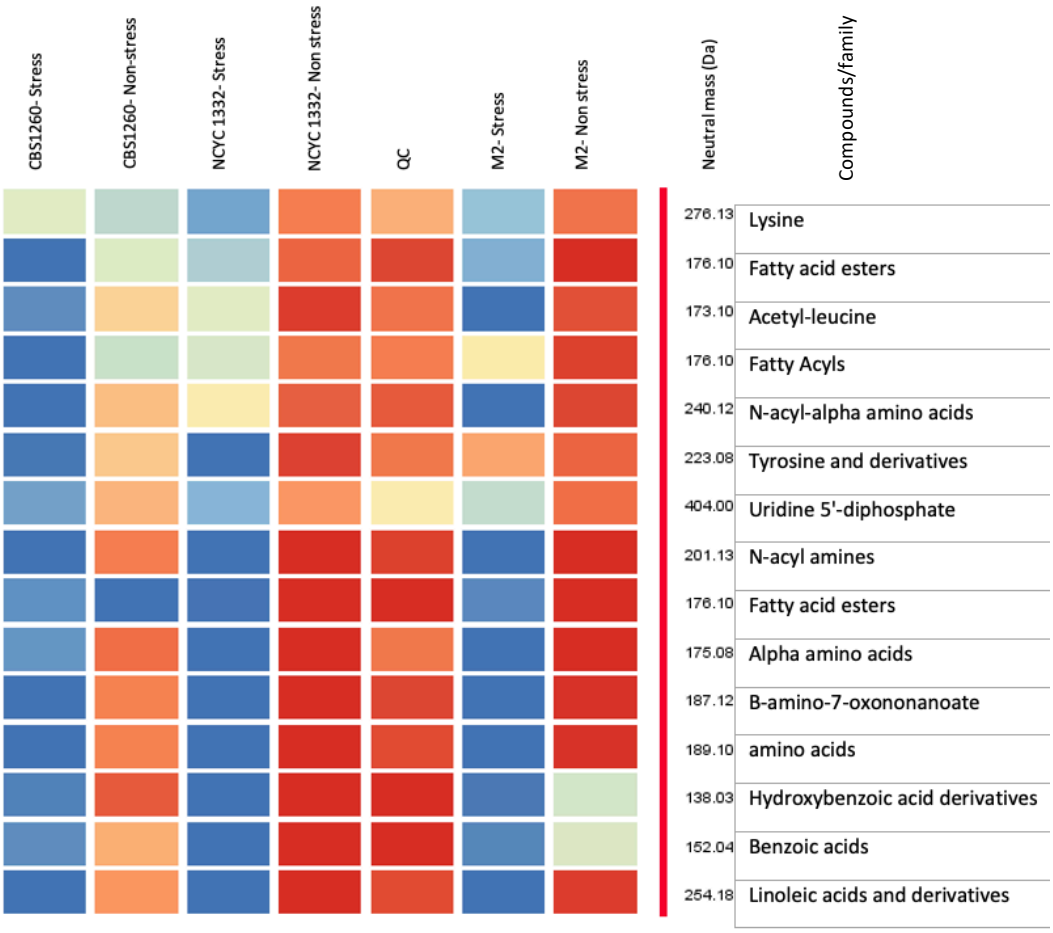

Figure 6.5. Analyte abundance as determined using reverse phase chromatography in negative mode. The heat map presents both the most abundant analytes detected in non-stressed cell populations and those positively identified with a fold change of greater than 15x in non-stressed cell populations. Neutral mass of each analyte is presented in Da and the QC control sample included.

6.3.6 Analysis of compounds down-regulated in response to stress using Hydrophilic Interaction Chromatography

Hydrophilic interaction chromatography (HILIC) was also used to assess the presence of compounds with significant fold change in abundance between stressed and unstressed yeast populations. The obtained heat map shown in Figure 6.6 presents the data obtained for analytes that were both significantly down-regulated (>15x fold change) in stressed cell populations and those that were found in high abundance in all of the unstressed cell populations. From this heatmap it is evident that many of the obtained analytes displayed a similar degree of downregulation in the stressed populations for each strain. This includes the analytes of mass 144.01, 102.03, 466.31, 90.03 and 108.06 Da, which correspond to the compounds 2-hydroxy-4-oxoglutarate, erythrose, cholesterol sulphate, aldose, and cresol. The non-stressed CBS 1260 cell population again appeared to show much greater variation in the abundance of analytes that were more uniform in non-stressed populations of NCYC 1332 and M2. An example of this can be seen in the analytes of mass 148.07, 175.08 and 226.11 (Da), corresponding to mevalonate, an unidentified fatty acid, and carnosine, which were all highly down-regulated in stressed populations of NCYC 1332 and M2, but not for strain CBS 1260. This variation within strains NCYC 1332 and M2 can be observed by the range of colours (dark red in M2 for most analytes) in comparison to the heatmap for unstressed CBS 1260 cells, which remained blue. However, in general most of the analytes obtained from this heat map were uniformly down-regulated in stressed cell populations. In summary, these compounds consisted predominantly of monosaccharides such as aldose, erythrose, 3-deoxytetronate, mevalonate and dextrose, in addition to amino acid derivatives such as malonamate, and carboxylic acids including 2-hydroxy-4-oxoglutarate and succinate. These compounds may be down-regulated as a result of being consumed in order to produce compounds useful in the yeast stress response. For example erythrose may be down-regulated as a result of consumption in the pentose phosphate pathway, which has a role in maintaining redox homeostasis and production of NADPH required for lipid synthesis (Schaaff-Gerstenschläger and Zimmermann, 1993; Juhnke *et al.*, 1996; Fakas, 2017). This matches with a theory that is widely accepted, suggesting increased energy

consumption is common among stressed yeast populations as a result redox homeostasis is imperative (Piper, 1995). In addition, it is known that mevalonate is consumed in order to produce sterols for increased membrane integrity (Caspeta *et al.*, 2014).

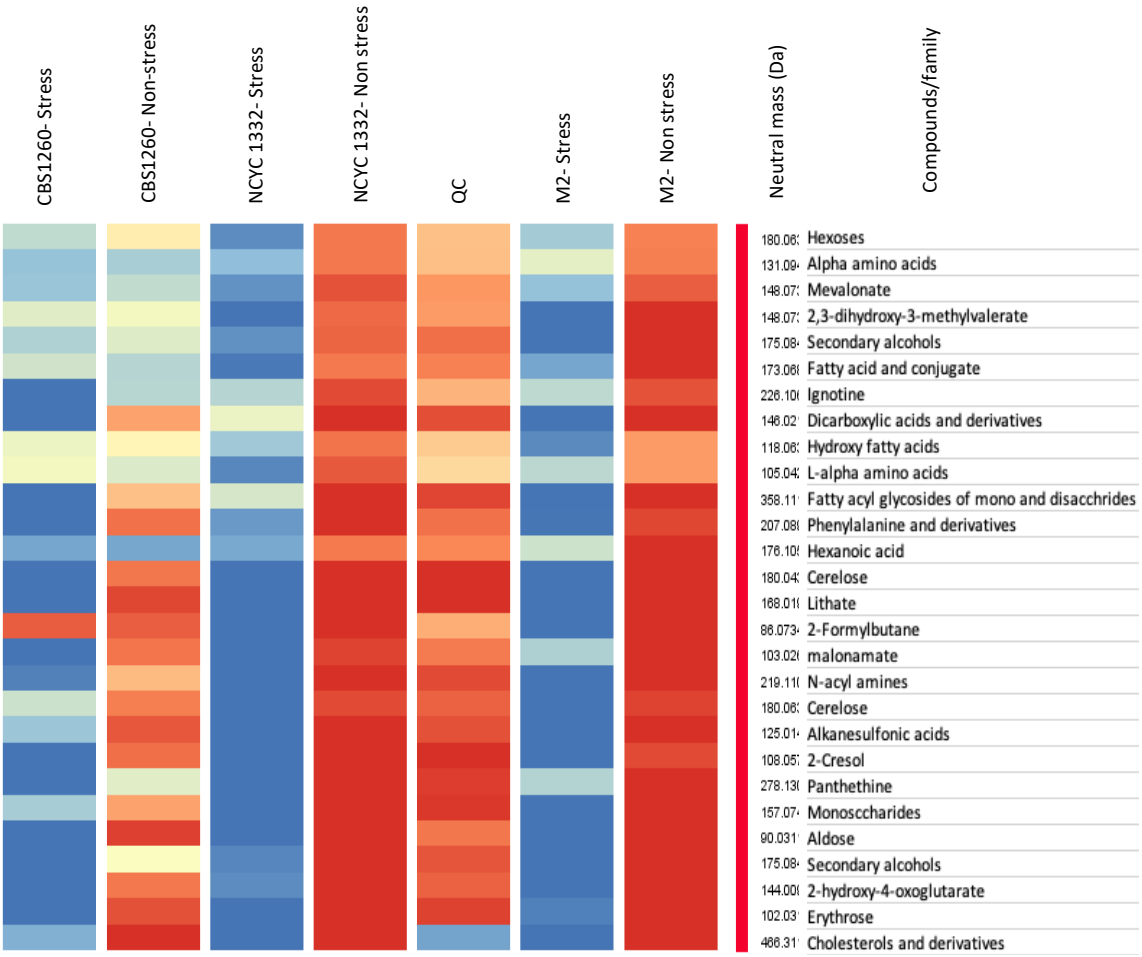

Figure 6.6. Analyte abundance as determined using HILIC chromatography. The heat map presents both the most abundant analytes detected in non-stressed cell populations and those positively identified with a fold change of greater than 15x in non-stressed cell populations. Neutral mass of each analyte is presented in Da and the QC control sample included.

The data presented in this section details the compounds successfully identified in both stressed and unstressed cell populations. Those with significant upregulation (>15x fold change) and those at naturally high abundance within each cell population were selected for analysis. By using reverse phase chromatography, in positive and negative mode, and HILIC, a large number of analytes up-regulated in stressed cell populations were identified. In addition, compounds significantly down regulated in stressed cell populations were also identified. Interestingly, many of the most significantly up-regulated compounds in stressed cell populations consisted mainly of fatty acids, amino acids and their derivatives, and the stress protectants sorbitol, glycogen and trehalose. However, differences in the degree of upregulation of these compounds were identified between the strains, showing variation in stress response. Furthermore, this metabolomic analysis also revealed compounds that were down-regulated in stressed cell populations, which appeared to mainly consist of amino acids and their derivatives, albeit a distinctly different group of amino acids than those identified to be up-regulated. These analytes related specifically to the amino acids leucine, valine, lysine, isoleucine and phenylalanine. As mentioned previously, it may be that these essential amino acids were prioritised in order to produce proteins for responding to stress, in addition to the possibility for amino acid leakage through damaged cell walls (Salgueiro *et al*., 1988; Piper, 1995). This is an interesting phenomenon since a different group of amino acids and their derivatives were found to be upregulated in stressed cells, suggesting that these were in fact not subject to 'leakage'. This may indicate that only certain amino acids are susceptible to leakage, possibly due to their size, polarity or location within the cell. Alternatively, it may be that all amino acids leak to some extent, however the upregulation of production is sufficient enough to negate this in some instances. Irrespective, the analytes and compounds identified in this section give further insight into the yeast stress response, and potential sources of stress response heterogeneity. However, to obtain further information at the molecular level, single cell gene expression was applied in the following sections, focusing predominantly on gene activity responsible for the production of the key compounds identified here.

6.4 Assessment of stress response heterogeneity using single cell RNA sequencing

The data obtained in the previous section demonstrated the presence of significant stress response heterogeneity. Further to this, the metabolomics data obtained indicated that certain intracellular compounds were up-regulated in stressed yeast populations, providing a potential list of genetic sources that could be used to assess the variation in expression of stress related genes for each of the studied strains. It should be noted that only strains CBS 1260 (high heterogeneity) and M2 (low heterogeneity) were used here, for the experimental reasons explained previously (Section 6.2).

6.4.1 Whole population gene expression profiling and cluster analysis

To obtain gene expression data for single cells within a yeast population, cells were lysed and RNA from each cell was barcoded, prior to sequencing as described in Section 2.15.2. This single cell gene expression analysis resulted in data from >50,000 reads per cell for each population. Given that greater than 500 cells were analysed for each set of conditions, this resulted in a large data set that could be used to make comparisons between strains. In all cell populations >99% of unique molecular identifiers (UMIs) produced were valid and a high genome coverage was achieved with around 6000 genes successfully being detected in each cell population. Consequently, a quantifiable measure of gene expression was obtained for each cell, therefore allowing stress induced heterogeneity to be observed.

The total gene expression data for individual unstressed cells belonging to strains CBS 1260 and M2 can be found in Figure 6.7(a-b). From these graphs, each coloured dot represents a cell, and the intensity of the orange/red colour is reflective of the total amount of gene expression (RNA) for each cell based on the total UMI count per cell. A darker red colour therefore portrays a cell with a higher UMI count (total gene expression) than those with a paler colour. As a result, it is evident from visual inspection that heterogeneity in gene expression was present in cell populations for

both strains, due to variation in colour. However, it could be seen that there were potentially more zones of varying expression present in the CBS 1260 population, matching the data from previous Chapters and indicating that this strain was indeed more heterogeneous.

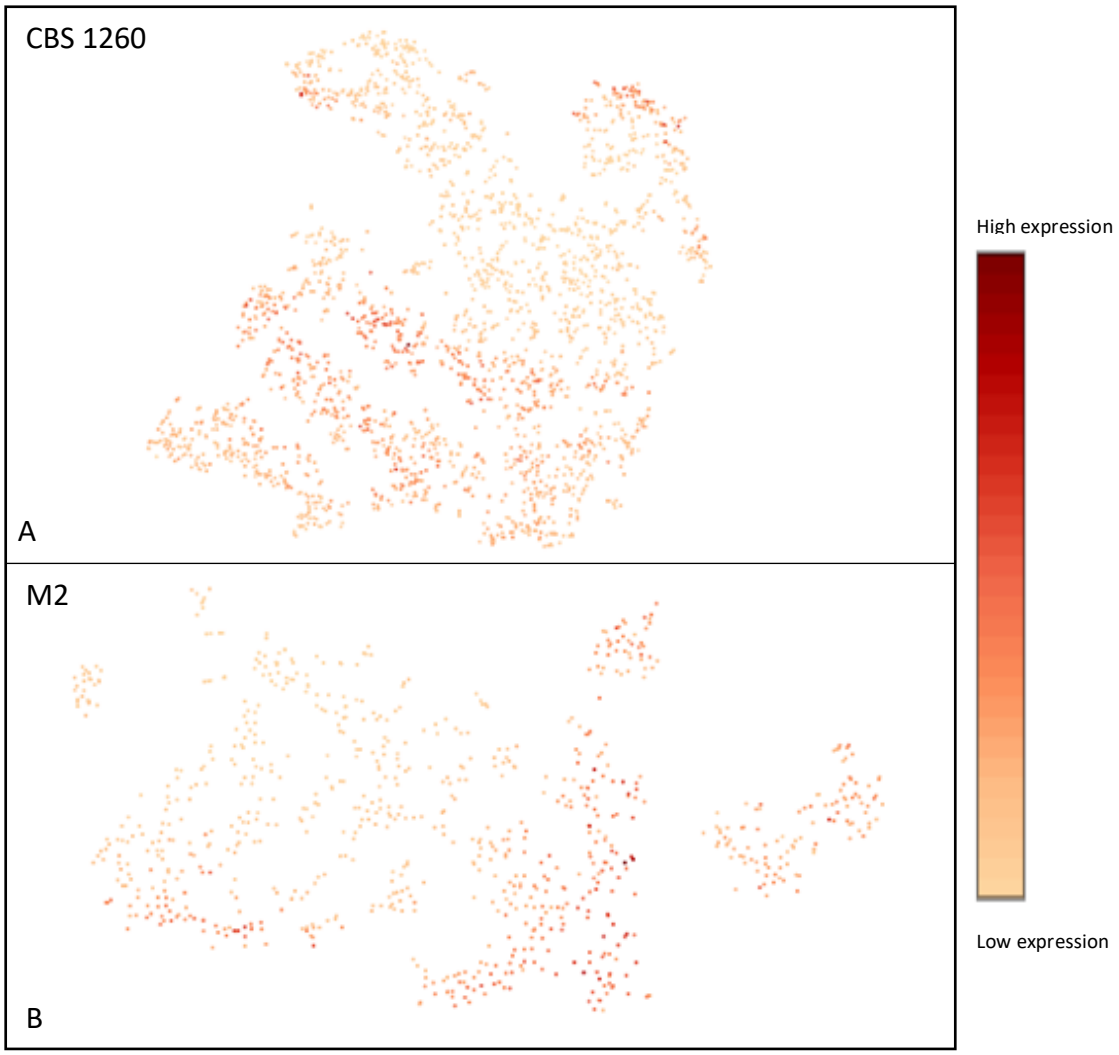

Figure 6.7. Gene expression intensity heat plot for single unstressed cells of CBS 1260 and M2. Each coloured dot represents a cell with the colour intensity representing the total number of UMIs per cell, relating to total gene expression.

The gene expression data for each cell was also pooled, such that cells exhibiting similar gene expression profiles were clustered together. This was performed using the loupe browser analysis software (10x genomics) for cluster analysis (Section 2.15.3). This cluster analysis for the unstressed cell populations of each strain is shown in Figures 6.8a-b, and visually demonstrates the presence of subpopulations of cells within isogenic yeast populations.

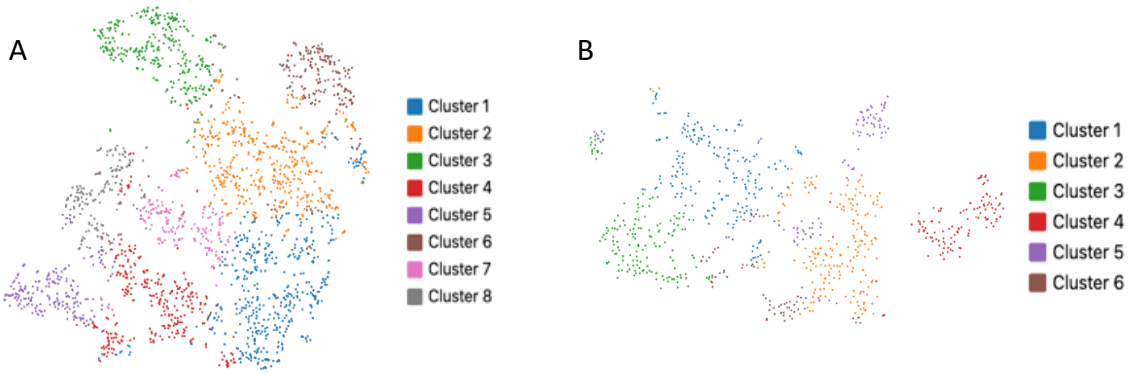

Figure 6.8. Unstressed cell populations clustered based on gene expression similarity. Unstressed CBS 1260 (A) and M2 (B) cell populations with divergent cell populations clustered into regions of similarity. 8 clusters of unique cells were found in CBS 1260 and 6 in M2.

