VARIATION WITHIN BREWING YEAST POPULATIONS

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Abstract

Harvesting yeast at the end of fermentation and using it to reinoculate a subsequent fermentation (serial repitching) is unique to the brewing industry. Despite its prevalence, this process can come at a cost; reusing yeast can negatively impact product and process consistency. In particular, fermentation completion time variability is an issue. In some cases, this variation is explained by differences in wort composition, raw materials or yeast viability, however it may also be the result of population shifts within the genome or phenome. Although much is already known about factors which impact the brewing yeast genome, phenotypic diversity within brewing yeast strains has not previously been explored. In this thesis I provide data to suggest that phenotypic heterogeneity (i.e. non-genetic variation) is evident in a range of brewing yeast populations, based on their sensitivity to brewery related stress factors.

Initially, the propensity for brewing strains to yield variants was explored in the lager yeast strains W34/70, CBS1174 and CBS1260 and the ale strains M2 and NCYC1332 using DNA fingerprinting techniques and giant colony morphology plating. Subsequently, phenotypic heterogeneity was assessed by determining resistance to key stress factors: ethanol, osmotic (sorbitol) and oxidative (H_2O_2) stress. Populations exposed to ethanol stress displayed the greatest differences in heterogeneity, while osmotic and oxidative stress elicited a more conserved response.

Of all of the strains, the lager yeast W34/70 was identified as being particularly phenotypically diverse. Further investigation into the cellular response of this strain to prolonged exposure to ethanol found evidence of a bet-hedging

strategy in the lager yeast W34/70, suggesting that the process of serial repitching could potentially select for sub-populations of cells thereby decreasing the populations overall heterogeneity. This theory was examined by fermenting W34/70 under low level ethanol stress over an extended period of time. After 18 days the population became more homogeneous in nature comprising mainly of highly resistant cells. However, upon removing the primary selective agent, ethanol, the measurement of heterogeneity proved that the investment in the switch was not permanent as after one normal, small scale fermentation the heterogeneity was found to be shifting back towards that of the control sample. The relationship between phenotypic heterogeneity and stress factor indicates that this concept is extremely complex in industrial systems. Nevertheless, the data presented here sheds new light on the potential root causes of viability loss, and why some strains are more suitable for certain fermentations.

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CHAPTER 1:

INTRODUCTION

1.1 History of Brewing

Consumption of alcoholic beverages is ubiquitous throughout all civilisations and without doubt pre-dates recorded history (Boulton and Quain, 2006). This can be attributed to a multitude of reasons; taste, ritual and (probably most notably) because of the bactericidal properties of ethanol produced during the fermentation process. This latter property, combined with the acidic nature of many other yeast metabolites, ensured that beverages like beer became the equivalent of sterilised water. Consumption of alcoholic beverages has therefore been seen to be beneficial from a health perspective, potentially saving people from contracting an array of potentially fatal diseases before the establishment of technologies to purify and treat waste water (Boulton and Quain, 2006). Although records of the first beer fermentations are bygone in prehistory it is feasible that it evolved alongside the development of cereal cultivation. Archaeological proof of this early relationship between human and yeast is the artefact 'Hymn to Ninkasi', a Sumarian tablet dating back to around 1800BC containing recipes and songs which celebrate a goddess of brewing (Lodolo *et al.,* 2008). Since this time, humans have been selecting and therefore domesticating yeast (which we now know to be *Saccharomyces cerevisiae*; the same strain which is also used in wine and bread making) based on their fermentation characteristics to produce the best tasting beer, which for much of history were ale-type products. Lager beer fermentations appeared far more recently in the 15th century but it took until the 19th century for these products to become popular and for the lagering process to become widely accepted as a brewing technique (Libkind *et al.,* 2011). Since then it has overtaken ale

production holding a 74.1% share of the total beer sales in the UK in 2016 (statista.com, 2018).

1.2 Overview of brewing process

The brewing process can be defined as the method by which the raw materials are converted into a sugar-rich substance known as wort and subsequently fermented into beer. Traditionally these raw materials comprise malted barley, hops, water and yeast. However, there is significant variation in practice, for example a range of cereal grains can be employed such as wheat, rye, oats, sorghum, rice or millet (Briggs *et al.,* 2004), and other flavouring can be substituted for hops. The consistent feature in all brewing fermentations is the use of yeast; although several different types of yeast can be employed, the most common are those belonging to the genus *Saccharomyces* (Section 1.3).

Yeast, a single-celled eukaryotic organism, are classified within the Fungi kingdom (Kutty & Philp, 2008). There are around 1,500 species which have been described in detail, although it is believed that this only represents in the region of 1 % of yeast species which exist in nature (Kurtzman & Fell, 2006). Yeast have been exploited for thousands of years, principally due to their capacity to produce ethanol and carbon dioxide, which are important in the production of certain foods and beverages, including bread, wine, distilled spirits, soy sauce and beer (Kutty & Philp, 2008).

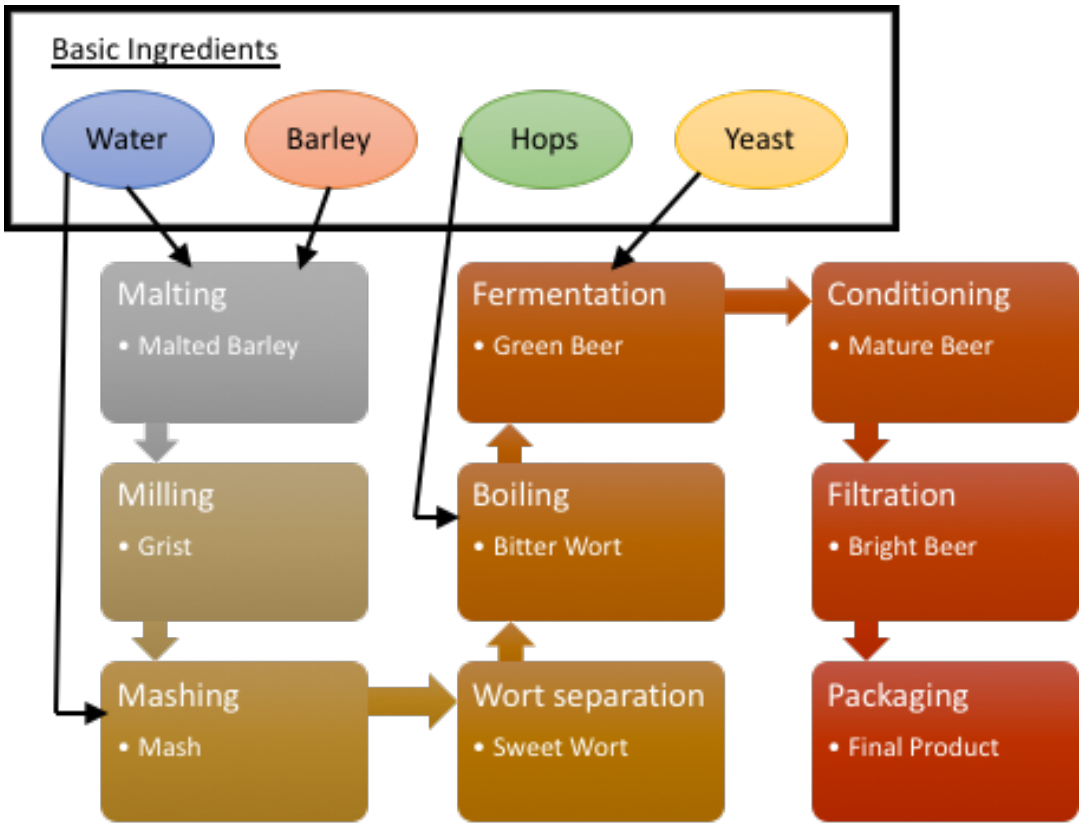


Figure 1.1 Basic overview of the brewing process.

1.2.1 Wort production

The cereal grain is treated in such a way that germination is initiated and then halted via heat treatment (kilning) (Briggs *et al.,* 2004) and is also termed the 'grist'. Germination is controlled by a series of steps involving wetting (steeping) and then exposing the grain to air at set temperatures (these conditions can change depending on the malt required). This hijacks the grains natural ability to mobilise its starch reserve via the activation of enzymes. Usually the grain would use this to begin its own development, but by halting this process at the right moment the starch and enzymes are held in a state which means they available for further extraction and break-down, releasing

the fermentable sugars which are used downstream (Boulton and Quain, 2006). This process is achieved by heating the malt in water known as mashing, and the resulting infusion is maintained at between 60-70^{o}C (Briggs *et al.,* 2004). The end result is a thick, porridge-like mixture which is allowed to stand so that a bed of husks (grist) is allowed to form. The liquid is run through this bed which acts as a natural sieve leaving the clear, sweet liquid known as 'sweet wort' to be collected into the copper vessel where it is subsequently boiled, often with hops (Briggs *et al.,* 1982).

1.2.2 Hops

As a flavour additive, hops play a crucial role in the final overall taste of the beer due to the release of bitter tasting resins during the boil. These hop compounds are present in the flowers of the hop plant, *Humulus lupulus*. Unhopped beer has been claimed to taste like an "ethanolic, sweetened and more acidic version of lemonade" (Verzele, 1986). Hops also contribute largely to the quality and stability of the beer. The resins released also provide foam stability and anti-microbial properties (Bamforth, 2003). For brewing the α-acid fraction is of most significance; these are isomerised during the boil to iso-α-acids making them more soluble (Vriesekoop *et al.,* 2012). The antimicrobial action of these iso-α-acids has been shown to work by disrupting intracellular pH, binding essential metal ions, and inducing leakage of the cell membrane. This combination of effects essentially causes starvation and inhibition of proton-driven active transport of nutrients (such as sugars and amino acids) across the membrane (Sakamoto and Konings, 2003). As a result other essential

cell processes such as respiration and protein/DNA/RNA synthesis are supressed. For this reason, the use of hops has to be controlled with care when producing lambic/sour beers; the majority of these beers depend on gram-positive bacteria (specifically hop resilient *Lactobacillus sp.*) to provide acidity (Tonsmeire, 2014), and these microorganisms can be susceptible to the antimicrobial action of the iso-α-acids.