The data obtained shows that cells in the CBS 1260 population were able to be divided into 8 clusters with similar gene expression, while cells from strain M2 were divided into just 6. This therefore demonstrates the presence of more distinctly different sub-populations in unstressed CBS 1260 cells when compared to M2. The total UMI count for each cell within a cluster was then expressed using violin plots, giving an insight into the range and average gene expression within each cluster. These data are presented in Figures 6.9(a-b) for unstressed cell populations of CBS 1260 and M2 respectively. The violin plots show the log2 total gene expression for cells within each cluster, indicating the range of expression, the interquartile range (IQR), and the median and mean expression within the cluster. From these plots, variation from cell to cell within each of the clusters is evident and, furthermore, there was significant

variation between each of the clusters identified. It can be seen that the violin plots obtained using CBS 1260 showed large differences in gene expression between clusters. For example, cluster 2 differed from cluster 6 in both the inter-quartile range, as well as the median and mean data, which showed a much greater range in cell-to-cell gene expression and average cell UMI count. While differences in IQR were present between clusters in the unstressed M2 strain, the variation was less pronounced, with only cluster 1 differing significantly in mean value. These violin plots establish the presence of sub-populations within the main population, based on individuals harboring more similar gene expression profiles. However, evidence also shows that within each cluster there was a large amount of cell-to-cell heterogeneity in total UMI count (gene expression), regardless of the strain. This is particularly emphasized by those which yielded an elongated violin plot. Consequently, it is hypothesized that differences in stress response heterogeneity between the strains may result not only from cell-to-cell variation, but from the formation of distinctly divergent cellular sub-populations.

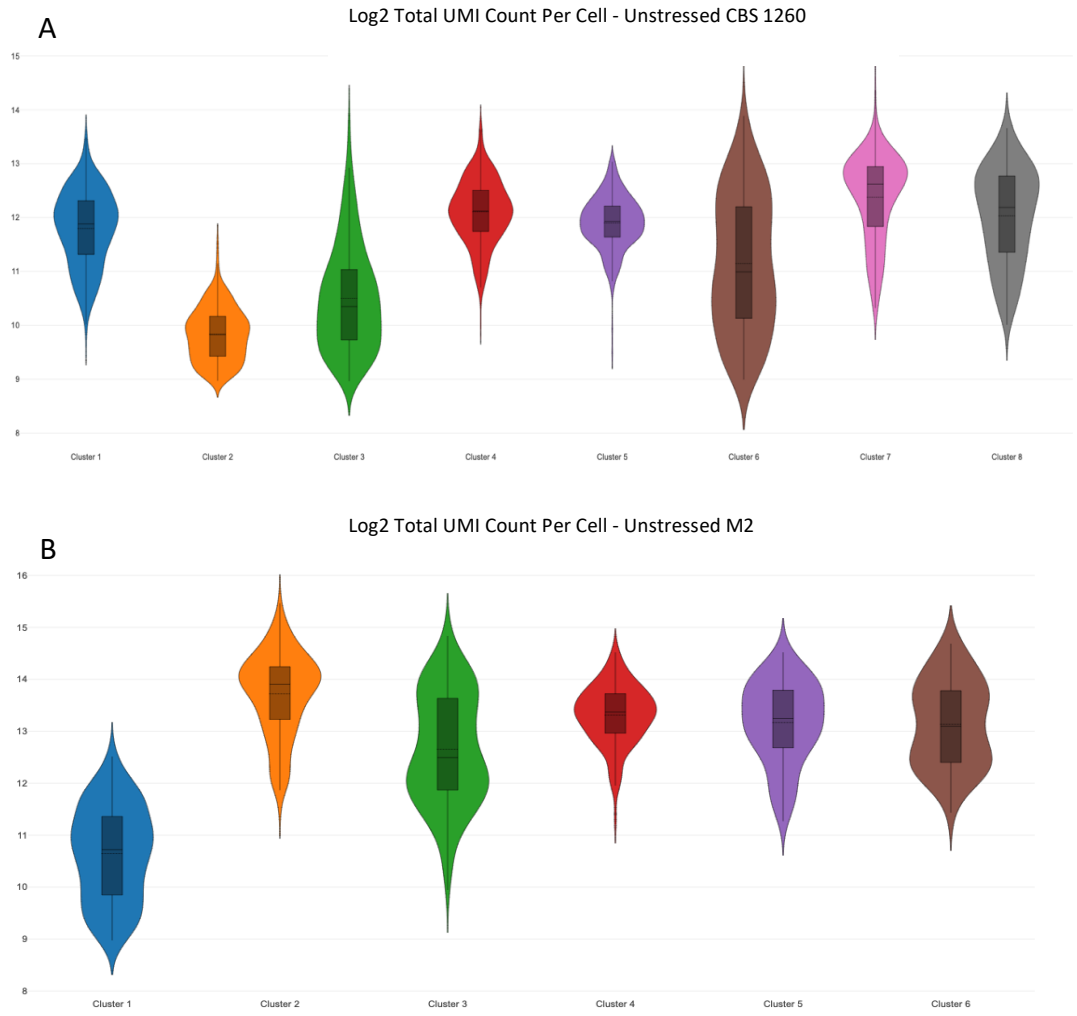

Figure 6.9. Violin plots obtained for the log2 total UMI count per cell within each cell cluster. A: CBS 1260, B: M2. Violin plots display the quartile 1 (Q1), Q3, IQR, median and mean data for each cluster.

6.4.2 Analysis of inter-cluster gene expression variation for unstressed CBS 1260 cells

By investigating the unique expression profiles of cells present in each cluster, greater detail could be obtained, revealing groups of genes that were preferentially up-regulated in cells of certain clusters over others. This analysis was able to highlight the unique gene expression profiles of cells within a cluster. In order to investigate the inter-cluster gene expression variation, heat maps were produced for the log2 fold change in gene expression between each cluster for all significant genes (i.e. genes with an average of more than one count per cell). These heat maps were used to identify genes that grouped together in terms of their expression profiles, and allowed comparisons to be made between clusters.

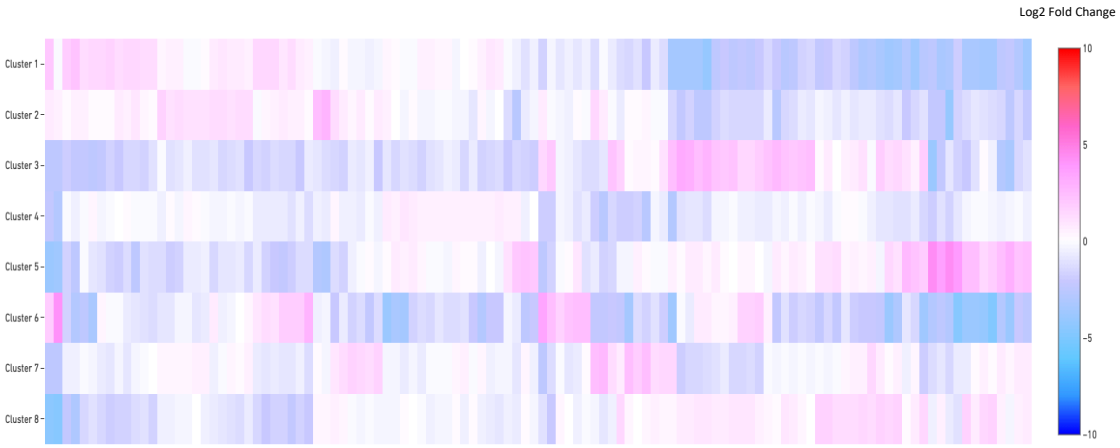

Figure 6.10. Single cell gene expression heat map for cell clusters within the unstressed CBS 1260 population. This heat map presents the log2 fold change for all significantly expressed genes with pink/red bars displaying upregulation and blue downregulation.

An example of this can be seen within the heat map produced for the unstressed CBS 1260 population. This data indicated that there was a large group of genes with similar expression profile in cell cluster 1 that were significantly up-regulated in comparison to the other clusters ($P<0.001$) (Figure 6.10). This large group of genes is evident at the top left of the heat map coloured pink, indicating a high level of gene expression. These genes include *SPG1, HSP30, DDR2* and *GAD1,* all of which are essential for the

yeast stress response (Paulo *et al.*, 2016; Moreno *et al.*, 2019). However, in cluster 1 there was also a large group of down-regulated genes (blue) in comparison to other clusters, including 3 and 5. Interestingly, the group of genes up-regulated in cluster 5 compared to cluster 1 consisted of *ADY2, ADH2, GPP1,* and *HXT2*, which are all essential genes in the ethanol biosynthesis pathway (Rodicio and Heinisch, 2009; Zhang *et al.*, 2017). This group also contained a high number of up-regulated genes involved in amino acid synthesis and cell wall structure. Another highlighted group of interest were the highly expressed genes observed in cluster 3. These consisted largely of genes encoding ribosomal proteins such as *RSP22A, RPL9A, RPL11A, RPS26B, RPL34A, RPL22A* and the cell wall structure related genes *ERG2 and CWP2*. This gene expression cluster analysis demonstrates the presence of significant gene expression heterogeneity among unstressed cells of the strain CBS 1260, providing evidence to suggest that the cells within each cluster exhibit their own unique metabolomic and stress related responses. As a result, sub-populations of cells with varying gene expression profiles and therefore varying physiologies are likely present.

6.4.3 The analysis of inter cluster gene expression variation for unstressed M2 cells

The same analysis described above (Section 6.4.2) was also performed using unstressed cells from the strain M2. This heat map (Figure 6.11) shows clear differences in single cell gene expression within each cell cluster. Cells in cluster 3 contain a high number of up-regulated genes compared to the other clusters, defined by the dark pink group on the heat map. This group of genes contained *HSP30, HSP104, SSA1, SSA2, SSA4, FES1, SIS1* and *BTN2*, all of which are involved in the heat shock protein response, responsible for the translocation, repair and release of misfolded and damaged proteins as a result of stress (Boy-Marcotte *et al.*, 1999). Interestingly this group of genes were down-regulated in all of the other clusters. The up-regulation of these genes in cluster 3, but not the other clusters may demonstrate the presence of a heterogeneous bet hedging strategy, such that a small sub-population of cells possess a heightened innate stress response to deal with any unexpected environmental challenges (Levy, *et al*., 2012). Cell cluster 4 similarly contained a group of up regulated genes, which were mostly down-regulated in the

remaining clusters. This group of genes contained *GPP1* and *GPP2*, responsible for glycerol biosynthesis, in addition to *GRE2* and *GID10*. All of these are stress response genes, in particular known to be up-regulated in response to osmotic stress. Furthermore, this group of cells also showed upregulation in expression of small nuclear and nucleolar RNAs, which are known to be utilised for processing pre-mRNA molecules and for the chemical modification of other RNAs (Tollervey, 1987).

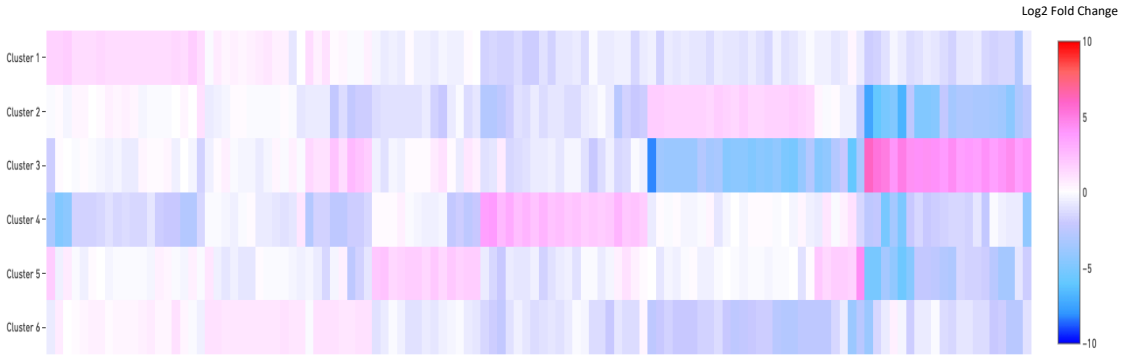

Figure 6.11. Single cell gene expression heat map for cell clusters within the unstressed M2 population. This heat map presents the log2 fold change for all significantly expressed genes with pink/red bars displaying upregulation and blue downregulation.

As expected, this data demonstrated that gene expression varied between cells from both strains. However, further to this, cells could be grouped into clusters of similar gene expression profiles, indicating distinct sub-populations of cells within a main population. Interestingly there is evidence to suggest that each of these sub-populations (clusters) has a unique role within the main population. This is demonstrated by the fact that certain clusters contained cells with up-regulated expression in genes with similar functions, such as those involved in stress response, even when cells were not stressed. This is an example of division of labour where sub-groups of cells have their own specific roles contributing to survival of the population as a whole (Wloch-Salamon *et al*., 2017).

6.5 Gene expression cluster analysis for stressed cell populations

In unstressed cell populations both strains demonstrated the distinct formation of unique clusters of cells containing cells with similar gene expression profiles. From this it was evident that the strain CBS 1260 naturally forms a greater number of sub-populations (clusters) than M2 based on their single cell gene expression. While inter-cluster variation was present for many genes within unstressed cell populations, this was also investigated for stressed cells to determine the impact of stress on gene expression heterogeneity. To generate these populations, strains CBS 1260 and M2 were cultured in wort supplemented with 5% ethanol (v/v), as described in Section 2.15.1. Cells within these populations were then analysed for their single cell gene expression (2.15.3). From this, the same visual presentation for unstressed cells was formulated for cell clusters in stressed cells. The dot plot in Figure 6.12(a-b) presents the cells within each strain population grouped into clusters with each colour representing a unique cluster based on gene expression similarity. These plots demonstrate that stressed cells of the CBS 1260 population (Figure 6.12a) form 5 clusters while M2 cells (Figure 6.12b) form 4. Again, this indicates that the strain CBS 1260 naturally forms a greater number of cell sub-populations containing unique gene expression profiles, irrespective of physiological state. It is interesting to note here that the number of clusters formed for both strains became reduced compared to the unstressed cells, which suggests heterogeneity decreases in response to stress, corroborating the data obtained in Section 5.6. This also supports a parallel investigation of brewing yeast strains from within the same research group (Brindley and Powell, In Preparation). To further assess the stressed cell clusters, violin plots presenting the range in total UMI counts per cell were produced, giving insights into both the range and average gene expression within each cell cluster. Figure 6.13(a-b) shows the Log2 total gene expression for cells within each cluster, indicating the range of expression, the interquartile range (IQR), and the median and mean expression within the cluster.

These plots show that within the CBS 1260 cell population there was significant difference between clusters (Figure 6.13a). For example, in mean gene expression, cluster 4 had a lower mean total UMI count than the other clusters. In addition, the length of the violin plots suggests a greater range in gene expression for cells within cluster 1, than cluster 4. While there were fewer clusters generated for stressed M2 cells (Figure 6.13b), there also appears to be differences between the clusters formed. For example, cluster 1 possessed a much lower mean gene expression and range in gene expression than cluster 2. These plots demonstrate that after exposure to stress both strains appear to generate sub-populations of cells which vary in both mean expression and the range of expression, with CBS 1260 forming a greater number of sub-populations overall. Despite this observation, it is hard to ascertain significant differences in population heterogeneity between the two strains. As a result, the unique expression profiles of cells present in each cluster were investigated in order to provide greater insight, revealing groups of genes that were preferentially up-regulated in cells of certain clusters over others.

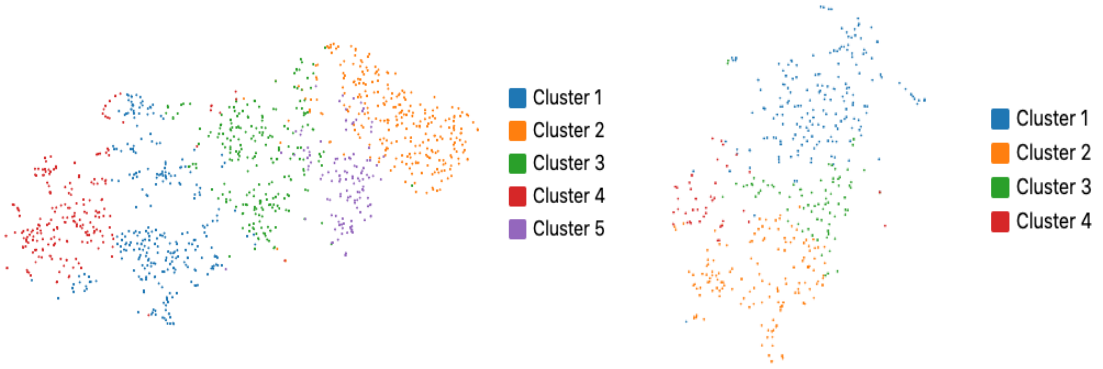

Figure 6.12. Stressed cell populations clustered based on gene expression similarity. Stressed CBS 1260 (A) and M2 (B) cell populations with divergent cell populations clustered into regions of similarity. 5 clusters of unique cells were found in CBS 1260 and 4 in M2.

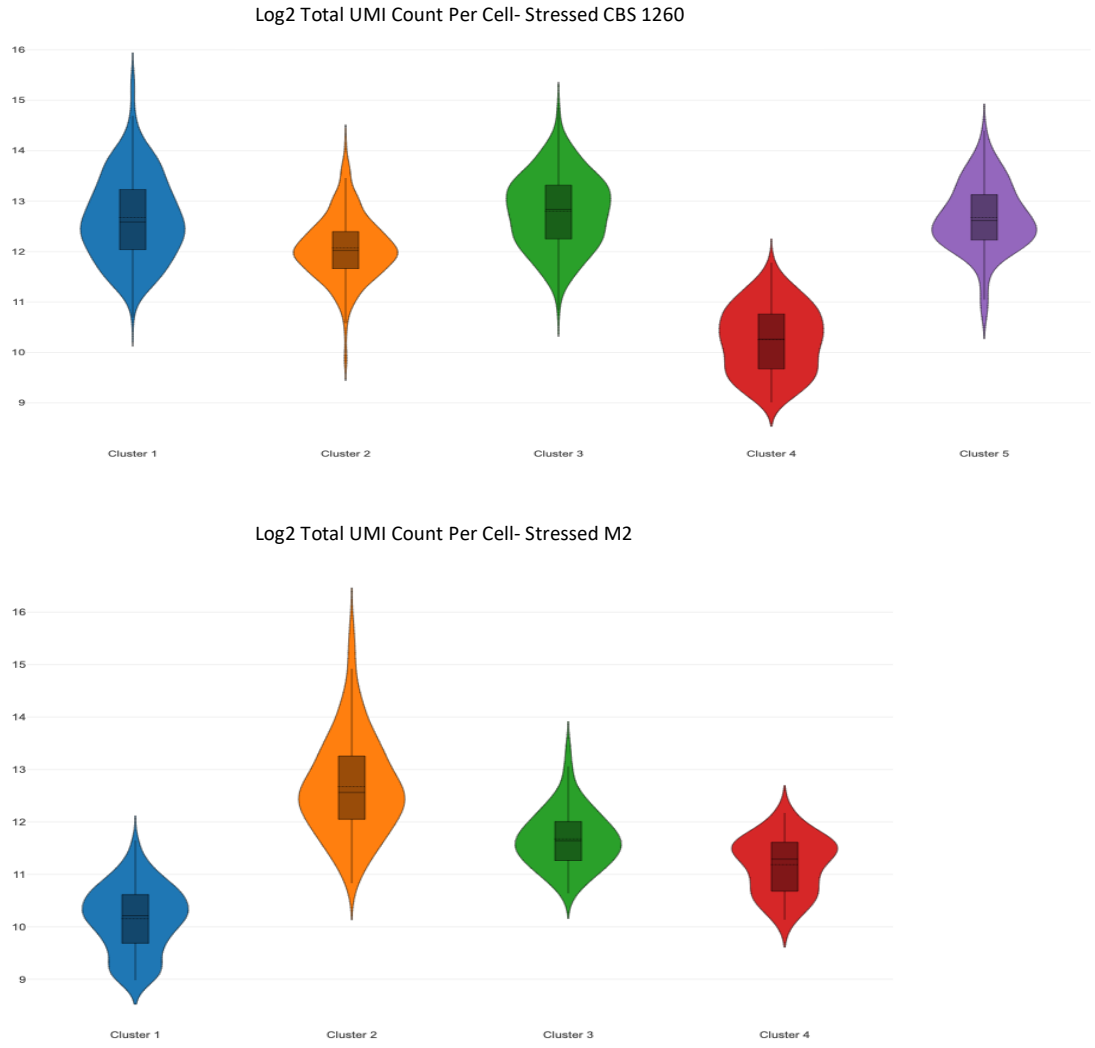

Figure 6.13. Violin plots obtained for the log2 total UMI count per cell within each cell cluster of stressed cell populations (5% v/v ethanol). A: CBS 1260, B: M2. Violin plots display the quartile 1 (Q1), Q3, IQR, median and mean data for each cluster.

6.5.1 Analysis of the inter cluster gene expression variation for stressed CBS 1260 cells

By investigating the unique expression profiles of cells present in each cluster, groups of genes were revealed that were preferentially up-regulated in certain cell clusters over others. In this way, the analysis was able to highlight the unique gene expression profiles of cells within a defined cluster. To portray inter-cluster gene expression variation, heat maps were produced for the log2 fold change in gene expression between each cluster for all significant genes. This was performed in the same way as for unstressed cell populations (Section 6.4), and the data analysed in the same way, based on cluster analysis.

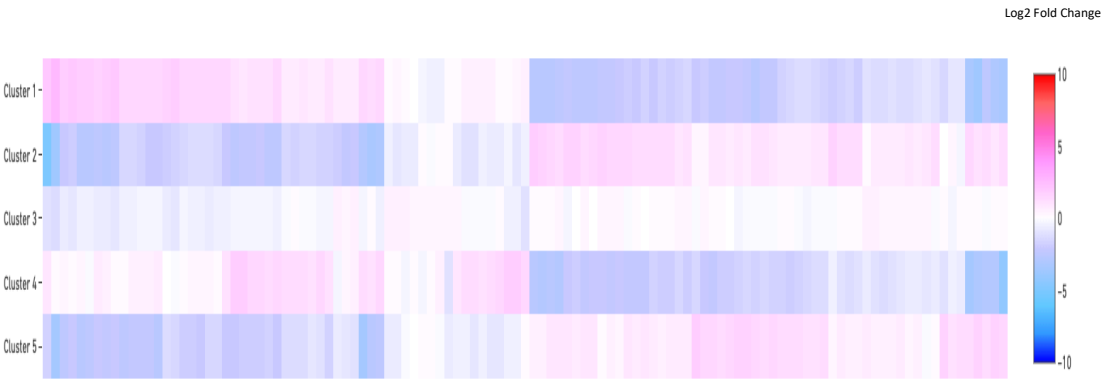

Figure 6.14. Single cell gene expression heat map for cell clusters within the stressed CBS 1260 population. This heat map presents the log2 fold change for all significantly expressed genes with pink/red bars displaying upregulation and blue downregulation.

When comparing gene expression within each cluster, it was evident that cluster 1 contained a large group of genes that were up-regulated compared to cells in the other clusters (Figure 6.14). The most up-regulated genes in cluster 1 were those involved in the ethanol biosynthesis pathway (*ALD2* and *GPP1*), both of which are also known to be up-regulated in response to stress (Pigeau and Inglis, 2005). In addition, the stress responsive gene *GRE2*, and the genes *ENA5, HXT5* and YAP1802 were also significantly up-regulated. Respectively, *ENA5, HXT5* and YAP1802 code for a sodium efflux protein, hexose transporter and a protein involved in clathrin cage assembly,

essential for intracellular trafficking (Verduyckt *et al.*, 2016). Interestingly the group of genes uniquely up-regulated in cluster 3 all encode for ribosomal subunit proteins (*RPS0B, RPL12A, RPS22A, RPS1B, RPL16B* and *RPL30*), an attribute also observed in unstressed CBS 1260 populations. There is evidence to suggest that the over-production of ribosomal proteins results in their secondary function as proliferation and apoptosis regulators (Chen and Ioannou, 1999; Naora and Naora, 1999). As such, there is a possibility that these cells have been programmed for stress induced cell death. Cluster 5 also contains an interesting group of highly expressed genes which were down regulated in cells from the other clusters. These genes consist of two distinct groups: *CIS3, SLR2, SCW10, PSA1, TOS1, PRY2*, which all encode constituents of the yeast cell wall crucial for integrity and optimal fluidity (Verna *et al.*, 1997; Rodríguez-Peña *et al.*, 2010; Heilmann *et al.*, 2013) and a second group: *HTB2, HHF2, HTA2, HTA1, HHF1* and *HTB1*, which encode for histone proteins that are responsible for maintenance and structural integrity of chromosomes, and for the regulation of gene expression (Alejandro-Osorio *et al.*, 2009; Weiner *et al.*, 2015). The upregulation of these histone proteins may be a direct response to stress, since histones are responsible for allowing the activation or repression of transcription in response to exogenous signals, shielding DNA from chemical stress, signaling damaged DNA for repair and aiding in arrangement of DNA repair complexes (Norris and Osley, 1987).

6.6 The analysis of inter cluster gene expression variation for stressed M2 cells

By analysing the gene expression heat map produced for cells of strain M2, grown in the presence of 5% ethanol stress, cell-to-cell variation was again apparent. This is demonstrated by the presence of groups of genes that were up-regulated and down-regulated in some cell clusters, but not in others (Figure 6.15).

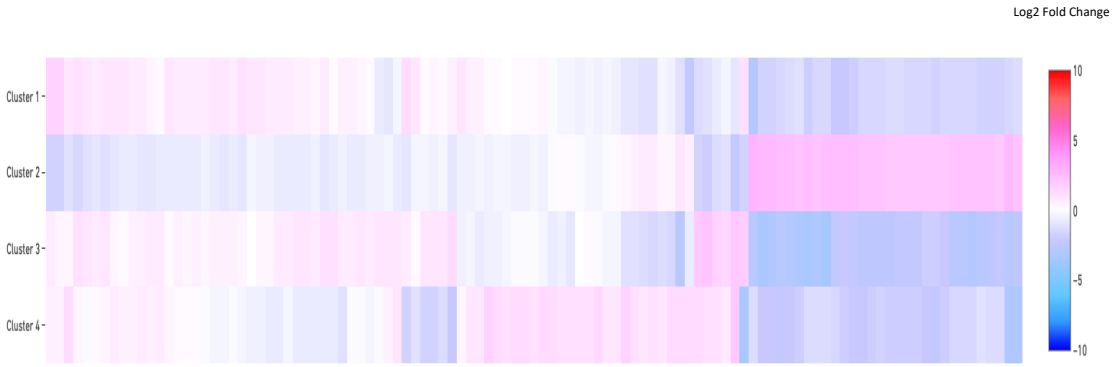

Figure 6.15. Single cell gene expression heat map for cell clusters within the stressed M2 population. This heat map presents the log2 fold change for all significantly expressed genes with pink/red bars displaying upregulation and blue downregulation.

Analysis of this data indicated that there appeared to be fewer unique groups of highly up-regulated inter-cluster gene groups in the M2 cell population, suggesting that a reduced cell-to-cell variation was present compared to stressed cells from CBS 1260. Irrespective, some interesting gene groups were still identified. For example, there was a large group of up-regulated genes in cluster 2 that were down-regulated in the remaining clusters. This group of genes consisted almost entirely of proteins that make up ribosomal subunits, such as *RPL10, ASC1, RPL19B, RPS27B, RPL6A* and *RPL38*. Cluster 4 also contained a group of genes that were up-regulated compared to the other clusters; while their fold-change was not as large as with previous comparisons, the differences were still significant ($P<0.01$). The up-regulated genes in this cell cluster included *ACC1, OLE1, FAS1* and *FAS2*, all of which are components of the fatty acid synthesis pathway. The production of long chain fatty acids and lipid storage is essential for maintaining lipid homeostasis, stores of which can be utilised to generate energy in times of stress and to regulate cell membrane integrity (David *et al*., 1998;

Van Roermund *et al.*, 2003). Furthermore, fatty acids and linoleic acids were both identified as up-regulated compounds in stressed yeast cells through the metabolomic analysis (Section 6.3). There was a small number of genes that were highly expressed in cluster 3 which were down-regulated in the other clusters. The genes in this group were *HHF2, HTA2, HTB, HTB1* and *HTA1*; the same group of histone proteins that were also observed to be up-regulated in sub-population in the stressed CBS 1260 yeast strain.

While this is not an exhaustive analysis on the constituents of each cell cluster, this investigation further demonstrates the presence of sub-populations of cells within all 4 sets of conditions (2 strains in stressed and non-stressed states). In addition, the gene expression profiles of these clusters show that yeast cells preferentially form sub-populations of cells with differing gene expression levels, despite having genetic uniformity. Cell clusters with their own unique gene expression profiles were present in both strains. However, these two yeasts were not genetically identical and are in fact of completely different species, therefore their genetic profiles are likely to be different. To test this hypothesis and the difference between these strains in their genetic stress response, their gene expression profiles were compared directly in both unstressed and stress states.

6.7 An assessment of contrasting gene expression between CBS 1260 and M2 cell populations

While the two strains studied here share many common traits that make them ideal for brewing, the expression of certain genes is likely to differ since they belong to different species. In order to assess the differences in single cell gene expression between the two strains, the average gene expression for all genes of unstressed cells were compared. By determining the genes with the greatest significant fold change in unstressed CBS 1260 cells compared to unstressed M2, an array of genes were discovered that were expressed to a higher degree in one strain than the other, revealing differences in gene expression profile. Furthermore, by analysing the genes that were most abundantly expressed in each of the unstressed cell populations, further variation between the strains could be obtained. The most significantly up-regulated genes by log2 fold change and those that were expressed most abundantly are presented in Table 6.2.

Table 6.2. A comparison in significant gene expression between unstressed CBS 1260 and M2. List of the most up-regulated and most abundantly expressed genes in each of the unstressed CBS 1260 and M2 cell populations.

CBS 1260 Up-regulated- unstressed		**M2 up-regulated- unstressed**	
Log2 fold change	**Gene function**	**Log2 old change**	**Gene Function**
RPL41B	Ribosomal subunit (Berthelot *et al.*, 2004)	EDC2	RNA bind protein (Steiger *et al.*, 2003)
FDH1	Formate dehydrogenase (Sakai *et al.*, 1997)	SPG5	Proteasome assembly (Hanna *et al.*, 2012)
MTH1	Glucose sensing signal transduction pathway (Lakshmanan, Mosley and Özcan, 2003)	SSA4	Heat shock protein (Hasin *et al.*, 2014)
HXT1	Low affinity glucose transporter (Roy *et al.*, 2014)	SPI1	GPI-anchored cell wall protein (Simoes *et al.*, 2006)
HIS4	Histidine biosynthesis (Arndt, Styles and Fink, 1987)	FLO11	Flocculation (Lo and Dranginis, 1996)
RPS30B	Ribosomal subunit (Chen *et al.*, 2021)	HMF1	P14.5 protein family (Oxelmark *et al.*, 2000)
HXT3	Low affinity glucose transporter (Roy *et al.*, 2014)	ICL1	Isocitrate lyase (Menendez *et al.*, 2003)

ARG4	Arginosuccinate lyase (Beacham *et al.*, 1984)	UTR4	Methionine salvage (Wakabayashi *et al.*, 2013)
ADE17	Purine biosynthesis (Gauthier *et al.*, 2008)	ZPS1	GPI-anchored cell wall protein (Frey and Eide, 2012)
PST1	Cell wall protein (Pardo *et al.*, 2004)	SBH2	Protein translocation into EPR (Vilardi *et al.*, 2014)
High Abundance	**Gene function**	**High Abundance**	**Gene function**
TDH3	Glyceraldehyde-3- phosphate dehydrogenase (McAlister and Holland, 1985)	TIP1	Cell wall mannoprotein (Kapteyn, Van Den Ende and Klis, 1999)
FBA1	Fructose 1,6-bisphosphate aldolase (Cieśla *et al.*, 2014)	HYP2	Translation elongation factor (Barba-Aliaga *et al.*, 2020)
CCW12	Cell wall mannoprotein (Narang *et al.*, 2008)	AHP1	Thiol specific peroxiredoxin (Trivelli *et al.*, 2003)
EGO4	Unknown	SOD1	Copper-zinc superoxide dismutase (Montllor-Albalate *et al.*, 2022)
ENO1	Enolase I- glycolysis (Brindle *et al.*, 1990)	TMA10	Unknown
TDH1	Glyceraldehyde-3- phosphate dehydrogenase (Delgado *et al.*, 2001)	HSP82	Heat shock protein (Erkine *et al.*, 1999)
RGI1	Protein involved in energy metabolism (Du *et al.*, 2020)	ALD6	Cytosolic aldehyde dehydrogenase (Meaden *et al.*, 1997)
HXT7	High affinity glucose transporter (Kasahara and Kasahara, 2010)	NCE103	Carbonic anhydrase (Götz *et al.,* 1999)
ADH1	Alcohol dehydrogenase (Pal *et al.*, 2009)	TSA1	Thiol specific peroxiredoxin (Shin *et al.*, 2005)
HOR7	Unknown	MAL32	Maltase (Dietvorst *et al.*, 2007)

By means of a summary, from this data it was evident that genes encoding proteins involved in glycolysis and alcoholic fermentation, amino acid synthesis, and cell wall production were all preferentially up-regulated in the unstressed CBS 1260 population compared to M2. It should be emphasized that this does not mean these genes are not expressed in the M2 population, but that they are expressed to a lower degree. Conversely, up-regulated genes observed in the M2 population (both by fold change and abundance) were more wide ranging, but primarily those involved in encoding proteins linked to the stress response (*SSA4, HSP82, AHP1* and *TSA1*), flocculation (*FLO11*) and production of cell wall constituents (*SPI1, TIP1*). This analysis was also performed on stressed populations to identify up-regulated genes across both populations by abundance and fold change (Table 6.3).

Table 6.3. A comparison in significant gene expression between stressed CBS 1260 and M2. List of the most up-regulated and most abundantly expressed genes in each of the stressed CBS 1260 and M2 cell populations

CBS 1260 Up-regulated- stressed		**M2 up-regulated- stressed**	
Log2 fold change	**Gene function**	**Log2 fold change**	**Gene Function**
FIT2	Cell wall mannoprotein (Protchenko *et al.*, 2001)	EDC2	RNA binding protein (Steiger *et al.*, 2003)
RPL41B	Ribosomal subunit (Berthelot *et al.*, 2004)	SPG5	Proteasome assembly (Hanna *et al.*, 2012)
MTH1	Glucose sensing signal transduction pathway (Lakshmanan *et al.,* 2003)	FLO5	Flocculation (Di Gianvito *et al.*, 2018)
GAL7	Galactose-1-phosphate uridyl transferase (Fridovich-Keil and Jinks-Robertson, 1993)	SPI1	Cell wall protein (Simoes *et al.*, 2006)
GPP1	Glycerol-3-phosphate phosphatase (Påhlman *et al.*, 2001)	ECM4	Glutathione transferase (Schwartz *et al.*, 2016)
ALD2	Cytoplasmic aldehyde dehydrogenase (Aranda and del Olmo, 2003)	TIR1	Cell wall mannoprotein (Kapteyn *et al.,* 1999)
GRE2	3-methylbutanal reductase (Guo *et al.*, 2014)	DDR48	DNA damage response protein (Treger and McEntee, 1990)
HXT3	Low affinity glucose transporter (Roy *et al.*, 2014)	PHD1	Pseudohyphal growth enhancer (Gimeno and Fink, 1994)
HCS1	Hexameric DNA polymerase (Biswas *et al.,* 2001)	GTT1	Glutathione S-transferase (Collinson and Grant, 2003)
PRM10	Pheromone- regulated protein (Heiman and Walter, 2000)	FLO11	Flocculation (Lo and Dranginis, 1996)
High abundance	**Gene function**	**High abundance**	**Gene function**
HSP12	Heat shock protein (Motshwene *et al.*, 2004)	21S_rrna	rRNA (Zinn and Butow, 1985)
PDC1	Pyruvate decarboxylase (Hohmann and Cederberg, 1990)	TIP1	Cell wall mannoprotein (Kapteyn *et al.,* 1999)
CCW12	Cell wall mannoprotein (Narang *et al.*, 2008)	ZPS1	GPI-anchored protein (Frey and Eide, 2012)
ENO1	Enolase I- glycolysis (Brindle *et al.*, 1990)	15s_RRNA	rRNA (Biswas and Getz, 1999)
TDH1	Glyceraldehyde-3- phosphate dehydrogenase (Delgado *et al.*, 2001)	HYP2	Translation elongation factor (Barba-Aliaga *et al.*, 2020)
TDH3	Glyceraldehyde-3- phosphate dehydrogenase (McAlister and Holland, 1985)	OLE1	Delta fatty acid desaturase (Stukey *et al.,* 1990)
CWP2	Cell wall mannoprotein (Li *et al.*, 2020)	NCE103	Carbonic anhydrase (Götz *et al.,* 1999)
CPR1	Cyclophilin (Kim *et al.*, 2010)	TMA10	Unknown

AHP1	Thiol- specific peroxiredoxin (Trivelli *et al.*, 2003)	SPI1	GPI-anchored protein (Simoes *et al.*, 2006)
GPM1	Phosphoglycerate mutase glycolysis (Heinisch *et al.,* 1991)	MTC7	Unknown

From the data displayed in Table 6.3, there was evidence to suggest that up-regulated and abundantly expressed genes in stressed CBS 1260 populations were again mainly those associated with glucose metabolism and glycolysis (*MTH1, GPP1, HXT3, ENO1, GPM1, PDC1* and *TDH1,3*). While many of these genes were common in both unstressed and stressed cell populations, there were slight differences with the focus on glycolysis appearing to be greater in response to stress. Similarly, there was an increase in up-regulated proteins directly involved in the yeast stress response (*GRE2* and *AHP10* and cell wall integrity). Interestingly, the most significantly up-regulated and abundant genes in stressed M2 populations comprised mainly cell wall proteins such as *SPI1, TIR1* and *TIP1*, the flocculins (*FLO5* and *FLO11*) and genes required for the production of the stress protectant glutathione (*ECM4* and *GTT1*). Despite these genes products being well linked to the stress response in yeast, the average cell expression of these genes was at a very low level in stressed CBS 1260 cells. For example, *ECM4* had an average gene expression of 0.04 compared to 5 in stressed M2 cells. Conversely, the stress response gene *GRE2*, up-regulated in CBS 1260 cells, had an average expression of 6.28, while in the M2 population expression was low (0.42). This demonstrates that the stress response in these two yeast strains is not identical, with each of them having a uniquely tailored approach to ethanol stress. This analysis demonstrated that in both stressed and unstressed cell populations, the strains showed very different gene expression profiles. Furthermore, it is interesting to note that this variation manifests itself at the cellular level, for example, both stressed populations showed an increased expression in cell wall mannoprotein synthesis genes, but the precise genes involved were different in each case.

By comparing the genes upregulated under each condition between the strains, differences in highly expressed genes could also be observed as described above.

There were however also some commonalities; genes such as *HSP12*, encoding the stress responsive plasma membrane protein (Praekelt and Meacock, 1990), were highly expressed in both stressed cell populations. An average cell expression of 85 was seen for strain CBS 1260 and 29 for M2, representing an increase from 16.6 and 4.2 respectively in unstressed cell populations. While the evidence suggests that stressed CBS 1260 cells express *HSP12* to a greater extent, it is important to note that the fold change in comparison to their unstressed counterparts is similar. This indicates that the up-regulation of the gene is a common response, irrespective of the final level of expression. This trend was also observed in genes such as *CCW12* (cell wall mannoprotein) and *TDH1* (glyeraldehyde-3-phosphate dehydrogenase).

The data presented here demonstrates both commonality and contrasting effects in gene expression up-regulation in stressed cell populations. For example, it would be expected that up-regulation would be similar in key physiological aspects such as cell wall integrity, the general stress response and glycolysis. Finally, while increases in gene expression on a population wide level give useful data for presenting key stress related genes of interest, analyzing the extent of gene expression from cell-to-cell may give greater insight into the presence of gene expression heterogeneity.