1.2.3 Introduction to fermentation

Fermentation reflects the process by which yeast converts the raw materials present in the wort into beer. The end product is dependant on great care being taken to ensure steps along the process within specification. Brewing yeast is 'pitched' or inoculated into the wort once it has cooled. It is paramount that the yeast is free from contamination and is in a good physiological state (Quain, 1986). The choice of yeast strain is of huge importance to a brewer as they are they are considered to be the flavour engine of the brewing industry (Meier-Dörnberg *et al.,* 2017). The wort is a rich source of nutrients, essential amino acids, inorganic acids and lipids (Bamforth, 2008). The resulting flavours and aromas are down to this synergy of wort composition and yeast strain. Initially the brewing yeast is exposed to oxygen and undergoes aerobic respiration. This is a requirement at the initiation of the process to provide the brewing yeast with the ability to synthesise sterols and unsaturated fatty acids which are essential for cell plasma membrane maintenance and function (Lorenz and Parks, 1991). This oxygen is quickly depleted and carbon dioxide concentrations rise to create an anaerobic environment (Dashko *et al.,* 2014). The principle role

of the brewing yeast culture is to produce ethanol, carbon dioxide and flavour compounds in the form of esters and higher alcohols during fermentation (Lentini *et al.,* 2003). This is characterised by corresponding changes in sugar concentration, along with a certain amount of yeast growth. Yeast contain a complex structure of metabolic pathways which they are able to turn on and off depending on nutrient and oxygen availability and the stresses imposed upon the cell (Piškur et al., 2006). Understanding these metabolic pathways is of great importance in order to create an optimal environment for the production of a high quality and consistent product. The suitability of these yeast for the fermentation processes is enhanced by their ability to perform this function in the aerobic and anaerobic environments (Piškur *et al.*, 2006), a peculiarity known as the Crabtree effect. Many *Saccharomyces spp.* have the ability to ferment sugars and accumulate ethanol and carbon dioxide even in the presence of oxygen providing them with a competitive advantage in beer brewing environments and are termed 'Crabtree-positive' yeast. Conversely, yeasts which do not show this ability are termed 'Crabtree-negative' yeast (De Deken, 1966; Pfeiffer and Morley, 2014).

Towards the end of fermentation, as sugars become a limiting factor, the yeast cells begin to clump together and form 'flocs'. This is a process known as flocculation (Section 1.4.3.2). This response to nutritional stress is beneficial to brewers as it allows for easy removal of the yeast cells (cropping) and it is then also possible for the yeast from the previous fermentation to be used in a subsequent fermentation (repitching) (Section 1.4.3). Equally yeast flocculation can cause issues in the process if it is initiated before the end of the

fermentation (that is before all of the fermentable sugars have been depleted). This can be a strain specific response and so can be hard to predict (Verstrepen *et al.,* 2003). Details regarding yeast and the mechanisms by which it is handled in the brewery are discussed in Section 1.3 and 1.4 respectively.

1.3 Yeast

Yeast are fascinating organisms. They underpin a diverse array of applications ranging from science, biotechnology, medicine and pharmaceuticals to food and beverage production. Nobel prize winners Leland Hartwell and Paul Nurse conducted work on yeast to discover key regulators of the cell cycle. Though, much of the initial academic work on yeast was conducted at the Carlsberg Laboratory in Copenhagen as early as 1883. It was in that year that Emil Christian Hansen used serial dilutions in of yeast to separate cells based on their morphological traits and revealed that different pure cultures of bottom and top fermenters (so called due to their flocculation profile) was the first real pioneer in the power of brewing yeast and its ability to give both unique and reproducible industrial fermentations (Rank *et al.,* 1988). Until comparatively recently in the history of beer, yeast has been known more for what it can produce rather than what it is (Robinow and Johnson, 1991). Due to its relative low cost as a raw material it can often get misused and economised (Jacques *et al.,* 2003). Quain (2006), elaborate on this further by describing this view of yeast to that of a 'supporting actor' in the theatre of brewing. However, yeast is the reason wort is converted into beer and its specific by-products are what

distinguish one beer from another (Lentini *et al.,* 2003). For this reason, the brewing yeast needs to be handled with care and its metabolic requirements need to be considered.

1.3.1 Physiology and characteristics

Since the establishment of yeast genetics by Øjvind Winge in 1935, the comparative research done into yeast physiology has lagged behind somewhat. The essential regulation of physiological aspects of yeast growth, metabolism, their interactions both inter- and extracellularly and how they ultimately die are still in need of further examination to be fully understood. The response induced by yeast in the presence of sugars and oxygen is fundamentally important. A central aspect of the brewing yeast genera is its ability to be 'facultatively fermentative' (Briggs *et al.,* 2004). This means they possess the ability to fully respire sugars to carbon dioxide, water and ATP via oxidative phosphorylation but can also, under the right environmental conditions, switch to fully ferment sugars to ethanol and carbon dioxide with energy being transduced by substrate level phosphorylation via pyruvate metabolism (Boulton and Quain, 2006). A simplified drawing of a yeast cell budding, and its internal components are represented in Figure 1.2. Yeast cell composition varies according to growth environment which includes the presence of oxygen, nutrient concentration, temperature, pH and metabolite build-up (such as ethanol) (Ratledge, 1991).

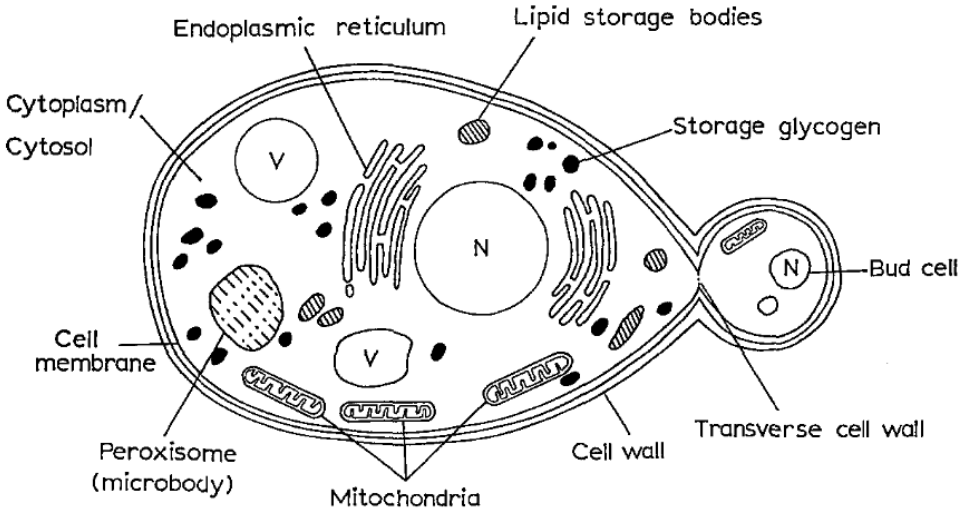


Figure 1.2 Simplified illustration of a typical yeast cell budding. N: nucleus, V: vacuole. (Ratledge, 1991).

1.3.2 Brewing yeast

The brewing process has long been tailored to ensure optimal performance of the brewing yeast and therefore ensure product quality (Quain, 1988). However with increasing pressures put on the brewing yeast to improve productivity, it is becoming apparent that there is more to the interactions between yeast genetics, physiology and environment than first thought. For example, studies conducted on high gravity fermentations found that the concentrations of esters causing off-flavours were disproportionately higher than those from a 'normal' gravity fermentation (Quain, 1988). This suggests there is an upregulation of stress related metabolic pathways in the presence of strong worts but further investigation is required to know the full extent these effects have on brewing yeast genetics and/or phenotype.

Brewing yeast cultures are primarily of the genus *Saccharomyces* and the cells, once inoculated (or pitched) into the wort, will transport sugars, nitrogenous molecules, vitamins and ions through their plasma membrane employing a number of metabolic pathways for growth and fermentation (Stewart, 2016). The yeast strain employed provides the unique characteristic flavours and aroma in beer (Knudsen, 1985) and is central to the whole fermentation process by influencing alcohol production rate by sugar uptake efficiency whilst also being responsible for the production of flavour compounds (Briggs *et al.,* 2004). Of course for the desired product, these metabolites have to be produced in the right quantities and ratios which is why brewers are concerned with optimising this balance (Bamforth, 2008). Meilgaard (1975) reported as many as 110 organic or short chain fatty acids in beer which have been found to be a product of yeast metabolism as well as being present in the wort initially (Boulton and Quain, 2006). Volatile esters are only found in trace amounts (ranging between 0.04 – 32 mg/l) however they impact enormously on the overall taste profile of the beer (Verstrepen *et al.,* 2003).

The selection of the yeast strain is primarily focused on genetic and phenotypic stability (Casey, 1996; Hammond, 1995; Powell and Diacetis, 2007), correct flocculation behaviour (Stratford, 1992; Verstrepen *et al.,* 2003) and its ability to fully attenuate (ferment available sugars) as quickly as possible (Boulton, 1991; Boulton and Quain, 2006; Gibson *et al.,* 2008). The yeast strains used in brewing are varied yet distinct. Historically the brewer would group these yeast based on their flocculation potential; top-fermenting ale yeast and bottom-fermenting lager yeast (Lodolo *et al.,* 2008).

1.3.3 Ale and lager yeasts

There are many differences between ale and lager production. Most of these are based around the yeast strain used and the fermentation conditions it requires. Typical differences are summarised in Figure 1.2. A yeast strain will be chosen based its expected fermentation properties and likelihood to produce a beer of a suitable composition (Boulton and Quain, 2006). The genotype of the yeast strain employed in fermentation of wort is vital due to its ability to produce desired flavour-active metabolites under specific conditions (Boulton and Quain, 2006).

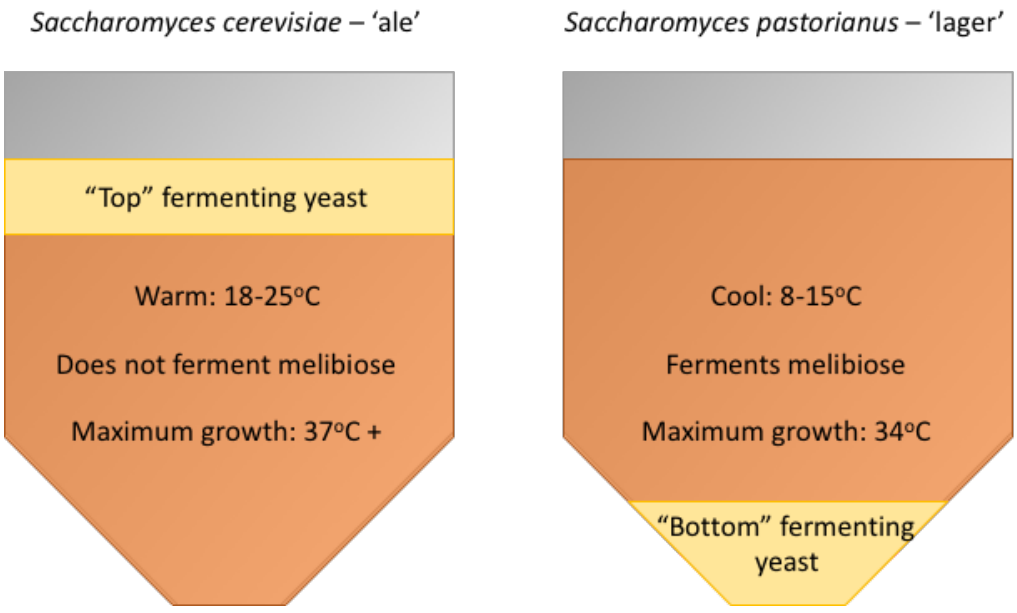


Figure 1.3 Illustration highlighting main differences between ale and lager yeasts (adapted from a table by Stewart (2016)).