6.8 The investigation of gene expression relating to compounds identified using Liquid Chromatography- Mass Spectrometry

The previous sections demonstrated large differences in gene expression profiles between the two strains in normal growth conditions, but more crucially in their response to ethanol stress. This data suggests that while the aim of each cell population may be to survive an imposed stress, their mechanism to achieve this can vary significantly. In Section 6.3, a group of compounds upregulated in stressed cells was identified using various modes of HPLC. To investigate these further, the most common groups of compounds upregulated in response to stress (identified in Section 6.3) were scrutinised for their potential genetic origins and any differences/similarities between the strains evaluated. This was performed in order to ascertain the source of

upregulation of these compounds, from which the identified genes could later be assessed for the impact on cell-to-cell heterogeneity.

6.8.1 Genes related to up-regulated analytes in response to ethanol stress

The group of compounds identified in reverse phase, positive mode chromatography that were up-regulated in stressed cell populations consisted of fatty acids and linoleic acids. As a result, genes involved in the fatty acids biosynthesis pathway were investigated for their single cell gene expression profiles. The genes *FAA2, OLE1, PXA2, TGL5, ELO1, ACC1, ELO2, FAS1, PXA1, ARE2* and *FAA4* were all significantly up-regulated in stressed CBS 1260 populations compared to unstressed ($P<0.001$). Similarly, the genes *FAA3, PXA2, FAS2, ARE1, ACC1, YEH2, FAA2, FAA4, FAA1* and *FAS1* were all significantly upregulated in stressed M2 cell populations ($P<0.001$). This again shows that the two strains have differing gene expression profiles while achieving the same outcome of increased fatty acid synthesis. In reverse phase negative mode, the sugars sorbitol, glycogen and trehalose were all identified to be up-regulated in stressed cell populations, confirmed based on the gene expression data obtained. The trehalose 6-phosphate synthase genes *TSL1* and *TPS2*, in addition to *GLC3* (glycogen branching enzyme), *GPH1* (glycogen phosphorylase) and *GSY1* (glycogen synthase) were significantly up-regulated in both CBS 1260 and M2 stressed cell populations. Further to this the genes *HXT13, HXT16* and *HXT17* were all significantly up-regulated in stressed CBS 1260 cells. These genes encode for sorbitol transporters, responsible for the uptake and growth on sorbitol (Jordan *et al.*, 2016). While glycerol functions as a better osmo-protectant, sugars such as sorbitol can also aid in the water balance in stressed cells (Shen *et al.*, 1999). The up-regulation in expression of these genes was not observed in the strain M2, where the average cell-to-cell expression levels were too low to be included in the analysis. However, the gene encoding for aldose reductase (*GRE3*), an enzyme that converts glucose to sorbitol (Konishi *et al.*, 2015) was significantly up-regulated in stressed population of both strains. In HILIC negative mode, fatty acids were again the main group of compounds up-regulated in stressed cell populations, which was also corroborated by the fatty acid synthesis gene expression data, with increased expression in both stressed cell populations.

6.8.2 Genes related to down-regulated analytes

The main group of compounds found to be down-regulated in stressed yeast populations detected in both positive and negative mode of reverse phase chromatography, consisted of amino acids and their derivatives such as acetyl-valine, acetyl-leucine, carnitine, lysine, isoleucine and thiamine. As such it was not surprising that the genes *ILV6* (BCAA synthesis), *ILV5* (BCAA synthesis), *BAT2* (BCAA aminotransferase), *ARO3* (aromatic amino acid biosynthesis) and *GCN4* (transcriptional activator of amino acid biosynthesis genes) were all significantly down-regulated in stressed CBS 1260 cells. Furthermore, the genes *BAT2, YAT1* (carnitine acetyltransferase), *CHA1* (hydroxy amino acid catabolism) and *PYC1* (pyruvate carboxylase) were all down-regulated in stressed M2 cell populations. While these two gene groups were different between the two strains, their general purpose of amino acid biosynthesis correlates. The differences are likely to be a result of the two strains genetic composition, resulting in the adoption of different strategies to achieve a similar outcome. In HILIC mode, among other amino acid derivatives, a group of monosaccharides including aldose, dextrose and erythrose were all found to be less abundant in stressed cell populations. This coincides with the data obtained from the single cell RNA sequencing that demonstrated a significant increase in genes involved in glycolysis such as *PDC1,5,6 ADH4, GPP1* and *ENO1*, in addition to sugar metabolism genes such as *GRE3* in stressed populations. It is likely that an increase in gene expression results in the utilization of sugar more readily, a trait often seen when stress is imposed, ultimately leading to a reduction in available sugars internally (Pan *et al*., 2019).

6.9 Identification of up-regulated genes in CBS 1260 cells in response to ethanol stress

In the previous sections it was demonstrated that gene expression alters when ethanol stress is induced, however the increase in expression of stress related genes was not the same between the two strains studies. Furthermore, the data showed the presence of divergent sub-populations with differing gene expression profiles in each strain under both stressed and un-stressed conditions. However, the presence of sub-populations with unique gene expression profiles appeared to be greater in the strain CBS 1260 than in M2. While the cluster analysis performed was useful for identifying the presence of sub-populations, more detail regarding cell-to-cell heterogeneity could be gained by analysing the expression of individual genes. In order to determine the genetic source of stress related heterogeneity, differences in gene expression between unstressed and stressed cell populations of each strain were investigated further. This firstly served as a method of detecting gene expression changes after stressed was imposed, but also attempted to reveal potential targets for investigating sources of heterogeneity in each cell population.

Firstly, a list of stress related gene targets was procured. To achieve this, stressed and non-stressed populations of CBS 1260 were compared by analysing the single cell gene expression of each population. The average gene expression (RNA content) for each gene over the cell populations were evaluated and from this, the top 50 most up-regulated genes (by log2 fold change) in the stressed cell populations were identified. It should be noted that genes with a low average count were not included, as a result only significant up-regulation was detected. The list in Table 6.4 presents the 50 most up-regulated genes in stressed CBS 1260 cells based on average single cell gene expression across the population.

Table 6.4. Top 50 most up-regulated genes (log2 fold change) in stressed CBS 1260 cells

Gene	CBS 1260_stressed Log2 Fold Change	Gene	CBS 1260_stressed Log2 Fold Change
GRE1	6.448576107	PUN1	2.902167285
ZRT1	6.070332002	RCR1	2.889835743
ZPS1	5.58008642	TIP1	2.839244373
TKL2	5.504527877	NCE103	2.83732687
PDC6	5.206146372	PDC5	2.810681357
RCK1	4.983509495	CTT1	2.800192245
NRG2	4.860068899	GND2	2.743542487
GID10	4.697301398	ALD6	2.739763441
ALD3	4.611632741	LEU2	2.72133633
ADH4	4.574742689	HBN1	2.702968182
NQM1	4.53017378	YHR033W	2.632313199
FIT2	4.518887383	MPC3	2.618568309
PNS1	4.461749963	HXT5	2.606963867
YHR210C	4.251178447	IMA1	2.518896423
GRE2	3.923247362	ALD2	2.502724606
ADH6	3.570351729	HXT2	2.475915382
YGR067C	3.562112552	ALD4	2.455728397
XBP1	3.387933731	SRF1	2.452802944
GPP1	3.364888762	YOR062C	2.450764462
SPG4	3.356466111	LAP3	2.450120674
ENA5	3.328666643	ERG3	2.447713199
PRY1	3.241656785	YDL124W	2.435093327
YBR285W	3.120803159	FMP48	2.426273313
PHM7	3.111371538	MSC1	2.326693949
CWP2	2.971234768	ZRT3	2.217145442

From this list, the most up-regulated gene in the stressed cell population was *GRE1*, a stress induced hydrophilin primarily known for its role in the desiccation-rehydration response (Dang and Hincha, 2011). The log2 fold change of this gene in stressed cells was 6.45 compared to unstressed cells. As a visual example this is represented in Figure 6.16(a-b), which presents the gene expression of *GRE1* for each cell in both unstressed and stressed conditions with the darker red colour depicting a greater degree of expression. In addition the second most up-regulated gene was *ZRT1*, a

membrane zinc transporter responsible for zinc uptake (Zhao and Eide, 1996), with a log2 fold change of 6.07, is also displayed (Figure 6.16c-d). While the culture conditions were not low in zinc, it is likely that the increase of this gene is due to the requirement for available zinc in stress response proteins such as Msn2/Msn4 which bind STRE elements in essential stress response genes, allowing their transcription (Watanabe *et al.*, 2011). In the unstressed conditions the average ZRT1 gene expression per cell was 0.17 (UMI count) and this increased significantly ($p<0.001$) to 11.49 in stressed cells (Figure 6.16 c-d).

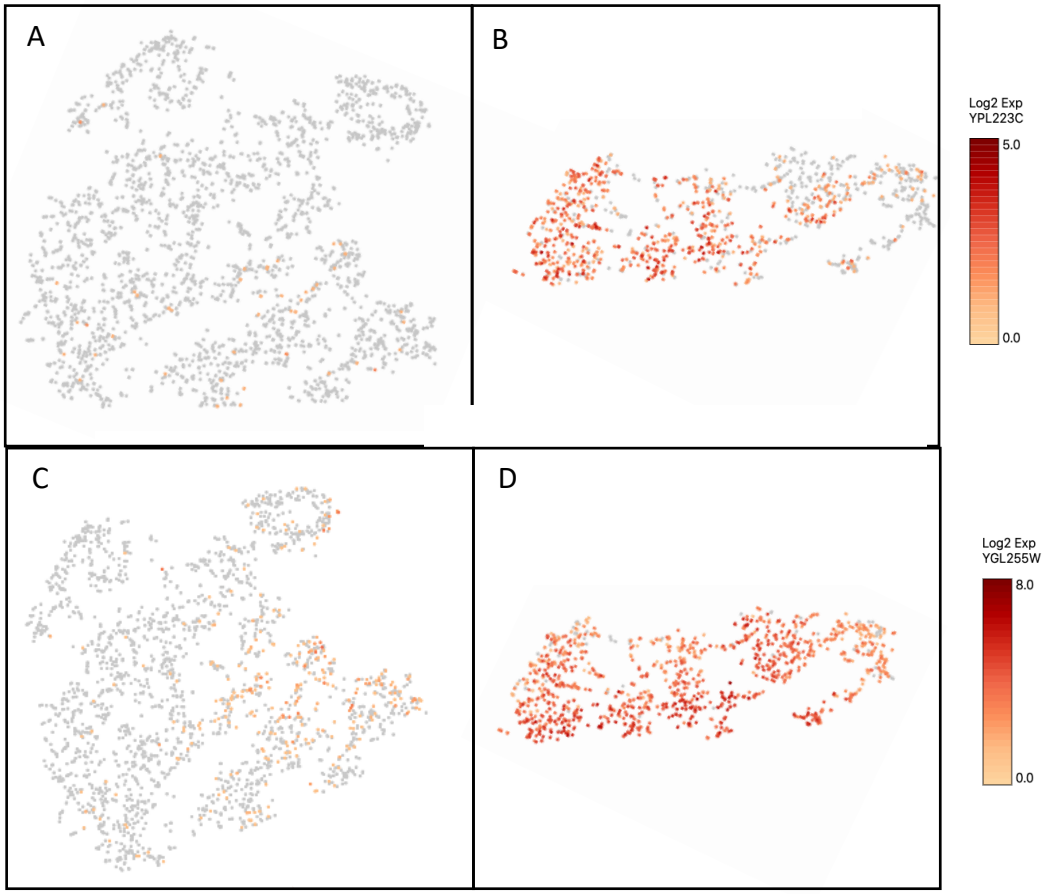

Figure 6.16. Gene expression intensity heat plot for the highest fold change genes in stressed CBS 1260. A: YPL233C (GRE1) gene expression intensity for unstressed CBS 1260 cells. B: YPL233C (GRE1) gene expression intensity for stressed CBS 1260 cells. C: YGL255W (ZRT1) gene expression intensity for unstressed CBS 1260 cells. D: YGL255W (ZRT1) gene expression intensity for stressed CBS 1260 cells. Each coloured dot represents a cell with the colour intensity representing the total number of UMIs per cell (log2 expression).

Many of the remaining genes in the 50 most up-regulated in stressed conditions (Table 6.4), were (as expected), stress response genes such as *RCK1* (protein kinase), *GRE2* (3-methylbutanal reductase), *SPG4* (high temperature survival protein), *GID10* (protein degradation), *CTT1* (cytosolic catalase T) and *ALD2* and *ALD3* (cytoplasmic aldehyde dehydrogenases). The other major groups of genes were those involved in cell wall integrity, including *FIT2* (mannoprotein), *CWP2* (mannoprotein), *RCR1* (plasma membrane ubiquitin ligase-substrate adapter), *TIP1* (mannoprotein) and *PUN1* (plasma membrane protein), and those involved in the PPP/glycolysis/fermentation pathways, including *TKL2* (transketolase), *PDC6* (pyruvate decarboxylase), *ADH4* (alcohol dehydrogenase), *GPP1* (glycerol-3-phosphate phosphatase) and *PDC5* (pyruvate decarboxylase). All of these therefore reflect processes that are essential in the yeast stress response and in defining the fitness of a cell.

In contrast, when investigating the 50 most down-regulated (data not shown) genes in the stressed CBS 1260 cell population, gene functionality was much more varied. These genes are those involved in functions such as copper transport (*CTR1*), amino acid synthesis (*DIP5*), mitochondrial function (*GCV2*), glucose transport (*HXT7*), and ethanol production (*ADH5*). This suggests that these functions were either not prioritized during stress induction, or may be down-regulated in stressed populations, perhaps due to DNA damage (Alexandre *et al.*, 2001; Voordeckers *et al.*, 2020). This has been shown to occur with mitochondrial function in response to ethanol stress, where irreversible damage can lead to the repression of mitochondrial genes. Furthermore the repression of these genes can lead to loss of mitochondrial function all together, leading to an increase in presence of petite mutations (Contamine and Picard, 2000).

6.10 Identification of up-regulated genes in M2 cells in response to ethanol stress

As for CBS 1260, the same analysis was performed using stressed M2 cell populations. The 50 most up-regulated (log2 fold change) genes identified in the stressed population, compared to the unstressed control population are presented in Table 6.5.

Table 6.5. Top 50 most up-regulated genes (log2 fold change) in stressed M2 cells

Gene	M2_stressed Log2 Fold Change	Gene	M2_stressed Log2 Fold Change
VEL1	9.54204702	YPL113C	2.70839726
TIS11	6.84015077	EXG1	2.69699365
LSO1	6.44466607	ILV5	2.61708183
SIT1	5.66271465	YER121W	2.59469098
FET3	5.31020085	ZAP1	2.57737629
FRE4	4.91715384	GTT1	2.54840775
RTC4	4.73712949	HSP12	2.52107403
FET4	4.11669999	INO1	2.49330668
TIR1	4.04352745	YML131W	2.44496505
ALD5	3.92866094	TDH1	2.3960298
RKM5	3.86600779	LEU2	2.39004451
MIN7	3.76103233	ARG4	2.36307052
BAT1	3.56369018	DDR2	2.35568928
ENB1	3.56266056	PUN1	2.25867019
PHO89	3.53892167	MEP1	2.23932295
VMR1	3.53740988	ARA2	2.2259304
NRG2	3.31633008	ATG41	2.20222704
ZPS1	3.18982206	PHM7	2.19040994
YDR034W-B	3.09133611	YMR181C	2.1891426
FTR1	3.08764972	PET20	2.16788466
THI4	3.0641439	ART10	2.15484572
CWP1	3.00293371	ENO1	2.12572383
GID10	2.96862414	ECM4	2.12084247
CSS3	2.93656966	NPC2	2.10924975
RAD27	2.75168896	PDR15	2.08891011

Interestingly, 8 out of the top 10 most up-regulated genes were involved in the response to iron and zinc starvation, including *VEL1, SIT1, FET3* and *FET4,* and *LSO1*. The remaining genes in this list consisted of those involved in cell wall and plasma membrane integrity, including *CWP1, HSP12* and *PUN1*, along with other stress response genes such as *DDR2* (DNA damage response gene), *PDR15* (plasma membrane ABC transporter), *GTT1* (glutathione S-transferase), *PET20* (mitochondrial petite protective protein). In addition, genes involved in glycolysis (*TDH1* and *ENO1*) and amino acid production (*LEU2* and *ARG4*) were up-regulated in stressed M2 cells, a trait also observed in stressed CBS 1260 cells. This provides further evidence that an increase in energy metabolism and protein production is needed for response to stress (Chandler *et al.*, 2004). Interestingly, genes crucial for mitochondrial function that were found to be down-regulated in stressed CBS 1260 cells, were up-regulated in M2. Specifically, the genes *MIN7, ALD5, BAT1, ILV5, THI4* and *PET20* were all significantly up-regulated. This suggests a key role of mitochondria in the yeast stress response for the strain M2, although it may also be indicative of superior mitochondrial health in this strain.

When analysing the top 50 genes that were most up-regulated by log2 fold change in the unstressed M2 population (data not shown), this group appeared to contain many genes involved in amino acid biosynthesis, including *CHA1, ARO10* and *LYS20.* Interestingly there was also a large group of genes that were up-regulated in unstressed conditions usually associated with the ethanol stress response, including *SSA2, FES1, SIS1, HSP42, SSA1* and *MDJ1*. All of these genes produce proteins that interact directly with the heat shock protein 70 (Hsp70) misfolded protein response (Haslbeck *et al.*, 2004; Buck *et al.*, 2007; Lotz *et al.*, 2019). Together these gene products are involved in protein folding in response to damage caused by stress. While this is surprising, proteins involved in this folding response are also required for the correct folding of new proteins. As a result, an increase in proteins that interact with Hsp70 may be caused by a rapid increase in growth rate and protein production in the cell population (Bonner *et al.*, 2000). In phases of rapid growth, protein production can increase in order to supply newly produced daughter cells with sufficient protein, and as a result require chaperone mediated folding and delivery especially for

transport to the cell membrane and mitochondria (Bonner *et al.*, 2000). Furthermore, it is understood that the chaperone ability of Hsp70 family is crucial for the transport of precursor polypeptides to the mitochondria and endoplasmic reticulum (Deshaies *et al.*, 1988). This may therefore also explain why several genes involved in cytoplasmic:mitochondria metabolite transport, protein folding and modification in the mitochondria are also up-regulated in unstressed M2 cells, including *YAT1, GCV3, CYC1, GCV2,* and *ADH3*. In addition, the protein *HSF1* is well understood to be responsible for the increased expression of protein chaperones like Hsp70, and is activated by the presence of unfolded proteins (Mifflin and Cohen, 1994; Bonner *et al.*, 2000). When analysing the average cell-to-cell gene expression of *HSF1*, a significant upregulation in stressed M2 cells was evident, therefore suggesting that the increased expression in the listed Hsp70 family of protein chaperones is likely to not be a reaction to stress and occurs as a result of a faster growth rate than cells in the stressed population.

6.11 Identification of the most abundantly expressed genes in CBS 1260 cells in response to ethanol stress

Measuring differences in average gene expression by log2 fold change over the entire population is useful for identifying genes that are the most up-regulated under stressed conditions compared to unstressed. However, a large fold change can still be achieved if the gene expression in unstressed conditions is very small, as such the relative expression in stressed cells could also be small. This was avoided to some extent in the previous analysis, since only genes significantly up-regulated ($P<0.001$) and expressed in both stressed and un-stressed cell populations were included. However, greater detail on the genes up-regulated in stressful conditions can also be gained by investigating genes that were both up-regulated in stress cell populations but also had a high abundance (RNA content) of gene expression within the population. A list of the 20 most abundantly expressed, up-regulated genes under stressed conditions for CBS 1260 is displayed in Table 6.6.

Table 6.6. Top 20 most abundantly expressed genes in stressed CBS 1260 cells.