Ale type beverages are likely to be the earliest type of beer to be produced by early brewers making the species *Saccharomyces cerevisiae* oldest domesticated brewing yeast (Gonçalves *et al.,* 2016). This early domestication would have begun with early brewers (and wine makers and bakers for that matter) realising that by 'backslopping' a proportion of the fermenting product into the fresh, unfermented food/drink would result in faster and more predictable fermentations (Gallone *et al.,* 2016). By keeping a proportion of yeast from a fermentation which had been deemed to have performed well, an isolated culture becomes further detached from its natural niche and more specialised in this new man-made environment. The domestication of *S. cerevisiae* resulted in highly adapted industrial strains which are genetically and phenotypically distinct from the wild type strains, particularly in the way they are able to ferment efficiently and produce specific ratios of flavour metabolites (Gallone *et al.,* 2016). As emphasised in Figure 1.3, ale type strains are known for rising to the top of a vessel upon completion of the fermentation hence their description as 'top-fermenters'. This occurs because of cell aggregates trapping CO_2 bubbles due to their hydrophobic nature resulting in them floating to the surface (Stewart, 2016). The term 'top fermenter' can be confused due to ale's more commonly being fermented in a cylindrical conical vessels in the modern brewery which promotes 'bottom cropping' in the cone of the vessel to ease the collection of yeast post-fermentation (Hammond, 1993). Ale-type strains (*S. cerevisiae*) are genetically more diverse than lager-type strains (*S. pastorianus*), ferment at higher temperatures (18-25^{o}C) and are able to be grown at higher temperatures (37^{o}C+) (Lodolo *et al.,* 2008; Stewart,

2016). Another phenotypic characteristic of ale yeasts in comparison to lager yeasts is their inability to utilise the disaccharide melibiose due to lacking in the periplasmic enzyme α-galactosidase which is produced by lager type strains (Boulton and Quain, 2006; Box *et al.,* 2012).

Unlike with the *Saccharomyces cerevisiae* fermented ale-type beers, lager beers have only been around relatively recently. Lager brewing emerged in Bavaria during the 15th century (Libkind *et al.,* 2011). Historically, before the invention of refrigeration, lager beer production would be suspended over the warmer summer months resulting in the selection of yeast strains more adapted to cooler temperatures (Dunn and Sherlock, 2008; Gibson and Liti, 2015; Wendland, 2014). It has been known for some time that lager yeasts, *Saccharomyces pastorianus*, are the result of hybridisation events making them alloploidy (Gibson and Liti, 2015). The hybrid appeared to be, based on DNA analysis, partly *S. cerevisiae* (possibly an ale strain) and a strain related to *Saccharomyces bayanus* (Nakao *et al.,* 2009; Rainieri *et al.,* 2003). However, *S. bayanus* itself is a product of interspecific hybrids (Gibson and Liti, 2015). More recently a wild strain, *Saccharomyces eubayanus*, was discovered by Libkind *et al.* (2011) in Patagonia which was a 99.5% match to the non-*S. cerevisiae* portion of the *S. pastorianus* genome (Baker *et al.,* 2015). Since this initial discovery rare isolets of *S. eubayanus* have also been discovered in Tibet (Bing *et al.,* 2014) and North America (Peris *et al.,* 2014). *S. pastorianus* can also be described as aneuploid as their chromosome number differs or contains break

points when compared to that of the wild strains they originate from (Dunn and Sherlock, 2008).

Within *S. pastorianus*, a group of interspecific hybrids, there are two sub-groups characterised on their genotypic and phenotypic homologies which also relate back to the geographic locations they originated from. Group I or 'Saaz' type lager yeasts were named after the region in Bohemia (now the Czech Republic) it was first utilised (Gibson and Liti, 2015) and was also used by the Carlsberg brewery in Denmark (Dunn and Sherlock, 2008). Group II or 'Frohberg' type lager strains were named after a region in Germany (Gibson and Liti, 2015) and were also used in the Netherlands, non-Carlsberg breweries in Denmark and North America (Dunn and Sherlock, 2008). Each group provides unique flavours and fermentation profiles. Both Saaz and Frohberg are sterile hybrids which could impose elevations in deleterious alleles arising in the presence of a selective environment, a function known as Muller's Ratchet (Baker *et al.,* 2015; Gabriel *et al.,* 1993; Muller, 1964). So called as it was Muller (1964) who observed this issue in relatively small asexual populations. Once a mutation occurs it is very rare that it will mutate back to its original form meaning no future offspring will ever produce fewer deleterious mutations than the mother cell. Saaz strains have a slightly improved capacity to grow at lower temperatures (10^{o}C) than Frohberg strains (22^{o}C) (Wendland, 2014). They also utilise sugars differently with Saaz having comparatively poor fermentation efficiency compared to Frohberg, partly due to their poor utilisation of maltose and maltotriose (Baker *et al.,* 2015) which make up 45-65% and 16-26% of all wort sugars respectively (Boulton and Quain, 2006). This

impact on the flavour and aroma as differing amounts of esters are produced (Gibson *et al.,* 2013). Nonetheless, the better attenuation achieved by Frohberg type strains may go towards explaining their dominance in modern industrial-scale brewing (Gibson *et al.,* 2013). Another important consideration is the difference between the extent of aneuploid in Saaz and Frohberg type yeast. Saaz strains have lost a significant proportion of the *S. cerevisiae* genome but retains virtually all that of *S. eubayanus*. Whereas Frohberg strains retains nearly all genetic content from both parents. In both cases it is the *S. cerevisiae* portion of the genome which shows more variation (both in terms of breakpoints and ploidy number) (Dunn and Sherlock, 2008). It has been suggested that due to the relatively low number of generations which have passed (a few thousand) since the hybridisation event which produced *S. pastorianus,* that the aneuploid genomic observations of genome loss, breakpoints and shuffling are most likely the very earliest type of genome rearrangements (Dunn and Sherlock, 2008). The loss of much of the *S. cerevisiae* genome from the Saaz type strains could be equivalent to the proposed rapid loss of genes that occurred in the ancestor of the *Saccharomyces* clade (branch) immediately after it must have undergone its first whole-genome duplication (Byrnes *et al.,* 2006; Dunn and Sherlock, 2008; Scannell *et al.,* 2006).(Byrnes *et al.,* 2006; Dunn and Sherlock, 2008; Scannell *et al.,* 2006)

The varying metabolic properties of the different brewing yeasts can provide huge diversity in the final product. In addition to this, the right brewing strain can also improve the quality of the final product, more so than any other facet

in the brewing process (D'Amore, 1992; Quain, 1986; Quain, 1988). More detail on the genetic make-up, resulting phenotype and the flux involved with brewing strain and environment will assist with future product development, quality and improve consistency and ultimately, profit margins for the brewer.

1.3.4 Industrial vs laboratory yeast strains

Laboratory strains of *Saccharomyces cerevisiae* occupy a completely different niche compared to their industrial counterparts. Non-industrial isolates have been selected based on their ability to proliferate quickly in nutrient rich media (Borneman *et al.,* 2011). When put under the same growth environments these strains will produce vastly different metabolic products (Mortimer and Johnston, 1986).

S. cerevisiae is widely used as a model organism in many areas of scientific research. In 1996 the yeast strain S288c was the first eukaryote genome to be fully sequenced (Goffeau *et al.,* 1996). It has been used in the field of metabolic engineering to better understand the processes and effects of genetic modifications such as gene deletion, insertion, over expression and modulation (Otero *et al.,* 2010). S288c was isolated through genetic crosses and selection was based on non-flocculence and the requirement of minimal nutrition for growth (Mortimer and Johnston, 1986). Yeast genomics has been built on this foundation of the S288c genome with the idea that this single consensus would offer a scaffold upon which other genomic sequences could be hung, built and compared (Engel *et al.,* 2014). Since those early days of genome sequencing it has become more clear that the genome of any given species (not just that of

yeast) can contain a great deal of complexity and diversity meaning the S288c reference genome can vary significantly from that of any given strain and can only offer a point of anchorage from which to explore metabolic and phenotypic variation (Engel *et al.,* 2014). One of the major differences between laboratory strains and industrial brewing strains when considering their genetic composition is the matter of ploidy. Cells of differing ploidy usually exhibit different developmental, morphological and physiological characteristics (Galitski *et al.,* 1999). Laboratory strains of *S. cerevisiae* are typically either haploid (contains a single complement of DNA) or diploid (containing a double compliment of DNA) whereas most brewing yeasts are polyploid (more than two compliments of DNA) or aneuploid (contain fractions of whole copies of the normal haploid DNA content) (Hammond, 1996). The use of haploid strains excludes a large portion of the industrial *S. cerevisiae* strains that have lost the ability to sporulate (this represents the vast majority of brewing yeasts) (Gallone *et al.,* 2016). Studies which use haploid laboratory strains rather than natural/industrial strains cannot explore fully the nature of brewing yeast such as the patterns of domestication like polyploidy, aneuploid and heterozygosity (Gallone *et al.,* 2016). Technologies associated with genome sequencing have advanced while costs have decreased meaning the genetic complexity of industrial brewing strains can be investigated by individuals and small groups in greater detail than ever before.

Despite the change in scale and modernisation of the process, beer production is essentially the same as it was before industrialisation. A proprietary strain of brewing yeast can be a closely guarded industrial secret as a brewery will be

aware of the characteristic properties their strain bestows on the final product (Boulton, 2015). In the context of progression, the industry has had to consider the concerns of consumers when it comes to the use of genetically modified yeast strains due to a general negative attitude regarding their use and the adverse opinion that a natural product like beer should not be tampered with genetically (Tenbült *et al.,* 2008). Genetically modified yeasts have never been used for commercial beer brewing (Boulton, 2015).

However, with fast and affordable genome sequencing tools at a researcher's disposal, there is an opportunity to analyse wild yeast isolates and new hybrids then link specific traits and phenotypic characteristics to the biological processes required during a brewery fermentation (Gibson *et al.,* 2017). This could offer genuine benefits through improved efficiency and greater product variety.

1.4 Brewery yeast handling

There are many stages of the brewing process where the brewing yeast needs to be managed. Industrial scale yeast management termed as 'yeast handling', includes the propagation, cropping, storage, pitching and repitching of the yeast (Lodolo *et al.,* 2008). The quality of the brewing yeast strain is influential upon final product quality and for achieving consistency from one fermentation to the next (Briggs *et al.,* 1982; Pickerell *et al.,* 1991; Powell *et al.,* 2000; Powell and Diacetis, 2007). After careful choice of the strain used in the brewing process, the upmost care needs to be taken to ensure there are no negative

impacts on the overall condition of the population due to poor handling prior to pitching in order to achieve the required flavours and aromas (Bamforth, 2008; Pickerell *et al.,* 1991; Quain, 1988). Brewing yeast which is ready to be pitched into a fermentation can originate from three different sources; a proportion of the previous fermentation can be added to the next, cells can be propagated freshly or some brewers, especially those operating on a smaller scale may wish to use dried yeast. In all cases upmost care needs to be taken in the maintenance of these cultures as they all have different requirements based on source, equipment and strain. It is the precision taken at the beginning of the process with all the raw ingredients which includes the yeast culture which will ensure consistency and therefore end product quality.

1.4.1 Storage

Brewers need to ensure the longevity of their brewing strain, after all, without it they cannot produce their signature beers associated with their brand. For this reason, the long-term quality of the production yeast strain are safeguarded by being maintained in either freezers at -70^{o}C or cryopreserved in liquid nitrogen, which equates to a storage temperature of around -196^{o}C (Hulse *et al.,* 2000; Wellman and Stewart, 1973). This is done to maintain the genetic integrity of the sample over extended periods of time. It cannot be presumed however that the samples removed from cold storage are the same as when they were deposited. To ensure the use of the correct strain (meaning it has not altered genetically during the freeze-thaw process nor is it a contaminant) the use of general microbiology and genetic techniques such as

DNA fingerprinting are implemented (Boulton and Quain, 2006; de Lopes *et al.,* 1998; Quain, 1986; Richards, 1967; Wightman *et al.,* 1996). These strains are then maintained on solid agar plates or slopes at 4^{o}C for up to 6 months for quicker and easier access. Master slope purity is ensured by spreading a single colony. Due to equipment availability and also for extra security production strains are regularly stored off-site at a regulated facility such as NCYC (National Collection of Yeast Cultures) (www.ncyc.co.uk) or CARA technology (www.cara-online.com) to name a few.

1.4.2 Propagation

Once removed from storage the yeast needs to be grown up in liquid media to a sufficient volume in order to sustain a full scale fermentation (Kennedy and Smart, 2000). This is done in a stepwise manner to avoid bacterial contamination and keep the cells in a non-stressful environment to produce inoculum which is in peak physical condition (Lodolo *et al.,* 2008; Voigt and Walla, 1995). Normally the production yeast will be grown in fresh sterile wort under aerobic conditions to encourage biomass production (Cahill *et al.,* 2000). For ease of use and storage, small-scale craft brewers often opt for the use of dried yeast which does not require intensive propagation steps or oxygenation (Quain, 2006). Once the brewer has the required yeast cell biomass there is a final check of the condition of the cells performed before use in a fermentation. The requirement is for the population to have a high percentage of viable cells as a successful output can only be generated by cells of high vitality. The culture

is now deemed ready to be added to the sweet wort for fermentation. This process is known in the industry as ‘pitching’.

1.4.3 Serial repitching

Rather than start afresh before every fermentation, it is common practice in breweries to remove and store the yeast at the end of fermentation for the use in subsequent fermentations (Boulton, 1991; Jenkins *et al.,* 2003; Powell and Diacetis, 2007; Smart and Whisker, 1996). To do this the brewer will extract the cropped yeast from the fermenter after cooling in two or three portions which are termed as ‘cuts’. The first cut comprises predominantly of dead cells along with other proteinaceous debris from the fermentation termed as ‘trub’ (Boulton and Quain, 2006). This first cut, and occasionally the uppermost (third) cut due to it containing a higher concentration of less flocculent yeast cells (O’Connor-Cox, 1997) is discarded as either waste, an expensive option, or it can be sold for animal feed and water-soluble vitamins (Kerby and Vriesekoop, 2017; Levic *et al.,* 2010). Spent yeast can also be sold as a source of human nutrition. Rather than the whole yeast cells, an extract is produced and used in yeast speads and flavouring (Kerby and Vriesekoop, 2017; Sombutyanuchit *et al.,* 2001). The second cut is heterogenic in nature as it consists of old, middle aged and virgin cells (Lawrence *et al.,* 2013). This portion of the cropped yeast is transferred to a sterile collection vessel and stored (usually at 2-4^{o}C) until it is required for repitching into a fresh fermentation. As storage conditions can only maintain the yeast and not improve them, it is best practice to refrigerate cropped yeast for as short a time as possible whilst mixing via an impeller or

through recirculation to minimise cellular damage (Loveridge *et al.,* 1999; O'Connor-Cox, 1997). To avoid yeast cell deterioration and possible contamination it is advisable to remove the yeast as soon as possible from the completion of one fermentation and then into the subsequent (O'Connor-Cox, 1997). Yeast sedimenting in the cone of cylindroconical vessels has been found to produce a stratification of aged cells (Kuřec *et al.,* 2009; Lawrence *et al.,* 2013; Powell *et al.,* 2004). This can lead to sub-populations of phenotypically and/or genotypically diverse yeast being selected for during the cropping process culminating in changes to fermentation performance in subsequent fermentations (Powell *et al.,* 2004). The repeated stress of being repitched along with the stress of being contained within a sediment at the end of fermentation in an environment of nutrient starvation, ethanol stress, osmotic and hydrostatic pressure whilst also being chilled (to aid clarification of the beer) leads to cell deterioration including the production of respiratory deficient (petite) mutants (Lawrence *et al.,* 2013; Powell *et al.,* 2004). An alternative method to traditional 'cool cropping' after the fermentation has chilled, is to 'warm crop' prior to chilling whilst some yeast is still in suspension (Loveridge *et al.,* 1999; Quain *et al.,* 2001). The prime time to do this is once all sugars have been utilised but the reduction of vicinal diketones (VDK) is yet to be completed (Loveridge *et al.,* 1999). The main drawback to this method is that it involves an extra cut being taken from the vessel; the warm crop and a second 'conventional' cut once the VDK levels have been reduced however the extra labour cost is returned in the form of process consistency due to the

collection of less stressed, more homogenous yeast from the cone (Loveridge *et al.,* 1999; Powell *et al.,* 2003; Quain *et al.,* 2001).

Consistency during fermentation creates the foundation for maintaining the quality of the beer produced and also for the recovery of good quality yeast which can be repitched into subsequent, consistent fermentations (Briggs *et al.,* 2004; Millar *et al.,* 2012; Quain, 1988). Yeast cultures are theoretically immortal, yet continuous stress imposed on a production culture over successive generations has been found to be the source of deterioration of production yeast (Powell and Diacetis, 2007; Smart and Whisker, 1996). Consideration of the effects of serially repitching yeast is not a simple one. Different yeast strains react differently to the variable stresses of a fermentation, wort composition and stochastic gene mutations (Gibson *et al.,* 2007; Powell *et al.,* 2000; Powell and Diacetis, 2007; Verstrepen *et al.,* 2005). For this reason, it is common practice to limit the number of times a yeast culture is repitched based on the strain and wort composition. Brewers tend to restrict the number of serial repitchings to 15-20 for ale strains and 7-15 serial repitchings for lager strains before reintroducing freshly propagated yeast into the process (Boulton, 1991; Powell and Diacetis, 2007; Smart and Whisker, 1996). A brewer may become aware of the affects of continual serial re-pitching if the production yeast deteriorates in condition due to changes in fermentation performance. This would become apparent due to a decline in fermentation performance from reduced utilization of nitrogenous compounds (Miller *et al.,* 2013), altered sugar assimilation rates (Jenkins *et al.,* 2003) and genetic drift (Casey, 1996; Gilliland, 1962; Gilliland, 1971; Gorter de

Vries *et al.,* 2019; Jenkins *et al.,* 2003; Lawrence *et al.,* 2013; Sato *et al.,* 2001; Wightman *et al.,* 1996). Furthermore, the number of times a yeast culture can be repitched can be influenced by process parameters. For example, the use of high gravity wort creates elevated stresses during fermentation (such as osmotic and ethanol) meaning the number of repitches that the yeast culture can undergo must be reduced (Stewart, 2009).

1.4.4 Fermentation

The aim of fermentation management is to control and standardise conditions so that the by-products of yeast growth and metabolism are produced within the anticipated specifications which includes time to complete. For this to happen the growth conditions must be tightly regulated. In addition to the carbon provided by the fermentable sugars in the wort, yeast also requires nitrogen, a range of metals (sodium, zinc, magnesium, copper, potassium, iron and manganese) and other inorganic nutrients (chloride, phosphate and sulfur) (Berry and Slaughter, 2003). The metabolic pathways induced by yeast during a fermentation are complex. The reactions required for cell growth, division and the fermentation process are driven by the conversion of the wort sugars into ethanol (Boulton and Quain, 2006). During a beer fermentation the production yeast will take up and metabolise the sugars with the major characteristic difference between ale and lager strains being the ability of lager yeasts to ferment melibiose (Box *et al.,* 2012; Olaniran *et al.,* 2017; Stewart and Russell, 1986). Glucose is the favoured carbon source for yeast and will readily metabolise it as an energy source over other sugars in the wort. However, the

sugar available in the largest proportion in the wort is maltose. The glucose available in the initial stages of the fermentation triggers a regulatory cascade causing a repression in the yeasts ability to uptake and metabolise maltose (New *et al.,* 2014; Rolland *et al.,* 2002; Verstrepen *et al.,* 2004). The proficiency in which a brewing yeast strain is able to switch between glucose and maltose utilisation is of upmost importance in the brewing industry. The pause in fermentation progression as this switch takes place is known as the "maltose lag" (Berry and Slaughter, 2003). A yeast strain exhibiting a smooth transition from glucose to maltose utilisation would be of value to the brewing industry however a strain which could utilise both these sugars simultaneously would be unaffected by the "maltose lag" hence increasing the rate of fermentation and therefore brewery productivity. This would rely on a yeast strain which maintains gene regulation of both pathways rather than repressing the uptake of the non-preferential maltose over glucose if it is available in the wort (New *et al.,* 2014). To visualise why glucose is a preferential carbon source, Figure 1.3 shows the metabolism of glucose and maltose in the glycolytic pathway which results in the production of ethanol, carbon dioxide and most importantly for the cell; ATP (adenosine triphosphate).