Gene	Average cell abundance-unstressed	Average cell abundance-stressed
HSP12	14.88101985	61.67817595
PDC- 1	17.24112072	56.32102483
ENO1	27.99143329	28.17341024
TDH1	25.0163616	27.95945803
AHP1	8.976167766	23.19940051
CPR1	6.02055718	22.18350682
ALD6	3.304848901	22.08563506
GPM1	13.51131213	21.08946746
ALD4	3.785715032	20.77764348
CWP2	2.350901081	18.44541256
MPC3	2.433345385	14.95161835
UBI4	10.00229858	13.91068769
HSP26	4.818214935	13.5844485
SOD1	5.999680725	13.33938975
MAL31	4.669956724	12.95928315
TSA1	4.945596691	12.79995703
unknown	2.960210483	12.39101998
IMA1	2.033154367	11.65887853
ZRT1	0.170550018	11.48817197
CDC19	9.78079586	11.08758058
PGK1	7.250498805	10.9578436
GPP1	1.048422623	10.80838052

The most abundantly expressed gene in CBS 1260, with an average expression of 62 per cell (stressed) and 15 (unstressed) was *HSP12*, a well-known stress response protein involved in maintaining membrane organization (Praekelt and Meacock, 1990). Genes involved in the glycolysis pathway such as *PDC1* (pyruvate decarboxylase), *ENO1* (Enolase 1), *TDH1* (Glyceraldehyde 3-phosphate dehydrogenase), *CDC19* (Pyruvate Kinase) and *GPP1* (glycerol 3 phosphate phosphatase) were all highly abundant and up-regulated in stressed conditions, correlating with the previous findings based on fold change. The up-regulation of these genes corresponds with the need for the stress protectant glycerol, and energy in the form of ATP both through glycolysis and by the production of pyruvate, which can be utilised in the glyoxylate cycle (Nevoigt and Stahl, 1997; van Rossum *et al.*, 2016). Within this list of most abundantly up-regulated genes are those involved in the stress response, including *HSP26* (membrane organization), *AHP1* (protective against oxidative stress), *TSA1* (cytoplasmic antioxidant), *ALD4* (mitochondrial aldehyde dehydrogenase) and *UBI4* (selected degradation of proteins). In addition, the gene encoding for a cell wall mannoprotein (*CWP2*) was also abundantly up-regulated to an average of 18 per stressed cell compared to 2.4 in unstressed cells. This correlates with the understanding that cell wall integrity and structural integrity are crucial in the stress response, in particular to chemical stressors such as ethanol (Nisarut *et al.*, 2022).

6.12 Identification of the most abundantly expressed genes in M2 cells in response to ethanol stress

This same analysis as described above was also performed using stressed M2 cells and the data is presented in Table 6.7. The most abundantly up-regulated gene in the stressed M2 population was *21S_RRNA*, with an average expression of 138 per cell, increasing from 57 in unstressed cells. This gene encodes for a mitochondrial 21S rRNA essential in the translation of mitochondrial proteins (Pintard *et al.*, 2002). As with stressed CBS 1260 cells, many of the up-regulated genes were involved in glycolysis and central carbon metabolism, including *ENO1* (a phosphopyruvate hydratase), *TDH1* (glyceraldehyde-3-phosphate dehydrogenase) and *PDC1* (pyruvate decarboxylase). In addition, the stress response genes *ALD6* (cytosolic aldehyde dehydrogenase), *ALD4* (mitochondrial aldehyde dehydrogenase, essential for growth on ethanol), *TSA1* (Thioredoxin peroxidase) and the cell wall/plasma membrane proteins *TIR1* (cell wall mannoprotein) and *HSP12* (heat shock plasma membrane protein) were all up-regulated, as expected based on previous findings.

Table 6.7. Top 20 most abundantly expressed genes in stressed M2 cells.

Gene	Average cell abundance-unstressed	Average cell abundance-stressed
21S_RRNA	56.7721158	137.684647
ZPS1	4.32074781	39.5060915
HSP12	6.77278142	38.9525777
ALD6	26.5791829	38.7077052
ALD4	21.101026	35.9783975
15S_RRNA	14.5774475	26.9257685
HOR7	23.8508725	26.7625201
ENO1	5.65721381	24.7372208
TDH1	4.32197844	22.7935456
TSA1	21.8437122	22.1533061
PDC1	9.94965709	21.3931811
OLE1	16.9894856	18.6128583
Unknown	7.10935919	18.5592925
CPR1	15.9828289	17.7787615
ZRT1	5.65598318	15.8019264
SPO24	12.930247	13.0726187
LSP1	9.05191129	12.7002085
UBI4	10.7938704	12.5981783
YDL124W	5.38709016	12.0523168
TIR1	0.72484206	11.9834464

6.13 Assessment of the gene expression for common stress related yeast genes

Analysis of both the most significantly up-regulated genes and the most abundant up-regulated genes in stressed conditions clearly identified an important role for many stress response genes. However a large number of expected stress related genes were absent from these lists. As a result, a literature search was also performed in order to identify yeast genes commonly found to be up-regulated under stress, in particular ethanol stress in other similar studies. A list of these genes can be found in Table 6.8, which includes the heat shock proteins (*HSPs: 10, 12, 26, 30, 31, 33, 42, 60, 78, 82* and *104*), the stress seventy subfamily A genes (*SSA: 1,2,3,4*), and the aldehyde dehydrogenases (*ALD2, 4*) and trehalose synthases (*TPS: 1, 2, 3*).

Table 6.8. Yeast stress related genes identified from the literature. Those highlighted in yellow are found significantly up-regulated in stressed populations of both strains. These genes were identified based on a variety of published works (Chandler et al., 2004; Kaino and Takagi, 2008; Stanley et al., 2010).

CTT1	KHA1	SSA3	PDI1	PMP1	ATG8	SPI1	SSA4
EUG1	HSP33	SSE1	HSP31	PBI2	GLK1	PFK26	GSY1
ALD2	HSP26	PRB1	GPM2	SPF1	OYE3	HSP60	HOR7
ALD4	DDR2	CCC2	PGK1	PYC1	GPH1	MSN2	HSP78
YPS1	LHS1	TSL1	TPS1	CDC19	TPS3	NTH1	HSP42
IMA2	PIC2	TPS2	SSA2	TDH1	APJ1	OYE2	GLG1
SOR1	PEX9	GLC3	UGP1	MSN4	GDB1	YGP1	HSP104
HSP12	AHP1	SIA1	CTR2	MCR1	HSP82	EMI2	CIT2
GPD1	YPS3	GRE3	CPR6	PGM2	HSP10	GIP2	STF2
GPP2	RPN4	DAK1	SSA1	UBC8	ERO1	ATG42	CIT1
YRO2	HXK1	STF1	CYC7	HSP30			

From this list, only those genes that displayed significant up-regulation common in stressed conditions ($P<0.001$) of both CBS 1260 and M2 population were taken forward for further analysis (highlighted yellow). As such, some stress related genes such as *HSP26* were not included due to low UMI counts; while a significant upregulation was observed in stressed CBS 1260 cells this was not observed in the M2

population. In addition genes such as *STF2* (oxidative stress), *CIT1 and CIT2* (osmotic and heat stress) (Lee *et al*., 2007; Pastor *et al*., 2009) and *CYC7* (hypoxia responsive) were also not included as a result of being down-regulated in ethanol stress conditions. It is likely that although these genes were not significantly influenced by ethanol stress, they may be responsive to other stresses that were not studied here. Within this list, genes such as *HSP31, HSP12, TSL1, TPS2, TDH1, ALD4, GLC3, GPH1, GSY1* and *DDR2* were all significantly up-regulated, as expected due to their commonality observed in many ethanol stress related studies. These genes encode a variety of heat shock proteins, proteins involved in trehalose synthesis, proteins involved in glycogen synthesis, and the general stress response protein.

6.14 The investigation of gene expression heterogeneity in stressed cell populations

The previous sections established a group of stress related genes that were commonly expressed in stressed populations of both strains. As a result, the cell-to-cell heterogeneity in expression of these genes was assessed directly in both yeasts, to gain insights as to whether they were also involved in the previously observed stress response heterogeneity. In order to measure the expression heterogeneity within stress induced genes, the normalized gene expression per cell was assessed using single cell RNA sequencing. By analysing the gene expression of individual cells and comparing variation for genes of interest between stressed populations of CBS 1260 and M2, a visual representation of heterogeneity could be obtained. In order to quantify this variation and give a value for gene expression heterogeneity for each gene; the inter quartile range (IQR) of gene expression over the cell population was assessed and subsequently divided by the population mean expression for that gene, thus allowing a comparison of heterogeneity between the two strains.

However, as there were more than 6000 genes detected in each strain at a significant expression level, the gene targets used for heterogeneity determination needed to be refined. In order to do this, the common genes identified in the top 50 most

significantly up-regulated (log2 fold change) genes under stressed conditions (Tables 6.4 and 6.5) were investigated. In addition, from the most abundantly expressed, up-regulated genes in stressed populations of each strain (Tables 6.6 and 6.7), common genes to both strains were also identified. Finally, from the genes identified in the literature to be important and up-regulated when exposed to ethanol stress, those found to be both significantly expressed and significantly up-regulated in stressed conditions ($P<0.001$) in both strains investigated here were also analysed. This accumulated group of up-regulated, stress related genes that were present in both strains were aggregated into one list and prepared for heterogeneity determination as in Table 6.9.

From this set of genes, differences in heterogeneity expression between the two strains were assessed. One of the genes identified to be commonly up-regulated in both strains was *HSP12*, encoding a plasma membrane protein involved in maintaining membrane organization under stress conditions (Parsell and Lindquist, 1993). By analysing the single cell gene expression of this gene for each cell within each stressed populations, a gene expression dot plot could be produced. From this plot, each dot represents a cell and the intensity of the red colouration corresponds to the amount of *HSP12* gene expression for that cell, with darker red colours symbolizing greater expression (Figure 6.17). It was apparent that cells within the CBS 1260 population presented a greater range in single cell gene expression than for strain M2, as demonstrated by the range of colour intensity for each cell. In order to assess this further, violin plots were produced to indicate the range in gene expression from cell-to-cell of each population.

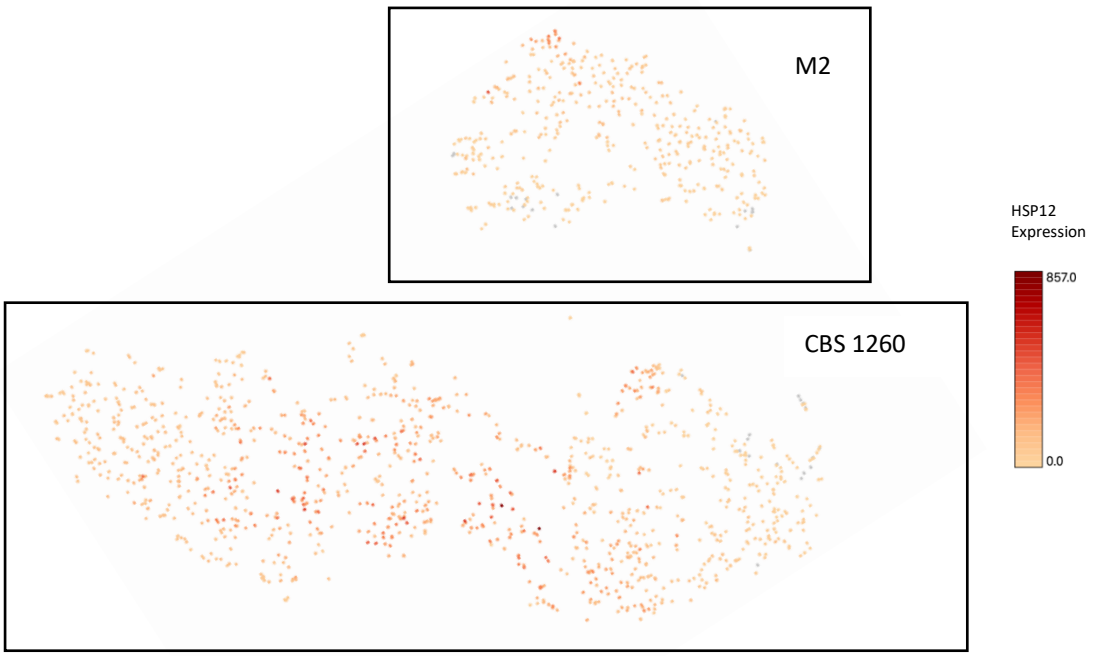

Figure 6.17. HSP12 gene expression variation among stressed M2 and CBS 1260 cells. The heat plots present cells from both cell populations where cells with a darker red colour are those with greater HSp12 expression compared to those exhibiting a lighter yellow colour

The violin plots for both strains demonstrate the range of gene expression for each cell population, with greater cell-to-cell variation demonstrated by more elongated violins and a larger inter quartile range. From the violin plots (Figure 6.18), it is evident that across the entire stressed population, the strain CBS1260 had greater cell-to-cell variation in expression than M2.

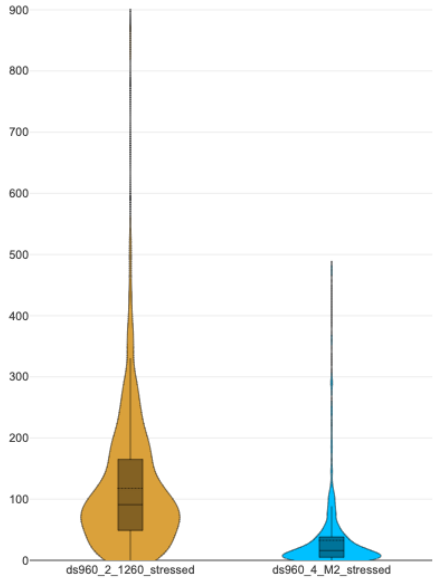

Figure 6.18. Violin plots for HSP12 gene expression in stressed cell populations. These violin plots present the gene expression variation among single cells of each strain. With the grey boxes represent the IQR obtained from Q1, Q3, mean and median.

When the variation in gene expression observed from these violin plots was calculated from the IQR and population mean, values of 1.4 (CBS 1260) and 1.17 (M2) were obtained, quantifiably demonstrating greater variation in the strain CBS 1260. While this data showed the cell-to-cell variation across the entire population, analysing the gene expression violin plots obtained from each pre-defined cell cluster for a given strain, allowed a greater insight into population heterogeneity. This revealed the presence of uniquely divergent sub-populations within each strain; the inter-cluster gene expression data for gene *HSP12* is presented in Figure 6.19(a-b) for both strains.

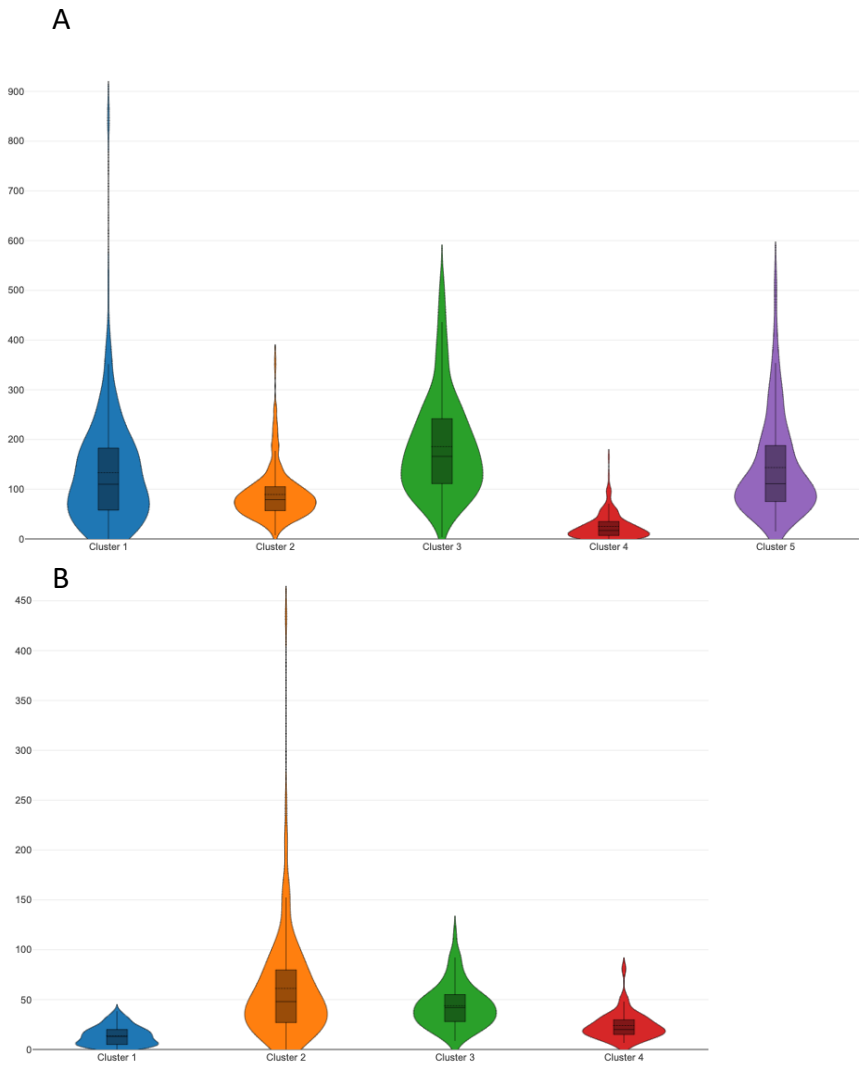

Figure 6.19. Inter-cluster variation for the expression of *HSP12* in stressed cell populations. The violin plots presented here demonstrate the range of gene expression for *HSP12* among cells of each pre-determined cell cluster A: CBS 1260 and B: M2. The grey boxes represent the IQR obtained from Q1, Q3, mean and median.

From these plots it can be seen that there is evidence of heterogeneity in both strains, demonstrated by differences in cell expression range within each cluster and the expression mean for each cluster. However, visually both the shape of violin plot and the relative mean expression in each cluster appear to vary more in the strain CBS 1260. This variation is further exaggerated by the presence of more clusters in CBS 1260, as a result of more uniquely different cell sub-populations being present. The gene *HSP12* is one of the most abundantly expressed genes in CBS 1260 population

with an average of 111 UMI counts per cell in cluster 3, 100 in cluster 5, 97 in cluster 2, 78 in cluster 1 and 77 in cluster 4. In stressed M2 populations the expression in each cluster is 40 in cluster 3, 29 in cluster 1, 16 in cluster 2 and 5 in cluster 4, thus demonstrating that both strains do exhibit inter-cluster heterogeneity. The analyses presented here were subsequently performed for the expression of all genes within the targeted list in Table 6.9, which contains the obtained values of cell-to-cell gene expression heterogeneity for each of the targeted stress induced genes, for both strains.