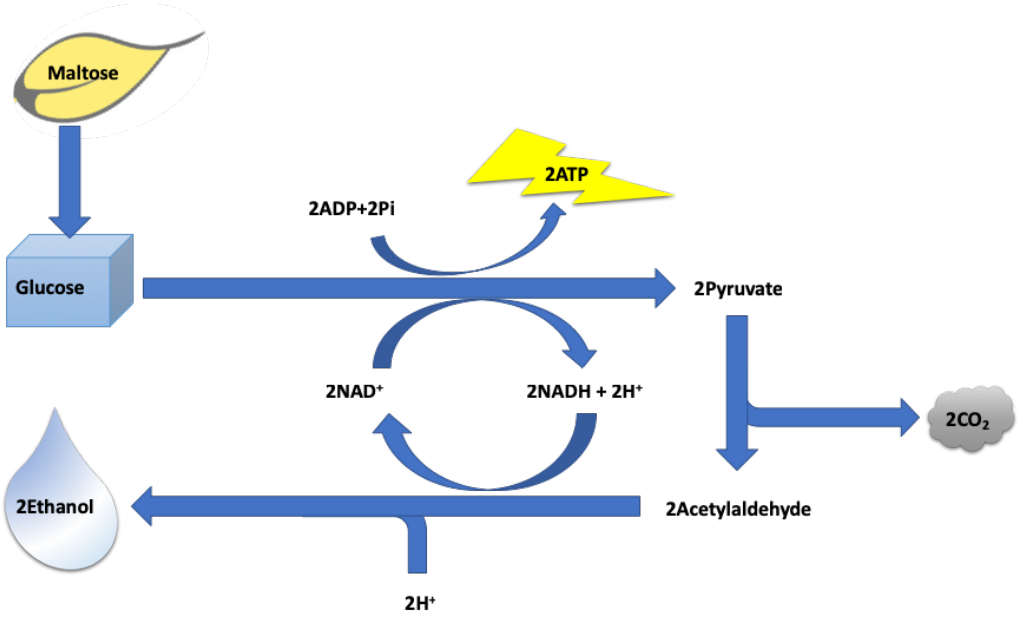


Figure 1.4 Simplified version of the glycolytic pathway. Starting from glucose as a carbon source creates the shortest and therefore the most efficient pathway. In order to breakdown maltose into glucose, the yeast must utilize the enzyme maltase. The production of ethanol is beneficial to the brewer however the product of value for the yeast is ATP.

The complete glycolytic pathway consists of ten chemical reactions in which two molecules of ATP are produced for each glucose molecule. Two of these reactions are reduction-oxidation (redox) reactions whereby the oxidation state of NADH (Nicotinamide adenine dinucleotide) is changed via the transfer of electrons (Dijken and Scheffers, 1986). NADH is oxidised by the loss of electrons whilst NAD^+ is reduced by gaining electrons. This constant reconstitution of NADH assures the continuation of the glycolytic pathway (Alba-Lois and Segal-Kischinevzky, 2008). ATP is widely known as the energy currency of life. During ethanol fermentation the yeasts' requirement for ATP

for growth and other metabolic functions will all derive from glycolysis (via the glycolytic pathway Figure 1.4). ATP synthesis is also controlled by the availability of ADP (adenosine diphosphate) in the cell making it a rate limiting step in glycolysis (Berry and Slaughter, 2003). Energy is released from ATP via hydrolysis (ATP + H2O $\rightarrow$ ADP + Pi) (Dahout-Gonzalez *et al.,* 2006). ATP is utilised by the production yeast during fermentation for biosynthetic and other energy-demanding reactions. The fact that *S. cerevisiae* and *S. pastorianus* can produce high yields of ethanol from glucose at such an efficient rate lead to its profuse use throughout the brewing industry. The conversion of wort sugars to ethanol is not a complete one though, even in non-stressful environments, with the actual amount representing around 88% of the theoretical (Boulton and Quain, 2006). The deficit can be explained by the yeast directing some carbon towards the production of new biomass and metabolic by-products which include glycerol, organic acids and flavour compounds (Berry and Slaughter, 2003; Dijken and Scheffers, 1986).

Yeast metabolise sugar for the production of ATP but the formation of ethanol and glycerol are integral to maintaining the redox balance. Ethanol ensures NADH is reoxidised whilst glycerol arises from excess NADH generated in the assimilation of wort sugars into yeast cell biomass (Dijken and Scheffers, 1986). Glycerol is an important osmoprotectant (Mager and Varela, 1993; Slaughter, 2003) and contributes to the body and mouth-feel of beer (Boulton and Quain, 2006). In addition to this, glycerol and fatty acids form one of many classes of lipid, glycerolipids, which are integral elements to yeast cell membranes (Klug and Daum, 2014). Lipids are components of cell membranes and can also be

stored in organelles for energy sources, structural elements, signalling molecules, or mediators of membrane fusion and apoptosis (Escribá *et al.,* 2008; Klug and Daum, 2014). Based on structure and function, lipids can be sorted into eight classes; fatty acids, glycerolipids, sterols, glycolipids, polyketides, sphingolipids and prenol lipids (Fahy *et al.,* 2011; Klug and Daum, 2014).

For membrane function, sterols and unsaturated fatty acids (UFA's) are of particular importance. Only very small quantities of UFA's are available in the wort so the cell must make up for the short fall by synthesising these lipids within the cell (Boulton and Quain, 2006). To do this, the cell requires a supply of oxygen. The implications of this is that synthesis of sterols and unsaturated fatty acids takes place during propagation and the initial phase of fermentation and are stored once the fermentation is underway. During fermentation the sterols required for cell membrane maintenance, permeability, fluidity, membrane-bound enzyme activity and growth are utilised from these stores (Lees *et al.,* 1995). Consequently, the quantity of stored sterol is a limiting factor for cell division as the production of a daughter cell significantly depletes the amount of sterol in the mother cell (Boulton and Quain, 2006). This highlights the need for oxygenation of the yeast culture during the repitching before the subsequent fermentation can take place (Boulton and Quain, 2006; Hammond, 2000; Hulse, 2003).

Growing yeast cells undergo an asymmetric form of cell division known as "budding" to generate a new 'daughter' cell (also known as a 'virgin' cell) from a 'mother' cell (Boulton and Quain, 2006). Once a site for bud development is

chosen the mother cell will establish an axis of polarity built upon an actin cytoskeletal framework (Pruyne *et al.,* 2004). Post bud emergence, the mother cell will restrict cell growth to the bud as the cell continues to duplicate and segregates organelles until the bud reaches a slightly smaller size than the mother. At this point mitosis and cytokinesis is initiated and the creation of a septum between the mother and daughter cells to complete the separation (Pruyne *et al.,* 2004).

Conditions must be acceptable in order for this replication to take place and as the virgin cell produces its first daughter cell it becomes itself a generation older, g1 (Kale and Jazwinski, 1996). This implies that a yeast cultures always contain the following proportion of cells; 50% virgin cells, 25% g1 cells, 12.5% g2 cells, 6.25% g3 cells and so on… (Powell *et al.,* 2003). As this theoretically makes the average age of a yeast culture very young, it could hypothetically be used indefinitely, i.e. be considered to be immortal (Powell *et al.,* 2000). In a fermentation environment, the reality is quite different. The production yeast within a fermentation will maintain a large proportion of young cells however stress related changes can accumulate including genomic mutations which can cause an overall loss of fitness (James *et al.,* 2008; Pedersen, 1994; Powell *et al.,* 2000). It is for this reason that fresh cultures are propagated and reintroduced into the fermentation procedure (Section 1.4.2). Finally, aging should be taken into consideration. The description of aging in yeast cultures is multifaceted, it comes in the form of 'replicative aging' i.e. the number of times a cell has undergone division, 'chronological aging' i.e. the time (in hours/days) a cell has been alive and can be referred to in the brewing industry

chronologically by the number of times a culture has been serially repitched (Powell *et al.,* 2000). Yeast are known to have a finite replicative capacity which is determined by strain and environment, this maximum limit is known as the 'Hayflick limit' (Barker and Smart, 1996; Hayflick, 1965). However under fermentation conditions, a yeast cell is likely to die of stress related causes rather than reach this maximum limit. During a typical fermentation a yeast culture will divide around 2-3 times (Powell *et al.,* 2003). All the yeast cells within a fermentation are not synchronised so division will not happen simultaneously for all cells (Millar *et al.,* 2012).

Physiological changes associated with cell aging are; increase in bud scars (Bartholomew and Mittwer, 1953; Egilmez *et al.,* 1990), increase in cell size, surface appearance becomes granular (Mortimer and Johnston, 1959), cell surface becomes wrinkled (Mortimer and Johnston, 1959; Müller, 1971) and finally the cell will become incapable of further replication, enter a physiological state of 'senescence' followed by death and lysis (Barker and Smart, 1996; Mortimer and Johnston, 1959). Cell death may also come in the form of necrosis due the accumulation of irreparable damage of intracellular components (Powell *et al.,* 2000). This has been found to be the case if yeast cultures are exposed to excess stress or repeatedly exposed to low-level stress (Moench *et al.,* 1995; Powell *et al.,* 2000).

Cells of the same genotype also demonstrate variation in their lifespan. Gene expression is known to vary over a cells lifespan (Egilmez *et al.,* 1989) which enables cells to produce the proteins/enzymes they need relevant to their current stage of life (Fleming *et al.,* 1988). Genetic and phenotypic variation

between yeast cultures leads to unpredictable fermentation performance meaning the profile of a fermentation containing repitched yeast can be altered compared to the previous time the yeast culture was used (Powell *et al.,* 2003). The proportion of young to old cells within a culture can contribute to changes in fermentation profile highlighting the importance of mitigating aged cell selection via the use of warm cropping (Section 1.4.3, Loveridge *et al.,* 1999; Quain *et al.,* 2001). Virgin cells have an extended lag phase, ferment at a slower rate, are smaller in size, have a reduced flocculation capacity which slows clarification, produces yeasty off flavours, causes filtration issues (Powell *et al.,* 2003) and are slower at recovering from cold shock (Smart, 2001) which is expected to be due to an energy trade-off between growth and cellular repair (Powell *et al.,* 2003; Smart, 2001). This is not to say that a fermentation containing only aged cells would be preferable, as they can flocculate early leaving less cells in suspension and causing the time for attenuation to increase (Barker and Smart, 1996; Powell *et al.,* 2003) and the genetic and physiological changes which occur can be unpredictable. Beer quality is actively influenced by the biochemical reactions and performance of the production yeast used during fermentation (Powell *et al.,* 2003). For this reason, aging needs careful consideration when choosing a yeast strain for a particular application as strain specific divisional capacity can be used to make assumptions about fermentation performance, for example, yeast with a lower Hayflick limit would be unsuitable for a chemostat fermentation (Barker and Smart, 1996; Powell *et al.,* 2000).

1.5 Brewing yeast quality

Production yeast should be in peak physical condition before being pitched into a fermentation. Specifically, the yeast culture should be high in both viability and vitality (and contain no contamination) (Boulton and Quain, 2006). Viability is the ratio used to measure the number of live cells with respect to the amount of dead. Vitality is the measure of the physiological condition of the live cells. The use of viability alongside the volume of cropped yeast can reflect the performance of the previous fermentation, for example a smaller than usual volume of cropped yeast which is low in viability would suggest that the previous fermentation performed poorly (Boulton and Quain, 2006; Mochaba *et al.,* 1998). Viability tests need to be quick and reliable, so brewers can make a judgement as to whether they use the culture in a fermentation (Briggs *et al.,* 2004). Vitality tests take the idea of viability one step further, the live proportion of cells may express differences in their physiology which would produce inconsistencies in fermentation performance and therefore beer quality (Boulton and Quain, 2006) and so a measure of vitality would give rise to a value which could be related to the predicted performance in a subsequent fermentation. Physiological function tests, such as the acidification power test, Intracellular pH test, fermentation capacity tests and membrane fluididty tests are likely to be some of the best ways of predicting the quality of the viable proportion of yeast cells cropped from a fermentation (Iserentant *et al.,* 1996; Lodolo *et al.,* 2007; Pickerell *et al.,* 1991; Weigert *et al.,* 2009) The vital condition of the yeast culture is important for predicting the success of a fermentation and is said to be conditional on the ability to initiate metabolism

rapidly once placed into nutrient rich environments (Kaprelyants and Kell, 1992; Lodolo and Cantrell, 2007), the ability to endure stress factors (D'Amore, 1992; Majara *et al.,* 1996; Moench *et al.,* 1995; Stanley *et al.,* 2010), storage conditions (Majara *et al.,* 1996; Sall *et al.,* 1988) and the overall physiological state of the population (Mochaba *et al.,* 1998). These elements are often put under more scrutiny when the culture has been serially repitched as repitched yeast can accumulate undesirable qualities (Briggs *et al.,* 2004; Powell and Diacetis, 2007; Quain *et al.,* 2001; Smart and Whisker, 1996).

1.5.1 Brewing yeast stress factors

In brewery fermentations there is a constant balancing act between controlling environments which are optimal for the production of high quality beer and those which optimally sustain yeast viability and vitality. However, it is usually the yeast culture which has to compromise with a sub-optimal environment meaning the process inflicts a variety of stressful conditions on the yeast cell. Briggs *et al.,* (2004) note that during an industrial fermentation brewing yeast undergo fluctuations in osmotic potential, ethanol concentration, oxygen concentrations, pH, temperature and nutrient availability. It is important to remember that as a unicellular organism which is non-motile, yeast will rely solely on physiological mechanisms to cope with these environmental changes (Gibson *et al.,* 2007).

These environmental changes are best described in the delineated graphs in Figure 1.5. Here it is possible to see that the brewing yeast undergoes these stresses in a consecutive order, most often in combination with another stress.

At the end of the fermentation phase the yeast is cropped and a fraction stored under beer at a low temperature (3-4^{o}C) until used in subsequent fermentations (Briggs *et al.,* 2004).

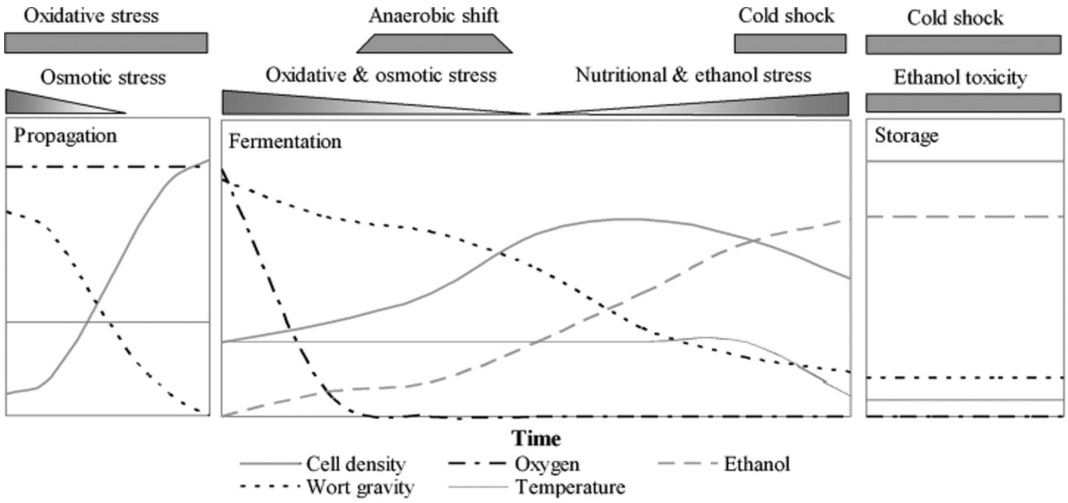


Figure 1.5 Schematic representation of the stresses imposed on yeast during propagation, fermentation and storage (Gibson *et al.,* 2007).

As mentioned previously, the extent of these stresses can be exacerbated by certain brewing techniques such as serial repitching. The act of serial repitching repeatedly exposes the culture to the same sequential stresses which can lead to a build-up in undesirable physiological qualities (Barker and Smart, 1996; Briggs *et al.,* 2004; Powell and Diacetis, 2007; Quain *et al.,* 2001), genetic mutations (Powell and Fischborn, 2010; Powell and Diacetis, 2007), and can cause a sub-population of genetic/phenotypic variants to be selected for (Ito *et al.,* 2009; Powell *et al.,* 2004, 2003; Rando and Verstrepen, 2007). In the case of chemostat fermentations, the yeast culture is held in the exponential phase

for extended periods adding a time element to the nutritional and ethanol stresses encountered.

1.5.2 Oxidative stress

Due to the presence of oxygen at the beginning of a fermentation being fundamental for the synthesis of unsaturated fatty acids and sterols, the wort is fortified with oxygen prior to yeast inoculation (Boulton and Quain, 2006). However, contrariwise to this, the presence of oxygen can lead to the generation of reactive oxygen species (ROS), a normal consequence of yeast cellular metabolism under aerobic conditions (Gibson *et al.,* 2008). Brewing yeast is also aerated (oxygenated) during the propagation stage. After the brewing yeast has been inoculated into the wort, oxygen is consumed within the first twelve hours of fermentation creating an anaerobic environment for fermentation to continue under until completion (Briggs *et al.,* 2004). Common ROS generated within the yeast cell under these aerobic conditions include the super oxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) (Beckman and Ames, 1998; Gutteridge and Halliwell, 2000). These act to oxidize nucleic acids, proteins, lipids and carbohydrates, resulting in damaged membrane activity and cellular functions (Belinha *et al.,* 2007). Mitochondrial damage can also prompt the generation of respiratory deficient 'petites' (Gibson *et al.,* 2008). As a consequence of this, the number of times a particular batch of yeast can be cropped and re-pitched may be determined by its ability to mitigate the effects of internal ROS production following every round of oxygen exposure (Gibson *et al.,* 2008).

1.5.3 Osmotic stress

Brewing yeast experience osmotic stress both during propagation and fermentation. At the start of these process steps the high concentration of sugars in the wort causes an imbalance between intracellular and extracellular osmolarities, known as hyper-osmotic stress. The extent of hyper-osmotic stress is exacerbated when using high gravity worts. The brewing yeast needs to cope with the changes in osmotic pressure throughout fermentation as the concentrations between the solutes within the cell shift between lower to higher than that in the surrounding environment. The yeast will work to keep a positive turgor but avoid water fluxes either into (during hypo-osmotic stress) which would cause the cell to burst or out of the cell (during hyper-osmotic stress) causing plasmolysis and dehydration (Kempf and Bremer, 1998). Because yeast do not possess the active transport mechanisms required for water, the correct turgor is sustained by the control of a group of osmotically active solutes in the cytoplasm which are either synthesised within the cell or accumulated via uptake from the environment (Kempf and Bremer, 1998). Two of the most important osmo-protectants in brewing yeast are trehalose and glycerol (Hounsa *et al.,* 1998). Under moderate osmotic stress yeast cells will synthesise glycerol from carbon sources available in the environment, however, if the carbon has been depleted then the yeast must hydrolyse glycogen and/or triacylglycerol into glycerol (Hounsa *et al.,* 1998). Trehalose acts as a storage carbohydrate by accumulating in the yeast cell during its stationary phase. It is mobilised during periods of growth initiation to provide

yeast with a carbon source (Hounsa *et al.,* 1998) but is also significant for the survival of cells which are under more severe osmotic stress, cells exposed to toxic chemicals (including high ethanol concentrations), dehydrated cells and cells exposed to heating/freezing by preserving protein folding (Levy *et al.,* 2012). Tsl1, a protein involved in the synthesis of trehalose, was found by Levy *et al.,* (2012) to be more prevalent in slow growing yeast cells within a clonal population which, in turn, have a higher replicative age.

1.5.4 Ethanol stress

Ethanol is a highly important compound to consider when dealing with yeast vitality during and after a fermentation. Ethanol is a high value product and the primary compound produced in almost all brewery fermentations so the synthesis of ethanol from fermentable sugars by brewing yeast cannot be prevented or bypassed. As the fermentation progresses, ethanol concentrations rise to increasingly toxic levels; under normal fermentations this range of ethanol concentrations can be 3-6% (v/v) although high gravity brews can rise to over 10% (v/v) (Briggs *et al.,* 2004). Ethanol toxicity on the yeast cellular membranes seem to be the major target for damage but other specific effects have been summarised by Gibson *et al.,* (2007) in Table 1.1.

Table 1.1 Table of cellular targets for ethanol toxicity (Gibson *et al.,* 2007).