Table 6.9. Stress induced genes common in both strains. The Table presents the values for cell-to-cell variation (IQR/Mean) of each of these genes in the stressed populations of CBS 1260 and M2

Gene	CBS 1260 cell-to-cell variation	M2 cell-to-cell variation	Gene	CBS 1260 cell-to-cell variation	M2 cell-to-cell variation
PHM7	1.5	1.3	CTR2	2.24	1.14
PUN1	1.5	1.25	PRB1	1.5	1.27
DDR2	1.4	1.2	PGK1	1.37	0.84
PDC1	1.32	1.17	YDL124W	1.34	1.23
ENO1	1.33	1.15	ATG8	1.3	1.37
TDH1	1.34	1.1	GSY1	1.28	1.36
CPR1	1.32	1.15	GPH1	1.45	1.39
ALD6	1.4	1.3	TPS2	1.85	1.5
GPM1	1.34	0.8	ALD4	1.32	1.36
ALD4	1.32	1.35	EMI2	1.32	1.29
CWP2	0.82	0.67	HSP31	1.88	1.18
UBI4	1.37	1.27	GRE3	1.5	1.43
TSA1	1.36	1.28	PDI1	1.5	1.28
YNL208W	1.34	1.22	PGM2	1.3	1.17
ZRT1	1.38	1.22	PBI2	1.3	1.11
CDC19	1.27	1.08	GLC3	1.53	1.22
PGK1	1.37	0.84	TPS1	1.47	1.28
PST2	1.37	0.84	PFK26	1.16	1.8
SCW4	1.32	1.33	RPN4	1.57	1.02
PEP4	1.34	1.35	HOR7	1.41	0.98
TFS1	1.38	1.15	GPD1	1.45	1.38
TMA17	1.34	1.24	TSL1	2	1.23
HSP12	1.4	1.17	YGP1	1.43	1.14
DDR2	1.41	1.24	DAK1	1.39	1.45
CCC2	2.08	1.56			

Interestingly, almost all of the genes shown in Table 6.9 displayed more cell-to-cell variation in the stressed CBS 1260 cell population. There were those with particularly large differences in heterogeneity observed, such as the genes *CCC2* (copper transporting ATPase), *CTR2* (low affinity copper transporter), *HSP31* (Methylglyoxalase) and *TSL1* (subunit of trehalose 6-phosphate/phosphatase complex). Contrary to this, there were a small number of genes within this group that were more heterogeneous in stressed M2 cells, including *ALD4* (mitochondrial aldehyde dehydrogenase), *GSY1* (Glycogen synthase), *PEP4* (vacuolar aspartyl protease) and *SCW4* (cell wall protein). This further emphasizing the complexity of heterogeneity, indicating that these genes do not contribute to the observed stress response heterogeneity in CBS 1260 cells, but may be a source of the lesser degree of heterogeneity present in M2 cells.

The gene *PGK1* displayed one of the largest differences in the degree of heterogeneity between the two strains, with variation values of 1.37 (CBS 1260) and 0.84 (M2). As a result, the gene expression in each cluster of these stressed cell populations was further analysed. The stressed CBS 1260 population had an average cell *PGK1* expression of 40 (cluster 1), 4.8 (cluster 2), 25 (cluster 3), 7 (cluster 4) and 9 (cluster 5). Comparatively, there was less inter cluster variation in the M2 cell population with values of 1.7 (cluster 1), 13 (cluster 2), 6 (cluster 3) and 0.6 (cluster 4). Further to this, the range of gene expression within each cluster was lower in M2 clusters than CBS 1260, implying that the presence of divergent and unique sub-populations was greater for CBS 1260, as presented in the violin plots in Figure 6.20(a-b).

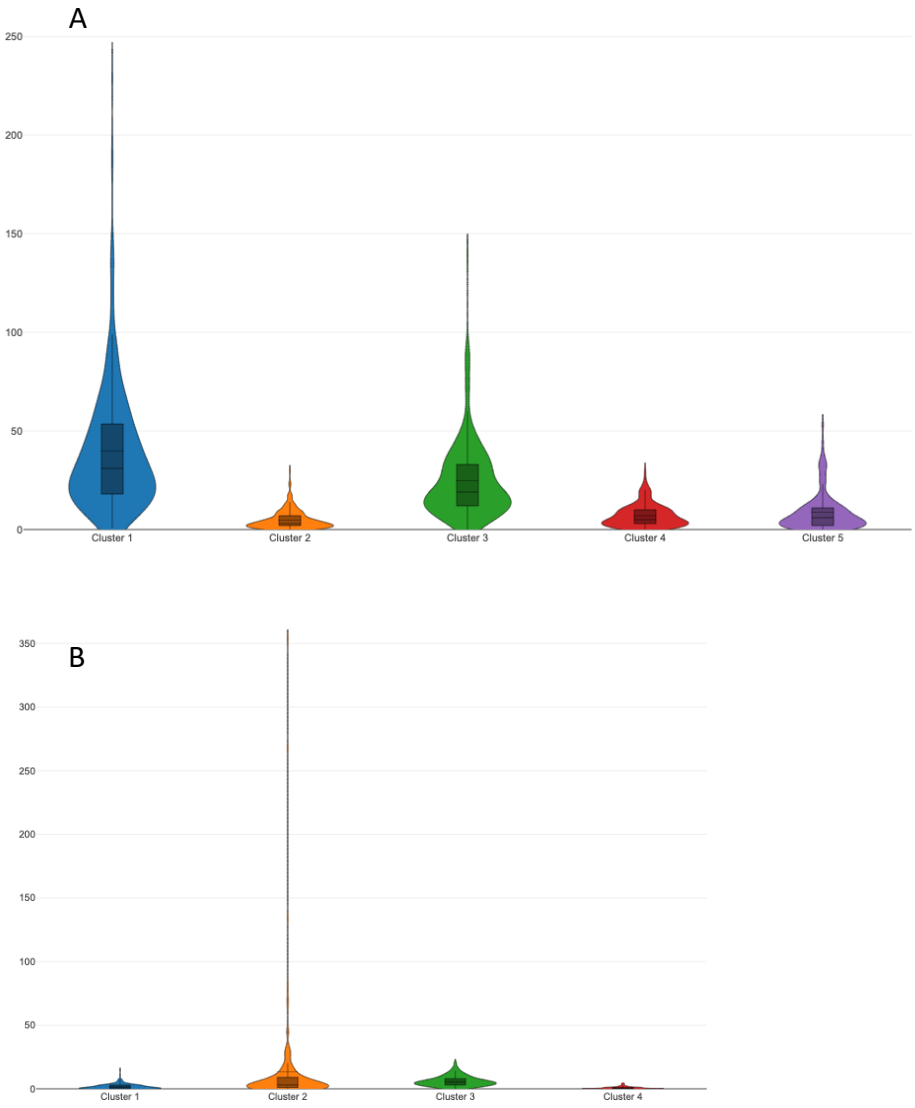

Figure 6.20. A presentation of inter-cluster variation for the expression of PGK1 in stressed cell populations. The violin plots presented here demonstrate the range of gene expression for PGK1 among cells of each pre-determined cell cluster A: CBS 1260 and B: M2. The grey boxes represent the IQR obtained from Q1, Q3, mean and median.

Interestingly cluster analysis for most of the genes in Table 6.9 presented similar gene expression profiles. Greater average gene expression and greater variation was observed in clusters 1, 3, 5 for stressed CBS 1260 cells and greater expression in clusters 2 and 3 in M2 cells. This suggests that the upregulation of these stress induced genes did appear to be preferentially expressed in certain sub-population over others,

an artifact with greater significance in CBS 1260 cells. It should be noted that while the degree of heterogeneity in many of the genes in Table 6.9 was higher in CBS 1260 than M2, the difference in variation values for a large number of genes between the two strains was often relatively low. There were however, some genes such as PGK1, HSP31 and TSL1 that exhibited much greater differences in degree of variation between the strains. Combining this with the finding that many of the cells expressing highly upregulated genes in response to stress were found in cells of specific clusters, therefore, the overall heterogeneity is likely a result of the accumulation of these small differences in gene expression heterogeneity amounting to a large degree of phenotypic heterogeneity and the formation of distinctly divergent sub-populations.

6.15 Heterogeneity assessment of gene-related compounds discovered using LC-MS

The yeast metabolomic data obtained in Section 6.3 presented many compounds that were up-regulated in cell populations stressed with ethanol. These compounds consisted of fatty acids and linoleic acids, in addition to sugars such as trehalose, glycogen and sorbitol. These compounds were subsequently used to identify stress related genes from which cell-to-cell variation in each of the stressed populations was assessed to determine whether these genes were contributing to the observed stress response heterogeneity. Genes related to these compounds were identified in Section 6.8 and included the genes *FAS1, FAA2, PXA2, ACC1, FAS2, TSL1, TPS2, GLC3, GPH1, GSY1* and *GRE3*.

Consequently, the gene *FAS1*, related to fatty acid synthesis, and highly up-regulated under stressed conditions for both strains was assessed for the presence of cell-to-cell heterogeneity. Using the same analysis as in Section 6.14, the cell-to-cell heterogeneity was shown to be slightly increased in the strain CBS 1260 compared to M2 (variation values of 1.2 and 1.05 respectively). In order to gain greater detail in the presence of heterogenous sub-populations, the pre-determined clusters were analysed for each of the stressed cell populations. Interestingly, when analysing the respective violin plots for each cluster in stressed populations of both strains, it was

apparent that both yeasts displayed a significant degree of inter-cluster heterogeneity (Figure 6.21a-b).

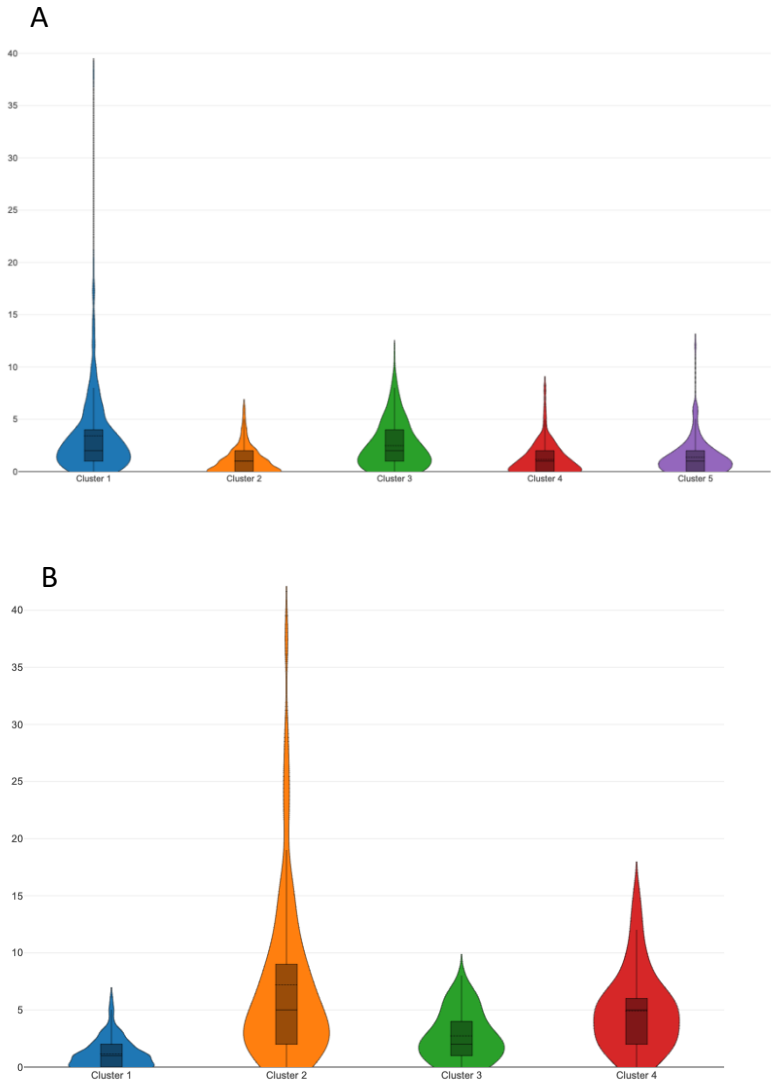

Figure 6.21. A presentation of inter-cluster variation for the expression of *FAS1* in stressed cell populations. The violin plots presented here demonstrate the range of gene expression for *FAS1* among cells of each pre-determined cell cluster A: CBS 1260 and B: M2. The grey boxes represent the IQR obtained from Q1, Q3, mean and median.

From this analysis, it can be seen that the IQR varied more between clusters belonging to M2 than CBS1260, this is represented by both the elongation of the violin plots and the length of the grey boxes representing the IQR. Furthermore, the mean gene expression in each cluster varied more in stressed M2 cells than in CBS 1260 cells. This implies that while this gene may be essential in the ethanol stress response, it was not a cause of the increased heterogenous observed in CBS 1260 in previous sections. Interestingly, most of the genes involved in fatty acid synthesis (such as *FAS1, FAA4, ACC1* and *PXA2*), although up-regulated in stressed populations of both strains, were not a significant source of heterogeneity in CBS 1260 cell populations. This indicates that fatty acid synthesis and the cellular content of fatty acids also may not be a significant cause of ethanol stress response heterogeneity.

Trehalose was another abundant and up-regulated compound in stressed cell populations, as identified by metabolomics (Section 6.3). As such, the gene *TSL1*, encoding for the large subunit of the trehalose 6-phosphate synthase/phosphatase complex, was investigated and seen to be up-regulated in both stressed cell populations. The upregulation of this trehalose gene displayed a significant increase in expression in stressed CBS 1260 cells, with a log2 fold change of 1.03 ($P<0.001$). In contrast, the upregulation of this gene in stressed M2 cells was small and not significant, with a log2 fold change of 0.14. When comparing the gene expression variation between the stressed populations of each strain, the CBS 1260 cells again appeared to exhibit a greater degree of heterogeneity, with a cell-to-cell variation of 2.02; compared to 1.23 in stressed M2 cells. The cell cluster expression data for the *TSL1* gene in stressed cell populations is presented in Figure 6.22(a-b) and demonstrates significant inter-cluster variation for both strains.

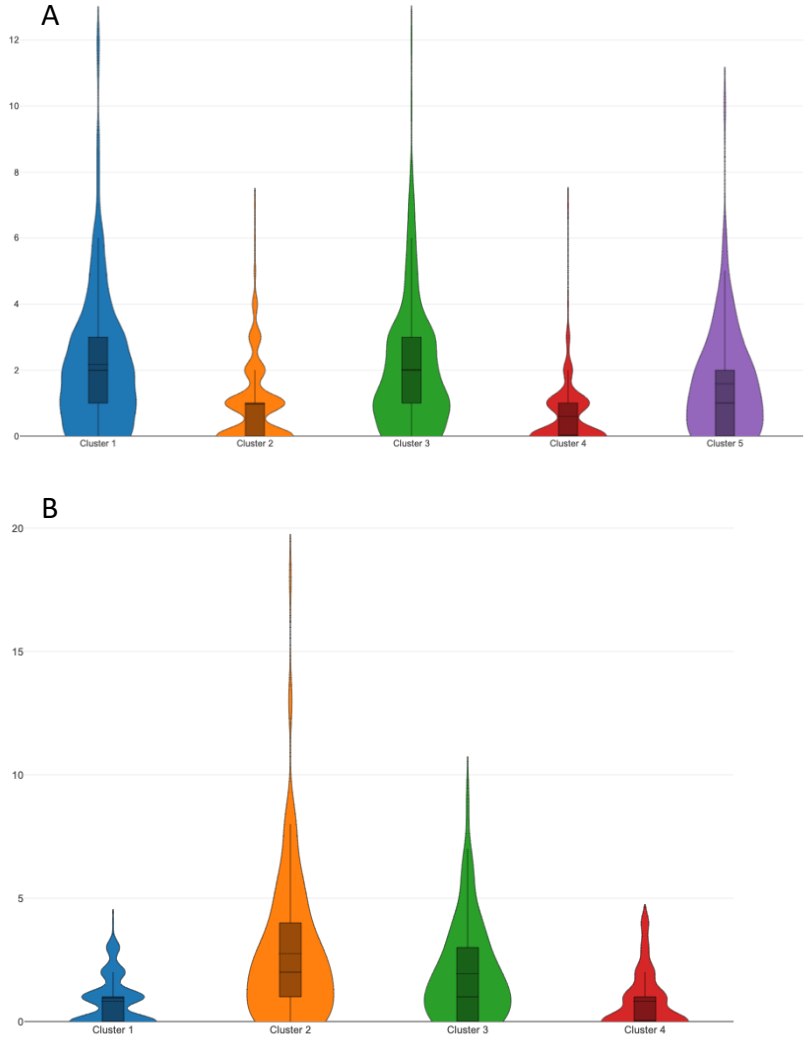

Figure 6.22. A presentation of inter-cluster variation for the expression of *TSL1* in stressed cell populations. The violin plots presented here demonstrate the range of gene expression for *TSL1* among cells of each pre-determined cell cluster A, CBS 1260 and B, M2. With the grey box representing the IQR obtained from Q1, Q3, mean and median.

In both strains, the violin plots suggest a split of cell sub-populations with high expression and those with comparatively lower expression for *TSL1*. The mean cell expression of this gene in the cell clusters for the stressed M2 population was 0.8, 2.8, 1.9 and 0.8 respectively, revealing the presence of 2 clusters with cells of higher expression, and two with low-average expression. This was also seen in stressed CBS 1260 cells, with mean expressions of 2.2, 1.0, 2.0, 0.6 and 1.6 in respective clusters. This again demonstrates that heterogeneity and the formation of divergent sub-populations with varying gene expression characteristics is present in both strains.

However, since the increased number of uniquely different clusters was greater in CBS 1260 than M2, this provides further support that CBS 1260 shows greater heterogeneity compared to M2. Interestingly, an almost identical pattern of gene expression was observed for the gene *TPS2*, a phosphatase subunit of the trehalose-6-P synthase/phosphatase complex, which is crucial for the storage of trehalose in response to stressful conditions (Data not shown).

Another stress related compound, glycogen, was also found to be up-regulated in stressed cell populations in the metabolomics study. As such it was not surprising that the gene *GLC3*, encoding a glycogen branching enzyme essential in stress induced glycogen accumulation (Ruis and Schüller, 1995), exhibited an increase in expression in stressed cell populations for both strains. Analysing the single cell gene expression for the *GLC3* gene revealed differences in gene expression profile between the two strains (Figure 6.23a-b). The average cellular gene expression of this gene increased from 2.26 (unstressed) to 4.36 (stressed) for CBS 1260 and from 4.35 (unstressed) to 5.59 (stressed) for M2 cells, therefore displaying both significant upregulation in both strains and high average cellular abundance of RNA for this gene. The variation in cell-to-cell gene expression again quantified, resulting in a variation of 1.15 for stressed CBS 1260 cells and 1.02 for M2. This indicates similar degrees of heterogeneity between strains, while marginally increased values between CBS 1260 cells.

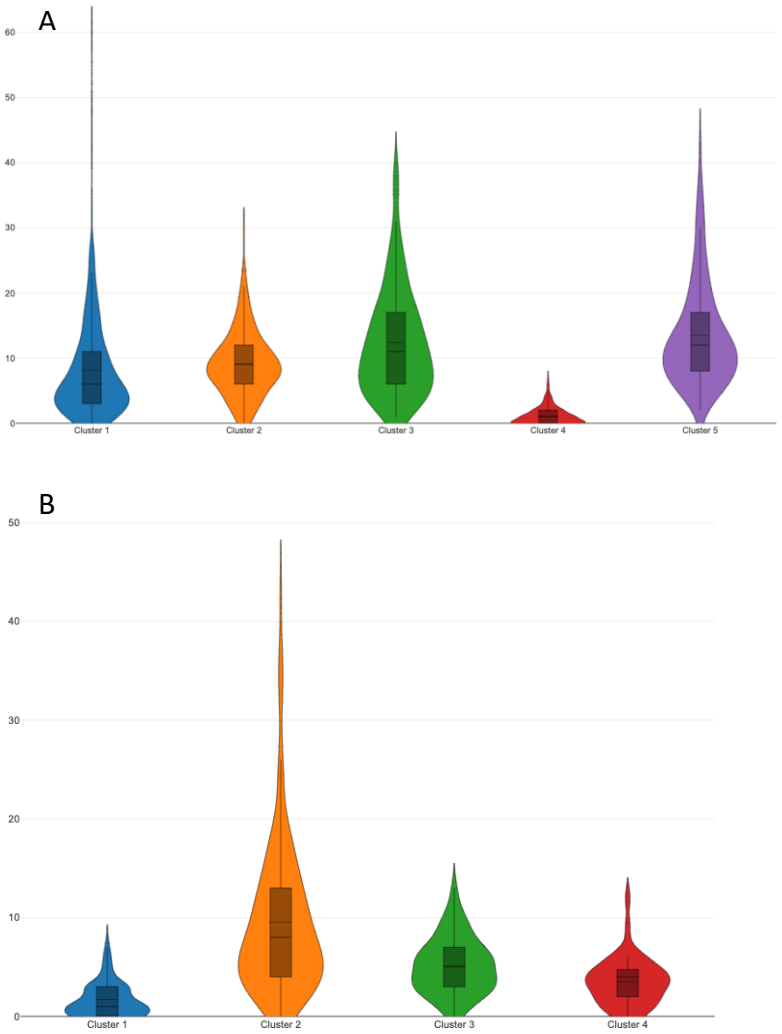

Figure 6.23. A presentation of inter-cluster variation for the expression of GLC3 in stressed cell populations. The violin plots presented here demonstrate the range of gene expression for GLC3 among cells of each pre-determined cell cluster A, CBS 1260 and B, M2. With the grey box representing the IQR obtained from Q1, Q3, mean and median.