Effect of ethanol toxicity on yeast physiology	Source
Growth inhibition / reduced cell size	Canetta *et al.,* 2006
Reduced viability, reduced respiration and glucose uptake	Pascual *et al.,* 1988
Reduced fermentation rate	Fernandes *et al.,* 1997
Enzyme inactivation, lipid modification, loss of proton motive force across the plasma membrane	Mizoguchi and Hara, 1997; Petrov and Okorokov, 1990
Increased membrane permeability	Marza *et al.,* 2002
Lowering of cytoplasmic pH and the induction of respiratory-deficient mutants	Chi and Arneborg, 1999; Ibeas and Jimenez, 1997; Jiménez *et al.,* 1988

A crucial observation from Table 1.1 is that repeated exposure to ethanol during fermentation can have an additive effect for these cellular targets including an increase in the abundance of respiratory deficient 'petite' cells. If at a high enough concentration in the re-pitching inoculum, petites can reduce fermentation rates in addition to producing unfavourable flavours (Debourg *et al.,* 1991; Ernandes *et al.,* 1993; Morrison and Suggett, 1983; Šllhánková *et al.,* 1970). Ristow *et al.,* (1995) found that ethanol is not an influential mutagen of yeast mtDNA which has lead to the suggestion by Ibeas and Jimenez, (1997) that petite induction via ethanol toxicity is solely down to the damage induced

on the mitochondrial membranes. To highlight this an ethanol tolerant yeast strain was compared to a strain which was sensitive to ethanol (Chi and Arneborg, 1999). Here the tolerant strain was found to have comparatively low levels of the petite mutation, relatively high ergosterol/phospholipid ratio, high phosphatidylcholine content and a high long-chain fatty acid component. Gibson *et al.,* (2007) theorises that it is the long-chain fatty acids which have a role in neutralising the fluidising affect of ethanol on the membranes.

1.5.5 Temperature stress

As mentioned previously in Section 1.5.1, during the industrial scale beer production process there are times when the production yeast must be chilled/cooled (2-4^{o}C). This time in storage allows for vessel cleaning and provides flexability in the supply chain (Somani *et al.,* 2012). Maintenance of the yeast in storage is required so that highly viable and vital yeast can be pitched into the subsequent fermentation (Section 1.5). A thermal downshift to 4^{o}C results in an increased expression of genes linked to the synthesis and degradation of trehalose and glycogen (Kandror *et al.,* 2004; Murata *et al.,* 2006). Somani *et al.* (2012) found strain specificity was required for storage temperatures. For example they found that the lager strain W34/70 benefitted from being stored at 10^{o}C by increasing initial sugar uptake once pitched into a fermentation. Trehalose content has been correlated with thermotolerance in against heat stress (Hottiger *et al.,* 1987). Heat shock upon being pitched into fresh wort is more of an issue in ale fermentations (20-25^{o}C) rather than lager fermentations (10-15^{o}C) however the heat shock response can also be of

significance as there is a functional overlap with the response from ethanol exposure (Piper, 1995).

1.5.6 Nutrient Limitation

When nutrients become limiting, as they do towards the end of a fermentation, yeast responds by ceasing growth and entering into stationary phase (Gasch and Werner-Washburne, 2002; Werner-Washburne *et al.,* 1993). In this stage the population is mainly unbudded, virgin cells (G_o) (Werner-Washburne *et al.,* 1996). Entry into this phase involves a complex metabolic network of reactions so that the yeast can survive until they are returned to an environment with sufficient nutrients for growth (Werner-Washburne *et al.,* 1993). Again, it is the reserves of glycogen and trehalose which stabalise the yeasts membranes and maintain cellular function during nutrient limitation as carbon energy reserves (Lillie and Pringle, 1980).

1.5.7 Yeast stress response

Yeast cells have developed both transient and long-term responses to cope with highly diverse environmental conditions. These defensive mechanisms in yeast are initiated upon sensing environmental changes in the aim of protecting itself by mitigating the stress and maintaining its most critical processes. These mechanisms associated with adaptation are what lead brewers to choose certain yeast strains in the first place. However, as the industrial process becomes more intensified (such as with the use of very high gravity brewing) it is these cellular responses which are causing unpredicted changes in the final

product. Fluctuations in the external environment such as those described in Section 1.5.1 can result in a variety of cellular perturbations that disrupt enzyme activity, metabolic fluxes, destabilise cellular structures, perturb chemical gradients and lead to overall cell instability (Gasch, 2003). It has been shown that the cellular responses triggered by *S. cerevisiae* are unique to the stress it is exposed to with genes not only being induced but also repressed creating the ability for the cell to make finely tuned adjustments in response to the environmental shifts (Causton *et al.,* 2001). An example of this is the yeast cells entry into stationary phase in the event of carbon limitation. The speed at which yeast sense the lack of fermentable carbon and then react with an adaptive response is central to ensuring their survival. Kuhn *et al.* (2001) describe the overall drop in transcription and translation rates in the event of diminished fermentable carbon sources being related to downregulation in cell cycle, growth and protein synthesis however there was upregulation in respiratory metabolism. The transcriptional regulation is slower than translational regulation. Translational regulation of proteins synthesised from mRNA allows for more instantaneous responses via the rapid increase/decrease of specific proteins (Kuhn *et al.,* 2001). This means that although the cell is restricting protein translation towards growth it can still translate proteins which are essential for survival. Cells exposed to comparatively mild stresses can induce resistance to not only the stress factor used but can also develop cross resistance against other stresses (for example a mild dose of heat stress provides resistance to more severe heat, osmotic and oxidative stress) (Mager and Ferreira, 1993). This increased resistance can also

be passed on to the next generation which in turn increases the chances of the strain to tolerate future encounters to a stress (Berry and Gasch, 2008). This demonstrates that there are central molecular mechanisms which act as a first line of defence against stress as soon as it is detected which protects the cell and allows for more specific responses to be induced to counteract the stress (Gasch and Werner-Washburne, 2002; Ruis and Schüller, 1995).

There are two major response pathways which brewing yeast initially utilise whilst under the pressures of a stressful environment. The heat shock response (HSR) and the general/global stress response (GSR). The HSR is composed of several proteins known as heat shock proteins (HSP's). The HSR is the most highly conserved genetic system across many organisms not only yeast and have all been found to be present at normal temperatures and therefore play vital roles in normal cell function (Lindquist and Craig, 1988). Another clue to the relationship between HSP's and other stress responses is evident in cells which enter the stationary-phase. They have been observed to be more thermotolerant than when actively growing suggesting a similar response to nutrient limitation has been induced by the cells as would be to heat stress (Schenberg-Frascino and Moustacchi, 1972). The GSR is also activated under a multitude of environmental stresses; oxidative, non-optimal temperatures, exposure to toxic compounds, osmotic imbalance, pH and fluctuating nutrient levels (Ruis and Schüller, 1995). These evolutionary adaptation allows the yeast to respond to the environment in a non-specific manner rather than perish. Cellular proliferation can then be maintained whilst a specific response to the particular environmental stress is activated (Ruis and Schüller, 1995). Induction

is rapid and intense highlighting them as emergency responses (Lindquist and Craig, 1988).

1.5.8 Yeast cell variation

Brewing yeast populations are usually considered to be homogeneous, both genetically and phenotypically, due to the way they proliferate via budding. This theory has perhaps been exacerbated by process measurements being taken as an average of the whole population thus overlooking the variation which may be occurring at single cell levels. Mechanisms responsible for maintaining the genome are allowed to alter for the achievement of contradictory outcomes: to maintain the genome unchanged and to acquire mutations which allow for environmental adaptation (Skoneczna *et al.,* 2015). Spontaneous mutations play a fundamental role in evolution (Kunz *et al.,* 1998) and populations require a margin of genetic variability to allow for changes to or new environmental conditions (Skoneczna *et al.,* 2015). Types of spontaneous mutation include: replication errors, error-prone repair of DNA lesions (provoked by endogenous factors such as ROS or metabolites), unequal segregation of chromosomes during mitosis, DNA synthesis errors and the movement of transposable elements (Adams *et al.,* 1992; Kunz *et al.,* 1998; Shigenaga *et al.,* 1994; Skoneczna *et al.,* 2015; Wilke and Adams, 1992). The types of DNA mutation and their descriptions are summarised in Table 1.2. (Clancy, 2008) however, terms such as single nucleotide polymorphism (SNP) and chromosome length polymorphism (CLP) are often used by biologists so summarise the effect a mutation has on the genome. These changes which

occur in the genome over time due to the continual exposure to fermentation stresses (Section 1.5.1-1.5.6) are referred to as ‘genetic drift’.

Table 1.2 Types of DNA mutation (Clancy, 2008).

Class of Mutation	Type of Mutation	Description
Point mutation	Substitution	One base is incorrectly added during replication and replaces the pair in the corresponding position on the complementary strand
	Insertion	One or more extra nucleotides are inserted into replicating DNA, often resulting in a frameshift
	Deletion	One or more nucleotides is "skipped" during replication or otherwise excised, often resulting in a frameshift
Chromosomal mutation	Inversion	One region of a chromosome is flipped and reinserted
	Deletion	A region of a chromosome is lost, resulting in the absence of all the genes in that area
	Duplication	A region of a chromosome is repeated, resulting in an increase in dosage from the genes in that region
	Translocation	A region from one chromosome is aberrantly attached to another chromosome
Copy number variation	Gene amplification	The number of tandem copies of a locus is increased
	Expanding trinucleotide repeat	The normal number of repeated trinucleotide sequences is expanded