By analysing the single cell gene expression of each unique cluster for the stressed cell populations, inter-cluster variation could again be observed in both strains. This inter-cluster variation was greater for the CBS 1260 strain, with average *GLC3* expression ranging from 1.1 in cluster 4 to 13.5 in cluster 5. In the stressed M2 population the range was from 1.7 in cluster 1 to 9.5 in cluster 2, with the other two clusters displaying similar expression profiles. Furthermore, an increased number of unique clusters were observed in stressed CBS 1260 cells, indicating the presence of more divergent sub-populations, with various gene expression profiles for *GLC3*. In addition,

it is evident from Figure 6.23(a) that each of the CBS 1260 clusters themselves exhibited a high degree of cell-to-cell gene expression variation, presented by the elongated violin plots.

This section provides further evidence for the existence of heterogeneity in stress related genes in each of the studied yeast strains. The genes investigated here were studied due to their link with stress induced compounds identified in the metabolomics study, and included genes related to fatty acid synthesis, as well as stress protectants such as trehalose and glycogen. When assessing these genes for the presence of cell-to-cell heterogeneity in expression, it was evident that the strain CBS 1260 exhibited greater variation in general. However, the genes related to fatty acid synthesis appeared to show less variation between the pre-defined clusters than for other studied genes, at least for CBS 1260. The key sub-populations identified in stressed M2 cells displayed inter-cluster variation for the expression of genes such as *FAS1*. This indicates that while the upregulation of these genes may be essential in the ethanol stress response, they may not be a significant source of stress response heterogeneity. The opposite was true for the stress response genes *TSL1* and *GLC3*, which showed a high degree of variation both within the population as a whole and between clusters for CBS 1260, suggesting the presence of distinctly different sub-populations of cells with unique gene expression profiles and a potential source of phenotypic heterogeneity.

6.16 Conclusion

In the previous Chapters, the data obtained demonstrated the presence of stress response heterogeneity, showing significant differences between strains for common brewing stresses such as ethanol and cold shock. In particular, the strain CBS 1260 displayed a high degree of heterogeneity, and the strains NCYC 1332 and M2, displayed an opposing survival strategy with significantly less heterogeneity. Subsequently, analysis of stress related cellular components, including mitochondrial number, mitochondrial membrane potential, internal membrane integrity and neutral lipid content, were all shown to exhibit increased cell-to-cell variation within the strain CBS 1260. Despite genetic uniformity in the studied strains, it is likely that this phenotypic heterogeneity may have an impact on strain performance. As a result, potential sources of heterogeneity were investigated at the metabolic and molecular level.

Initially, a novel metabolomic approach was employed to screen and identify compounds found in greater abundance in stressed populations of the strains CBS 1260 and M2. Intracellular compounds were identified using LC-MS in both reverse mode (positive and negative) and HILIC. Many of the identified compounds up-regulated in stressed cells were associated with synthesis of fatty acids, linoleic acids, sugars such as trehalose and glycogen, and a group of amino acids and their derivatives implicated in the ethanol stress response (Lomako *et al.*, 1993; Šajbidor *et al*., 1995; You *et al*., 2003; Bandara *et al.*, 2009). These identified stress related compounds presented potential sources of genetic interest that could be used to identify the root causes of ethanol stress response heterogeneity. To investigate this, single cell RNA sequencing was performed using stressed and unstressed populations derived from the highly heterogenous strain CBS 1260, and the less heterogeneous M2. Three categories of genes were identified based on 1) those expressed to a greater extent in stressed cell populations (this study), 2) stress induced genes taken from the literature, and 3) genes responsible for producing those compounds identified through metabolomics (this study). Consequently, this entire group of stress related genes were analysed in order to assess the presence of cell-to-cell variation in

gene expression. Many of these genes were associated with glycolysis and energy metabolism, cell wall structure and repair, heat shock proteins, mitochondrial function, and intracellular stress protectants. It was evident from this analysis that the two studied strains displayed many differences in the expression of genes that were up-regulated after exposure to sub-lethal ethanol stress. However, this was not entirely surprising, given their different genetic origins. However, there were several genes that were commonly up-regulated in both strains, and these were subjected to heterogeneity analysis.

The presence of heterogeneity was immediately evident by presenting the cluster analysis for unstressed cell populations. In total, 8 clusters of were identified with similar gene expression profiles for the strain CBS 1260, while only 6 were found for M2. Immediately, this implied the presence of a greater number of divergent sub-populations with unique gene expression profiles for CBS 1260 cells. In addition, the stressed populations also consisted of a number of unique clusters, with 5 clusters present in stressed CBS 1260 cells and only 4 for M2, further emphasising the presence of greater heterogeneity in the CBS 1260 cells. Interestingly the decrease in the number of clusters in response to ethanol correlates with previous findings that the degree of heterogeneity can reduce after prolonged stress, an artefact previously observed within our laboratory (Brindley and Powell, In Preparation) and in Chapter 5 here. This may be a result of those sub-populations with superior stress resistance being preferentially selected, therefore lowering the amount of heterogeneity. In addition, this may also be due to acquired stress resistance after long periods of ethanol exposure (Berry *et al.*, 2011).

The assessment of cell-to-cell gene expression variation in ethanol stress induced genes provided evidence to show that there was a degree of gene expression heterogeneity present in both strains. This suggests that gene expression variation between isogenic yeast populations is an innate property likely present in all yeast strains. However, the existence of a greater number of unique cell clusters and increased cell-to-cell gene expression in stress related genes in CBS 1260 strain indicates a greater degree of heterogeneity overall. While many of the differences in

gene expression variation between the two strains were small (such as in the genes *TSA1, TMA17, GRE3*), there were also a set of gene where a larger difference in heterogeneity was observed. For example, the genes *TSL1* and *HSP31* showed considerably greater variation in CBS 1260 cells than in M2. It is therefore likely that the opposing heterogeneity observed in the physiological stress response is not a result of a small number of specific stress related genes, but an accumulation of many small differences in gene expression over a large number of genes.

Furthermore, the data in this section suggests that the observed heterogeneity in stress induced gene expression is not a random trait but appears to have a notion of population wide organisation. This is demonstrated by the accumulation of cells with similar gene expression profiles into clusters. For example, among stressed CBS 1260 cells, most of the identified stress related genes were found to be up-regulated in clusters 1, 3 and 5. Conversely, there were genes expressed to a greater extent in cells of other clusters, such as genes encoding ribosomal RNA in cluster 4. This finding suggests the use of a division of labour strategy among this yeast strain, whereby certain sub-populations of cells appear to have their own unique role within the main population (Wloch-Salamon *et al*., 2017). These observations give greater insight into the presence and source of heterogeneity among brewing yeast strains, which may in turn impact suitability and versatility of a given strain for industrial fermentations. Furthermore, the presence of divergent sub-populations demonstrated by the clusters of cells with similar gene expression, could provide a method for population quality control (PopQC) (Xiao *et al.*, 2016). By targeting genes that are over-expressed in cells of certain clusters using genetic markers, it may be possible to isolate cells from sub-populations which exhibit desirable properties. In addition, as discussed previously, the observed stress response and its relative degree of heterogeneity is an innate, inheritable property. Therefore by isolating certain cells of interest it may be possible to produce a new yeast population with favourable and desired characteristics.

Chapter 7. Conclusions and future work

The brewing industry employ a range of yeast strains, primarily selected based on their favourable properties during fermentation. Brewing yeasts were historically categorised according to the type of fermentation that they were used in (lager or ale). However, current convention is that beers produced using *Saccharomyces cerevisiae* are designated ales and those made using *S. pastorianus* are classed as lager products. However, this is a gross simplification, and it is known that these groups comprise many strains which yield a remarkable range of flavour profiles, often due to individual differences in nutritional requirements and growth kinetics. Furthermore, the suitability of a strain for a particular process can be affected by its capacity to tolerate environmental stresses. As a result, both strain selection and strain improvement remain important strategies to maximise the success of current processes, through ensuring rapid process times and reproducible products. This is also relevant for new process and product streams, including the 'low and no' alcohol sector, and with regard to the challenges associated with very high gravity brewing.

To ensure that the 'best' yeast strains are employed in a given scenario, it is important that a complete understanding of the capacity of a strain to tolerant stress is achieved. This is because fermentation conditions present a unique challenge for yeast; fluctuating and intense levels of stress mean that responsive adaptations are required for the population to survive and to be successful. During the beginning of fermentation, osmotic and oxidative stress factors are high. As these reduce in intensity, ethanol stress and CO_2 pressure become dominant (Gibson *et al.*, 2007), followed eventually by nutritional deprivation. From a brewing perspective, this coincides with flocculation and sedimentation into the yeast cone, which itself presents a different set of environment challenges, including cold stress and continued starvation (Powell and Smart, 2004). Furthermore, the practice of serial re-pitching continually exposes a yeast population to successive stresses, while also potentially leading to sub-populations of yeast being preferentially selected during cropping (Powell and Diacetis, 2007).

While it is relatively common for brewers to understand the tolerance limits of a given strain, little is known about potential variation within a brewing yeast culture. In the wild it is known that microbial cell populations employ heterogeneous strategies of survival to overcome fluctuating, stressful environments. These evolutionary adaptations can be divided and described as 'bet hedging' and 'division of labour' strategies, which ensure the best possible chances of population survival under varying conditions (Levy *et al*., 2012; Holland *et al.*, 2014; Wloch-Salamon *et al*., 2017). With the complexity of stress factors observed in brewing fermentations, it is unknown whether yeast populations adopt one of these innate strategies of survival. Furthermore, it is possible that the presence and effect of heterogeneity in brewing yeast populations may be important factors when considering the suitability of a particular strain for a specific process, or to determine its versatility.

In this study, seven yeast strains were analysed, each differing in their genetic origin. Two traditional *Saccharomyces cerevisiae* ale strains were used, three lager strains (of both Saaz and Frohberg type) a Voss Kveik strain and a *Brettanomyces anomalus* (BA) wild yeast. These strains were selected to represent the diversity observed in current modern-day brewing, and to gain greater understanding of the brewing yeast stress response in general. Initially, each yeast strain was characterised using phenotype-based methods including permissive growth temperature analysis and growth kinetics in liquid media (Chapter 3). This was followed by genetic characterization to confirm speciation (using ITS PCR-RFLP) and to ensure strains were unique (interdelta PCR fingerprinting). Subsequently, each yeast was tested for its ability to tolerate a range of fermentation related stresses (ethanol, oxidative and osmotic), and two heavy metal stress factors (copper and zinc). This was performed to assess their capacity to survive under a range of stresses of industrial and environmental significance; heavy metal toxicity is an important consideration in yeast bioremediation and has already been studied extensively in relation to heterogeneity (Holland *et al.*, 2014). Stress tolerance was initially determined through analysis of colony growth on solid nutritional media, supplemented to provide the respective stress factor. This analysis provided useful insight into the maximum stress tolerance of each strain, based on the capacity to form colonies under increasing concentrations of stress. From this it

was evident that ale strains were generally more stress resistant to ethanol and osmotic stress, with lager strains more adapted to oxidative stress. Interestingly among the lager yeast, the strain W34/70 exhibited superior stress response across all brewing-related challenges, while the Kveik strain, known for its ability to ferment fast and withstand high levels of stress (Preiss *et al.*, 2018), exhibited a higher tolerance to all of the stress factors when compared to conventional brewing strains. Interestingly, this pattern was also observed in response to the heavy metals zinc and copper; the Kveik yeast exhibited greater tolerance limits than the remaining strains, which all showed similar, but lower, resistance. The tolerance of the Kveik strain is likely a major reason for the recent insurgence of this type of yeast in industrial brewing fermentation, since it is accepted that these yeasts are highly resistant to a large number of stresses (Preiss *et al.*, 2018; Garshol, 2020). The wild *Brettanomyces* strain had a relatively low tolerance to all of the stresses apart from copper toxicity, likely to be derived from its 'wild' nature (Holland *et al.*, 2014). This data also indicated large degrees of variability between the strains and revealed complexity in that tolerance to one stressor did not always imply a similar response to the other stress factors. It is known that there are generic stress response mechanisms which are triggered in response to a wide variety of stresses (Chatterjee *et al*., 2000), however the data presented here supports the observation that there is also a unique tailored response to each stress, and that this varies from strain-to-strain. Furthermore, the variation in tolerance between stress factors, and between strains, demonstrates the challenge that brewers face when selecting yeast strains for a particular fermentation. For example, stressful environments such as those found in high gravity brewing necessitate the use of strains that can survive elevated levels of multiple stresses. Simply put, it should not be expected that an individual yeast strain associated with a particular product will be able to perform as normal if changes are made to a fermentation regime.

Once it had been established that the stress response was variable not only between strains, but also between stresses, the extent of cell-to-cell heterogeneity within each population was investigated in Chapter 4. This was performed to gain a greater understanding of the stress response strategy employed by each strain and to more

fully understand its capacity to survive under adverse conditions. To quantify heterogeneity in response to stress, a cell cytotoxicity assay was developed based on MTT staining. This relied on viable metabolising cells within an initial cell 'seed' proliferating, allowing the occurrence of sub-populations of cells with different response mechanics to be revealed. The extent of these divergent sub-populations was found to vary between strains and also between stresses. This implied that each yeast exhibited a unique survival response strategy based on the stressor. The overall assessment gave a measure of phenotypic heterogeneity for each yeast; strain CBS 1260 displayed a very *high* degree of heterogeneity, while strains M2 and NCYC 1332 displayed a very *low* degree of heterogeneity in their response to ethanol. This finding suggested that those strains with a *lower* heterogeneity would contain cells with similar adaptation to an imposed stress, while those exhibiting *high* heterogeneity comprised a sub-population(s) of cells with varying ability to tolerate this stress factor. In other related industries, the presence of cellular heterogeneity in the stress response has been shown to be a significant issue in process performance and consistency, resulting in expensive and laborious downstream processing (Hitzeman *et al.*, 1983; Wang *et al.*, 1995; Alonso *et al.*, 2014). With the identification of stress related heterogeneity in the brewing yeast strains studied here, the question of whether this reflects a desirable trait was posed. It is interesting that despite years of domestication through forced evolution and adaptation to the unique challenges presented in brewing fermentations, a high degree of cell-to-cell variability was observed to be relatively common in industrially used brewing strains. This would suggest that stress response heterogeneity is an important attribute for a strains survival and that populations have adopted a bet hedging/division of labour strategy for survival. While beneficial from the perspective of the yeast cell, this may potentially occur at the expense of fermentation consistency, particularly when yeast are used in serial-re-pitching. This practice could theoretically select out certain sub-populations of cells, based on their survival characteristics, which could lead to unpredictability in other fermentation parameters such as flavour production and VDK assimilation.

In addition to differences in the extent of variation between strains, different stress factors also procured a different response. Some stresses elicited a high degree of variation, whereas others produced a similar effect on cell viability across a population. As such, potential sources of population heterogeneity and their causative agents were also investigated. This was achieved using fluorescent staining to analyse stress related cell targets, including membrane integrity (fluidity) and structure, mitochondrial biomass and health (MMP), lipid content and internal membrane integrity. This approach also revealed heterogeneity within yeast cell populations, and further demonstrated that the degree of heterogeneity varied between strains. The cell organelles targeted in this analysis demonstrated variation within cells of all the studied strains, however the degree of variation ranged between strains and did not specifically follow the same patterns of heterogeneity observed for stress factors. For example, the strains M2 and W34/70 were found to have a relatively low degree of heterogeneity in ethanol stress response, however the cell-to-cell variation in mitochondrial biomass were the highest among the studied strains. Conversely, these same two strains presented the lowest variation in neutral lipid content. This phenomenon indicates that the source and cause of stress response heterogeneity is far more complex than once thought. If yeast strains primarily utilised a holistic approach to stress using elements, such as the general stress response pathways, it would be anticipated that heterogeneity in organelle composition and integrity would match that observed in response to stress factors. The complex nature of stress response heterogeneity gives the impression that it is likely to occur a result of intricate differences in expression of a large number of genes that cause an additive affect (Gasch *et al.*, 2017). Irrespective, by obtaining greater insight into the yeast stress response, this may allow brewers to more accurately predict which yeast strains are suitable for particular processes, and may also uncover mechanisms that allow key characteristics to be controlled or harnessed in order to improve currently used strains.

Although heterogeneity was observed within the studied strains, the causes of this remained unclear. Previous studies have typically been performed on ‘standard’ cultures of microorganisms, containing cells of varying age and size (Holland *et al.*,

2014; Gasch *et al.*, 2017; Saint *et al.*, 2019). Although it is well documented that cell size and age vary within a population, the effect of these parameters on heterogeneity was investigated in Chapter 5. This was performed in an attempt to determine whether analysis of mixed aged cells showed the 'true' innate stress response heterogeneity, or whether daughter cells exhibited a different pattern of heterogeneity entirely. In this study, the strains CBS 1260 and NCYC 1332 were used, selected for their opposing heterogeneous survival strategies in response to ethanol stress and cold shock; CBS1260 displayed a high degree of heterogeneity and NCYC 1332 a low degree of heterogeneity. A novel method for yeast isolation was employed, allowing yeast cells that were uniform in age to be collected and analysed. Daughter cells were obtained immediately after budding and used to reflect the 'innate inherited traits', without impact from exposure to environmental conditions or aging, thus yielding a 'true' reflection of the heterogeneous stress response. Daughter cell fractions were obtained using a combination of fluorescent bud scar staining and fluorescence activated cell sorting (FACS) with flow cytometry imaging. Through this method, mixed aged (control) and daughter cell populations of each strain were obtained with high purity (>95%) and yield. These two fractions were then tested to determine firstly if there were different degrees of heterogeneity between each age fraction, and subsequently to determine if age itself was a major cause of population variation. As above, it was anticipated that analysis of daughter cells would reveal the 'true' or 'innate' heterogeneity, since analysis of stress tolerance would be observed before any age-related effects or undefined environmental factors had affected a cells individuality. Interestingly, stress response heterogeneity determination of the purified daughter cells revealed similar degrees of variation to their mixed aged counter parts, suggesting that heterogeneity is an innate inherited property and not a characteristic acquired over time due to age related factors or environmental influence. However, in contrast to mixed cells, the *maximum* tolerance of daughter cell populations appeared to be reduced in comparison to mixed aged cells. This was particularly true for ethanol, which is arguably the most important industrially relevant stress factor. This finding suggests that while heterogeneity is an innate artifact in yeast cells, the maximum tolerance is likely something that is acquired

during somatic growth, perhaps due to exposure to different stress factors leading to adaptation over time (Berry *et al.*, 2011).

Following analysis of the effect of age on stress tolerance heterogeneity, cell fractions were also analysed for stress related cell targets as in the previous chapter (Chapter 4). In this way, mitochondrial biomass, MMP, internal membrane integrity and neutral lipid content were evaluated for mixed aged and daughter cell populations. The data obtained provided evidence to show that strain CBS 1260 in particular yielded a greater degree of heterogeneity for these cell targets, matching the response to stress factors and showing that cells were compositionally variable. Although it is unknown whether these differences were the *cause* of heterogeneity or an artefact of a heterogenous *response* to stress, it is interesting to note that the degree of physiological heterogeneity within CBS 1260 decreased with both age and exposure to sub-lethal ethanol stress (5% v/v). This was true for neutral lipid content and mitochondrial biomass as indicated by Nile red and MitoTracker green staining respectively. This reduction in heterogeneity observed after exposure to sub-lethal ethanol stress could be a result of underperforming sub-populations of cells effectively being selected out, thus decreasing the overall heterogeneity of the population. While this presents questions over the resulting consistency of fermentation if heterogeneous strains are used in serial re-pitching, it does however provide an opportunity. With the use of sub-lethal stress, it may be possible to alter the dynamics of a yeast strain; by selecting out underperforming sub-populations the resulting cells are theoretically more adapted to the imposed stress. This could provide a method of obtaining unique sub-strains that can be tested and selected for enhanced fermentation characteristics. On the other hand, the analysis of mitochondrial membrane potential (MMP) and internal membrane integrity revealed an unchanging, high degree of heterogeneity in the CBS 1260 populations regardless of age or stress state. This suggests that both MMP and internal membrane integrity are cell attributes unaffected by cell age and exposure to stress. However, given that mitochondria health/number and neutral lipid content were significantly different from cell-to-cell, these findings may provide an indication of cause and effect in stress response heterogeneity. It should be noted that these structures represent only a

small number of the potential stress-related targets that could be investigated. As such in the future other stress related cell targets such as the cell membrane and wall structure (Navarro-Tapia *et al*., 2018; Nisarut *et al.*, 2022), cell protectants such as trehalose and glycogen (Cahill *et al*., 2000; Bandara *et al.*, 2009) could also be directly investigated for their impacts on heterogeneity.

A greater insight into physiological aspects of stress response heterogeneity was achieved through analysis of yeast cell metabolomics (Chapter 6). This data revealed a large group of compounds in which production was upregulated in stressed cell populations. Interestingly, many of these compounds were associated with fatty acid biosynthesis, energy metabolism (through glycolysis and alcoholic fermentation), and the production of stress protectants such as trehalose, glycogen, glycerol, and sorbitol. The increase in fatty acid content and associated derivatives is likely derived from the requirement of these in maintaining membrane fluidity and structure upon exposure to ethanol stress (Alexandre *et al.*, 2001; Li *et al.*, 2010; Ishmayana *et a*l., 2017). The increase in compounds associated with energy metabolism concur with the observation that the requirement for ATP is increased in the response to stress, likely due to increased metabolic demands in order to survive (Piper, 1995; François and Parrou, 2001). In addition the upregulation of the stress protectants trehalose, glycogen, glycerol and sorbitol were all expected commodities based on current literature (Nevoigt and Stahl, 1997; Cahill *et al*., 2000; Bandara *et al.*, 2009). The expected upregulation of these stress related compounds firstly confirmed the efficiency of the developed method to identify stressed cell populations, but also further emphasised the complexity of the ethanol stress response. The upregulation of a wide range of compounds apparent in stressed cells suggests a dramatic shift in gene expression upon exposure to stress. Furthermore, the observed differences in the compounds that were upregulated between the strains suggests that different brewing yeasts have their own unique method of responding to stress, with a similar intended outcome. However, irrespective of this complexity, analysis of analyte upregulation after exposure to stress presents a useful methodology of quantifying metabolites in response to an external stressor/signal that could be useful in other studies. Firstly, comparing the compounds obtained with those upregulated in

response to other brewing stresses such as osmotic and oxidative stress could give greater detail into both the general stress response and the unique tailored response to each stress. Furthermore this could be a useful technique to identify compounds of interest in the bioenergy and pharmaceutical industries, such as the accumulation of useful lipids that could be used for biofuel production (Zhang *et al.*, 2011; Cheng *et al.*, 2021). Using this method, it would also be possible to screen different chemical inducers for positive impacts on useful compound upregulation. The metabolomic methodology developed here could also be utilised within the brewing industry to test wort conditions and determine the effect (regulation) of desirable (or undesirable) flavour compounds, without the use of expensive and laborious reference standards. This study also highlighted a set of compounds found to be produced at lower concentrations in stressed cell populations. This group consisted mainly of certain essential amino acids and their derivatives, and a group of non-essential monosaccharides. This suggests that, upon exposure to stress, the production of certain proteins (most likely those associated with rapid growth and biomass production) may be arrested, confirming previous reports (Simpson and Ashe, 2012; Sun and Gresham, 2021). Furthermore it is well known that exposure to sub-lethal ethanol stress can result in membrane alterations that cause amino acids to leak out of the cell membrane (Salgueiro *et al*., 1988).

The use of metabolomics to analyse key compounds thus provided novel insight into the brewing yeast ethanol stress response, with data that has not previously been considered within the industry. This method provided a quantitative assessment of the precise cellular compounds and metabolic processes upregulated upon exposure to ethanol stress. The complexity of the compound upregulation demonstrated in Chapter 6 suggests that the yeast stress response is derived from complex and unique gene expression alterations within cells of each strain. Therefore, to complement the metabolomic work, single cell RNA sequencing was applied to the same brewing yeast cultures. Single cell RNA sequencing is a relatively new gene expression technology that extracts and sequences the mRNA present in single cells within a population. As a result, this approach can reveal intricate differences in gene expression between individual cells within a genetically uniform population (Gasch *et al.*, 2017). In a

previous study using this technology to analyse brewing yeast populations (Jariani *et al.*, 2020), large amounts of information were gathered firstly displaying the upregulation of genes upon switching between media types and the subsequent investigation of cell-to-cell heterogeneity. In Chapter 6, single cell gene expression data was first analysed to discover genes that were significantly and abundantly upregulated when exposed to sub-lethal ethanol stress (5%). This analysis was performed using the designated 'heterogenous' strain CBS 1260, as well as the comparatively 'homogeneous' strain M2. In this way, genes involved in energy metabolism, amino acid synthesis, the ethanol stress response, cell wall protein production and stress protectant synthesis were investigated. As expected, these were all shown to be essential for survival in ethanol stress (Piper, 1995; Grant *et al*., 1997; Parrou *et al*., 1997; Chandler *et al.*, 2004; Nisarut *et al.*, 2022). However, differences were discovered in the precise genes that were upregulated in response to stress between the two strains, with some genes being highly upregulated in one strain but not the other. Although, the general trend towards increasing cell wall/membrane integrity, energy metabolism, amino acid synthesis, trehalose synthesis and glycogen synthesis in response to stress all appeared to be common outcomes. The data showed that, while the outcomes were broadly similar (e.g producing trehalose), the mechanism (through gene expression) to achieve these goals was different between strains. This was a perhaps an expected trait, due to different genetic origins of the two strains. However, it was interesting to note that there were many upregulated genes in common between the two strains; these were taken and further evaluated for variation in single cell gene expression, therefore providing a method for gene expression heterogeneity determination.

The data obtained through single cell gene expression analysis further demonstrated that strain CBS 1260 harbored the propensity for greater cell-to-cell variation based on the pattern of genes upregulated during stress. This was further emphasized by quantifying the presence of unique cell clusters within populations, based on gene expression profiles, and comparing these between strains. In both stressed and unstressed conditions, strain CBS 1260 exhibited a greater number of unique cell clusters, indicating the presence of more divergent sub-populations with unique roles

within the main population. Interestingly it was also observed that cells with higher expression levels in stress related genes appeared to group within specific clusters in both strains, indicating that heterogeneity within a population may be directed and not random *per se*. This also suggests that individuals within these clusters may have a unique role in ethanol stress resistance from the perspective of the population in general. This finding may support evidence that strains do indeed utilise both bet hedging and division of labour strategies to survive. However, irrespective to this, it is suggested that the observed combination of an increase in the number of cell clusters, and the accumulation of stress related genes with greater cell-to-cell heterogeneity, are likely the cause of the variation in ethanol stress response observed within strain CBS 1260.

The results in Chapters 3, 4 and 5 demonstrate the complexity of the yeast stress response and the unpredictable and somewhat random nature of heterogeneity. However, the data presented in Chapter 6 indicates that differences in cell-to-cell gene expression are the likely source of heterogeneity, and that this may be less random than previously thought. While expression of specific stress related genes may vary between strains, those upregulated after exposure to stress all appeared to group in specific cell sub-populations, implying some level of organization with regard to the observed heterogeneity. Furthermore, while heterogeneity in stress related gene expression was present in both the highly heterogeneous strain CBS 1260 and the less heterogeneous M2, the occurrence and number of these unique and organised sub-populations was the major influential factor of stress response heterogeneity. This had a larger impact than the differences in gene expression heterogeneity among a specific set of genes, and interestingly the propensity to form unique sub-populations of cells is an attribute often referred to in many heterogeneity studies (Bishop *et al.*, 2007; Holland *et al.*, 2014). However, the actual visualisation of sub-groups is rarely observed on such an intricate level. Usually the identification of heterogeneous sub-populations relies on the use of genetic engineering and fluorescence markers such as GFP to present groups of cells differing in just one cellular aspect at a time (Attfield *et al.*, 2001). However, the techniques applied in Chapter 6 allow visualisation of sub-populations formed as a result of the gene

expression of thousands of genes at the same time. This could have major implications when considering impacts on a variety of bioprocesses. The presence of sub-populations with unique gene expression profiles could be a source of significant inconsistencies between brewing fermentations. However, these sub-groups of cells could subsequently be isolated to produce unique sub-strains of yeast with desirable properties which could be assessed for fermentation performance with potential applications. The methods developed and utilised in Chapter 6 present a powerful tool for screening yeast strains based on their metabolic profiles and measuring epigenetic differences between cells of an isogenic population. In this study, this was utilised to measure and identify the source and extent of heterogeneity in gene expression of stress related genes. However, this could be used to screen and compare a range of specific genetic targets tailored to the study.

The data obtained in this study provides evidence for the presence of significant cell-to-cell heterogeneity in brewing yeast populations in response to stress. Through applying cell sorting techniques, physiological analysis, metabolomic profiling, and single cell RNA sequencing, causes and effects of heterogeneity were also investigated. While the data obtained provides new learnings from a brewing yeast perspective, the stochastic nature of gene expression is a trait that has been observed previously in other studies (Gasch *et al.*, 2017; Jariani *et al.*, 2020). However, here we have been able to investigate the fundamental causes behind what makes a strain heterogeneous in nature. For example, both the increase in cell-to-cell variation in gene upregulation and the greater propensity to form unique sub-populations of cells provide key rationale as to why strain CBS 1260 has a significantly greater degree of heterogeneity in response to ethanol stress. Interestingly, the gene expression data also demonstrates that a large amount of cell-to-cell heterogeneity remains in stress induced genes within a designated 'low heterogeneity' strain (M2). The observation that this type of yeast also contained a significant number of divergent sub-clusters of cells with unique gene expression profiles indicates that variation is likely present within all yeast strains. Based on this, it can be concluded that a truly homogenous culture may be almost impossible to obtain. These findings lead to the conclusion that heterogeneity is not caused by a small number of specific genes responsible for large

differences in stress response, but rather the accumulation of many genes with expression levels that are slightly more heterogeneous in one strain than another, leading to an increased number of divergent sub-populations.

The techniques utilised and developed in this study firstly demonstrate a powerful method of screening yeast strains based on their ability to tolerate stress. In addition, the data obtained using metabolomics and single cell RNA sequencing provides a useful tool for identifying key metabolic differences between yeast strains, while also being able to compare epigenetic variation between individual cells. Therefore, It is possible that the precise level of detail obtained with regard to the yeast stress response in this study could be used to aid in the selection of yeast strains. For example, there is a desire within the industry to produce multiple product streams from a single yeast strain, while simultaneously 'intensifying' fermentations using high gravity worts or by applying hotter conditions, both of which can procure a stress to yeast cultures. This, coupled with the fact that yeast are subjected to multiple consecutive stress factors during yeast handling, suggests that genetic and phenotypic plasticity would procure an advantage in industrial scenarios. However, while it is likely that heterogeneity may aid the survival of a strain exposed to stress, it should be noted that the degree of heterogeneity declines after being exposed to stress, which indicates that in reality the scenario is complex. In effect, stress itself causes a population to become more homogeneous, at least temporarily. This hypothesis is supported by observations that a smaller number of unique clusters were identified in stressed cells based on gene expression data, as well as physiological data that showed variation in mitochondrial mass and neutral lipid content were reduced in cells exposed to ethanol stress. This may be caused by underperforming sub-populations of cells being 'selected out', leaving only cells with the necessary adaptation to survive the stress, thus reducing the number of divergent sub-populations. However, when combining this finding with the fact that the degree of heterogeneity observed within a strain varies depending on the stress factor, it is possible that this could lead to inconsistencies in performance, albeit depending on the inter-relationship between stresses associated with fermentation and yeast handling. In contrast, when considering more homogenous strains, a lower degree of

heterogeneity coupled with a superior maximum tolerance, may actually prove to be beneficial. In this scenario the likelihood of sub-populations of cells being selected out is less likely, potentially leading to a more consistent fermentation. This rationale can be applied to a hypothetical situations whereby two strains exhibit a similar maximum average ethanol tolerance of 10% (v/v). However, one strain may contain cells that can survive from 5-15% with an average of 10%, while the other contains cells that can all survive until precisely 10% ethanol is exceeded. In this instance, when a fermentation reaches 5% (v/v) ethanol, many of the cells in the former (heterogeneous) population may die, leading to a lower cell count and potentially altered beer flavour due to the presence of autolysed dead cells. Cells from the more homogenous strain may all survive and thrive in the environment, leading to a more consistent process. Such insight into the stress response aid understanding of industrial yeast strains by providing greater detail into the attributes of individual cells, complementing more conventional 'population wide' tolerance data.

The occurrence of heterogeneity within the studied strains does appear to have an element of randomness. At least, based on our knowledge of the heritage of industrial yeast strains, the reason why one particular strain has developed to be heterogeneous and others less so remains unknown. It could be that within yeast strains, two divergent strategies of surviving stress have developed independently, irrespective of whether they are ale or lager strains. The result would be that some strains preferentially use heterogeneous strategies of survival to overcome stress, while others adapt to highly stressful environments leading to a lower degree of heterogeneity. It may be that this ‘decision’ was formed early in the domestication of each strain and was dictated by the type of fermentation environment in which it commonly resided. Those strains with greater heterogeneity may have been used in fermentations with less stress, as a result maintaining the innate stress response heterogeneity. Conversely, strains lacking heterogeneity may have been exposed to higher concentrations of stress continuously, therefore adapting to these conditions and traits becoming genetically ‘fixed’ over time.

7.1 Future work

While the data obtained here demonstrate the occurrence and root causes of population variation, the direct effects of heterogeneity on fermentation performance and consistency remain largely unknown. This could be investigated by utilising both the high and low heterogeneity strains employed in this study in fermentations trials where cells are exposed to high levels of stress. From this, it would be interesting to determine the attributes of the final beer, including residual sugar content, ABV, VDK production profiles, and sensorial qualities. The impact of serial re-pitching on process and product consistency would also be useful to determine. For example, a yeast crop could be re-pitched over consecutive generations to observe whether a finished product is more consistent when produced by a more or a less heterogeneous yeast culture. Similarly, it should be noted that while the data obtained using single cell RNA sequencing provided a useful insight into the heterogeneity of stress related gene expression, over 6000 genes were assessed for both stressed and unstressed cell populations. As such, there is a wealth of data that remains unexplored. It would be valuable to fully examine aspects of this data to determine variation in other parameters associated with industrial fermentations, including key metabolic enzymes, as well as genes involved in flavour synthesis. In particular, those known to be involved in higher alcohol production (Ehrlich pathway) (Ravasio *et al*., 2014), ester production (esterase genes)(Fukuda *et al.*, 1998b, 1998a) and amino acid production, including the valine synthesis pathway for VDK production (Villa *et al.*, 1995). Furthermore, analysing flocculation gene characteristic would provide an interesting view of the variation in this attribute between stressed and unstressed cells (Bony *et al*., 1998; Halme *et al.*, 2004; Verstrepen *et al*., 2004).

As discussed briefly above, one additional application based on understanding population variation is the potential for breeding novel and superior isolates. In similar studies examining heterogeneity, variation has been used as a method for 'population quality control' (Xiao *et al.*, 2016). Essentially, heterogeneity can be used to remove underperforming cells and/or to select for cells with desirable features. The clusters of sub-populations of cells revealed in the current study (containing higher expression

rates in stress related genes) could potentially be investigated further in order to identify unique and desirable genetic attributes. These could also serve to identify targets through genetic engineering or GFP tagging with fluorescence activated cell sorting which could be used to isolate specific cells from a population. As shown by our data, heterogeneity and stress response is an inherited trait and it would be expected that strains with greater heterogeneity may prove to be more useful as candidates for strain improvement.

While brewing yeast fermentation and its related stresses has been the main focus of study here, many of the methods developed and utilised could be transferred to other bioprocess. The production of bio-ethanol requires large volumes of ethanol to produced rapidly (Ruyters *et al.*, 2015; Kostas *et al.*, 2016). As such, cell populations that can produce large quantities of ethanol, but also have superior ethanol stress response are desired. The application of the heterogeneity and tolerance studies, coupled with gene expression analysis could be used to identify sub-populations of cells with both of these attributes. By combining this with FACS sub-populations of highly efficient sub-populations could be isolated and used to improve bio-ethanol production. Furthermore, this could also be utilised in the pharmaceutical industry where large quantities of specific engineered or non-engineered compounds are required. Heterogeneity could effectively be utilised to obtain cells with improved attributes based on the requirements of the process.

Brewers yeast are single cell organisms and as such it is not surprising that other related single celled microorganisms also display heterogeneity (Ackermann, 2015). However, while heterogeneity is present in these single celled organisms, it is unclear how heterogeneity may impact more complex structures, such as human tissue. There have been many studies assessing the heterogeneity of cells present in tumours, however these often investigate the different 'types' of cell within them, rather than differences in functional traits (Schelker *et al.*, 2017). Utilising the techniques demonstrated in this study, cell-to-cell heterogeneity could be investigated for genetically identical cells within human tissues and tumours. This could provide greater detail into their growth dynamics, survival, response to exogenous signals and

potential weaknesses. In addition there are many environments within the human body that are highly stressful, such as those experienced in the stomach (low pH) (Lund *et al.*, 2014) and those imposed when medicines are taken such antibiotics (Gardete *et al.*, 2006) and chemotherapy (Ding *et al.*, 2018). Heterogeneity is certainly present when cells respond to these stresses, demonstrated by the survival of some unwanted bacteria when exposed to antibiotics, and the survival of cancerous cells when exposed to chemotherapy treatments. While there are single cell heterogeneity studies in these areas of research (Lee *et al.*, 2014; Sánchez-Romero and Casadesús, 2014; Winterhoff *et al.*, 2017; Poonpanichakul *et al.*, 2021), combinations of metabolomics and single cell RNA sequencing are rarely performed. Many of the cell attributes and genetic targets focused in these studies are often pre-determined based on known genes. However, by screening cells for upregulated/downregulated compounds and highly upregulated/downregulated genes a new and novel list of potential sources of heterogeneity could be discovered. It is worth noting that this element of 'going in blind', and not solely focusing on genes already investigated, has historically lead to the discovery of many new cell attributes that challenge the current dogma, leading to significant advances in science.

Finally, in comparison to the number of yeast strains commercially available, only a very small number of yeast strains were investigated here. With the methodology for heterogeneity determination now fully established, this could be developed into a screening method to identify strains with extremes of heterogeneity and stress tolerance. While ethanol stress proved to be the overarching focus of this study, in particular in regard to the metabolomics and gene expression assessments, additional stress factors could also be incorporated in order to gain a more complete overview of the yeast stress response in relation to relevant industrial conditions. It is anticipated that such analyses would allow an even greater understanding of current and future industrial strains, allowing process efficiency to be enhanced and providing opportunities for new highly efficient product streams in the future.

8. References

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