Brewing strains can vary in their susceptibility to genetic drift (Casey, 1996; Gilliland, 1962; Gilliland, 1971; Gorter de Vries *et al.,* 2019; Sato *et al.,* 2001; Wightman *et al.,* 1996). Paquin and Adams, (1983) first studied adaptive genetic mutations by observing mutation rates over 2612 generations of 11 populations for two haploid and two diploid strains. Under the nutritional stress of glucose limitation, the frequency of adaptation was measured against canavanine, cycloheximide and 5-fluorouracil resistance (apart from in the diploid strains where canavanine resistance is recessive so cannot be monitored). Both diploid and haploid strains saw genetic changes occur "surprisingly frequently" however diploids did so at a rate of around 1.6 times more often per cell than in haploids. Powell and Diacetis (2007) conducted a study using two brewing yeasts which were exposed to the usual brewery stresses over the course of being serially repitched for a year. The ale strain was re-pitched 98 times into subsequent fermentations and the lager yeast was maintained in a brink (modified keg) with fresh wort added every two weeks for 135 cycles. DNA fingerprinting allowed for the stock stains to be compared to the strains at the end of this serial repitching evaluation as well as studying the phenotypic morphology of the colonies. Genetic fingerprinting of the nuclear DNA found no genomic variants. Similarly, there were only some changes in the macro-morphological characteristics of the ale strain indicating possible phenotypic variants. This suggests that these two yeast strains were highly suited to this brewing environment and that the repeated exposure to stresses involved with fermentation and storage were counterbalanced by the cells ability to provoke mechanisms which ensured their survival. There are a

few microbiological methods which aid in the detection of genetic variants such as the use of Triphenyl tetrazolium chloride (TTC) overlay agar, this redox indicator aids in the numeration of respiratory deficient mutants (ŠIlhánková *et al.,* 1970). Genetic variants may also change in colony morphology and topography when compared to the original strain on Wallerstein Laboratory Nutrient (WLN) agar (Powell and Diacetis, 2007). However, some of these phenotypic techniques can be variable due to the physiological state of the cell meaning the only way to be sure of an alteration to the DNA is to use a molecular method which studies it. Chromosome length polymorphism (CLP) can be detected by the use of pulsed field gel electrophoresis (PFGE) and is a technique used in the identification of *Saccaromyces* subspecies (Frezier and Dubourdieu, 1992; Hayford and Jespersen, 1999). PCR techniques have been used in the differentiation of closely related species such as those of the *Saccharomyces* sensu-stricto complex and have been successfully resolved via restriction fragment length polymorphism (RFLP) of the DNA that encodes the ribosomal RNA genes (5.8S) and the non-coding internal transcribed spacer (ITS) regions (Esteve-Zarzoso *et al.,* 1999; Fernández-Espinar *et al.,* 2000; Pham *et al.,* 2011; White *et al.,* 1990). RFLP of the ITS region highlights larger genetic changes based around a single locus and can therefore be deemed 'not powerful enough' for precise characterisation (Legras and Karst, 2003). DNA sequence analysis can provide definitive answers but is not always appropriate due to cost and time constraints. A study of *S. cerevisiae* DNA sequences found repeated elements of 0.3kb which flank the TY1 and TY2 retrotransposon or as an isolated element which are termed as delta (δ) elements (Legras and Karst,

2003; Ness *et al.,* 1993). Around 300 of these elements have been described in the genome of the yeast strain S288C making them ideal targets in the routine identification of genomic variation (Lavalliée *et al.,* 1994; Legras and Karst, 2003). With this method it is possible to visualise a great number of bands in the regions of 70bp up to a possible 4kb+ via gel electrophoresis (Legras and Karst, 2003). The interdelta PCR method and PFGE method are both capable of species and subspecies typing showing equivalent discriminatory power, however, the PCR technique obtains profiles which are more diverse and is quicker and less laborious than using PFGE (Hayford and Jespersen, 1999).

1.6 Epigenetics and Phenotypic heterogeneity

Epigenetics brings together the disciplines of genetics and developmental biology and was first coined by Conrad Waddington in 1939. Early epigenetic studies considered theories in development such as that early embryos are undifferentiated (Waddington, 1939) and that imaginal discs of *Drosophila* develop into specific adult structures (Hadorn, 1955). In both of these studies the researchers were able to relate genes and gene action to development (Hayford and Jespersen, 1999). Another example is that of stem cells, switches in gene activity associated with cell division mean a variety of new types of cell can be produced. In contrast to the developmental definition associated with epigenetics is the idea put forward by Russo *et al.* (1996) that epigenetics is the study of mitotically and/or meiotically heritable changes in gene function which cannot be explained by alteration in the DNA sequence (Bird, 2007). Several

studies how found links between environment or aging to long lasting epigenetic effects resulting in changes in phenotype (Bird, 2007; Fraga *et al.,* 2005; Wong *et al.,* 2005). The theories behind epigenetics, or phenotypic heterogeneity, can therefore have significant biological and evolutionary functions. In microbial populations clonal variation in gene expression confers a fitness advantage under environmental stresses (Levy *et al.,* 2012). This adaptation to change is based on a 'bet-hedging' strategy whereby a population maximises long-term survival by distributing risk among individuals (Kussell and Leibler, 2005; Levy *et al.,* 2012; Simons, 2009). Clonal populations of *S. cerevisiae* has been shown to exhibit striking phenotypic heterogeneity (Avery, 2006) however this has often been overlooked in typical studies which average out the total effect of the whole population thereby masking the possible effects of any sub-populations. Gene product fluctuations can correlate with cell cycle (Newman *et al.,* 2006) and cell size (Colman-Lerner *et al.,* 2005; Volfson *et al.,* 2005). Also, due to the budding nature by which *S. cerevisiae* divide, the cell is able to segregate certain molecular components between the mother and daughter cell (Liu *et al.,* 2010). This unequal segregation can drive the phenotypic changes associated with replicative age in a population (Avery, 2006).

Simple growth plate analysis is an excellent way to determine heterogeneity within a population. This quantitative measurement of the ability single cells have to form colonies when under a range of stresses and varying concentrations reveals the presence of any resistant sub-populations, or 'persisters' (Bishop *et al.,* 2007; Hewitt *et al.,* 2016; Holland *et al.,* 2014; Sumner

and Avery, 2002). An example of phenotypic heterogeneity in yeast populations is represented under heat shock (which has been closely linked with other stress resistance, such as ethanol, Section 1.5.4). Cells which grow more rapidly have been associated with reduced tolerance to heat shock (Elliott and Futcher, 1993) whilst slow growth has been linked with heat tolerance (Attfield *et al.,* 2001). This is due to the slow growing cells in the population being impeded by having to produce proteins, such as Tsl1; a molecular marker for slow growth which is involved in the synthesis of the protective carbohydrate trehalose and enables the cell to persist through the environmental perturbation (Levy *et al.,* 2012). In addition to this, cell-cell growth rates of wild type *S. cerevisiae* have been found to vary widely even in optimal growth environments (Levy *et al.,* 2012) meaning that the strategy truly is a bet-hedging one rather than being initiated in the presence of a stress factor. This means that phenotypic heterogeneity within a population provides a dynamic source of diversity without the need to induce genetic mutations allowing for rapid reversion to the original phenotype if appropriate (Avery, 2006). Sub-populations of better equipped cells are able to survive through extreme environmental changes as well as exploit new niches (Avery, 2006; Booth, 2002; Sumner and Avery, 2002) though this comes at the cost of optimal performance under non-stressful conditions (Levy *et al.,* 2012).

1.6.1 Generalist vs specialist cells

Holland *et al.* (2014) provided a schematic of how larger fractions of stress resistant cells within a population, making the population more

heterogeneous, is advantageous in natural environments which can undergo periods of environmental stress (Figure 1.5). This explanation of what happens in the natural environment can be easily extrapolated to that of an industrial fermentation. The previously described stresses (Sections 1.5.2-1.5.6) act as selective pressures upon the brewing yeast population, changing the homogeneity of the culture with every repitch into subsequent fermentations. Therefore, a change in the overall population phenotype would be attributed to the fluctuating and repeated stresses of industrial fermentations. This split of cells which perform optimally under non-stressful conditions and those which are able to persist under extreme stress can be described as specialists and generalists respectively. A specialist culture, much like that of the low heterogeneity strain described in Figure 1.5, has a narrower niche width and copes best under defined environments which do not fluctuate to extreme ranges (Kassen, 2002). These strains are less able to readily evolve as extreme environmental perturbations are more likely to cause cell death. A generalist culture, much like the high heterogeneity strain described in Figure 1.6, has a wider niche as it is able to cope in a wider range of conditions and can be commonly related to “a jack of all trades but a master of none” (Tienderen, 1991). The generalist culture uses a division of labour approach so that sub-groups of phenotypically diverse within a population perform subtasks which allows the population to grow in heterogeneous environments (Ackermann, 2015). Generalist cultures are able to evolve more readily (Kassen, 2002) but phenotypic plasticity comes at the cost of fast growth in non-stressful environments (Tienderen, 1991).

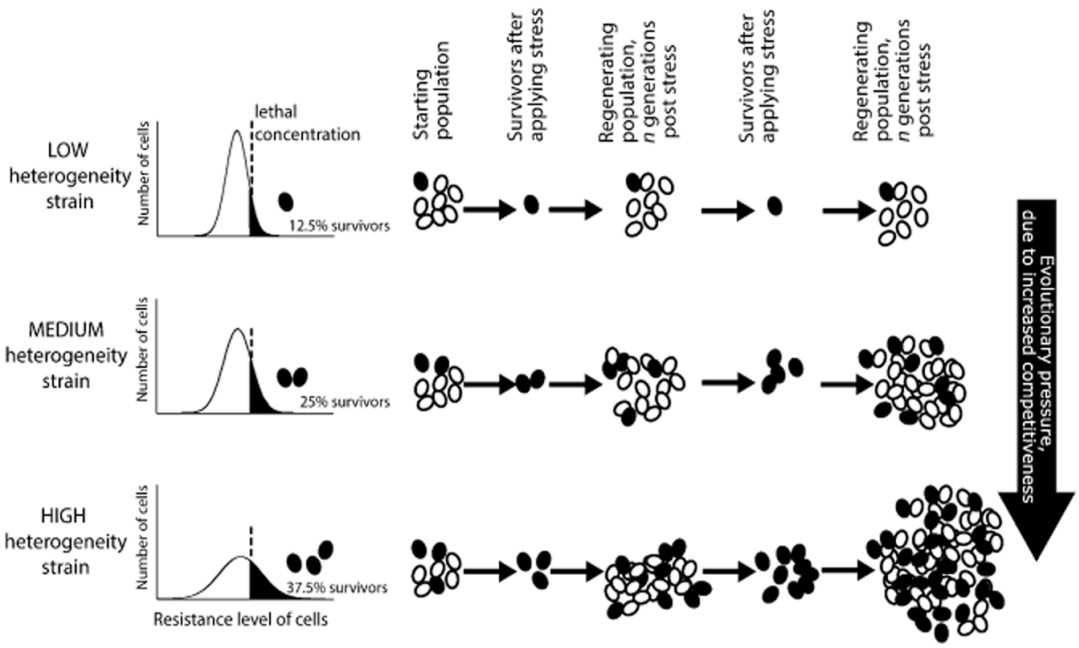


Figure 1.6 Selective pressure of stress factors favours heterogeneous organisms (Holland *et al.,* 2014).

The fundamental question then becomes; is high heterogeneity in brewing yeast a good thing? Homogeneous populations are less adaptable to process changes, but it may be easier to predict concentrations of metabolic by-products under that strains optimal conditions. High heterogeneity populations will contain sub-populations of cells which will slow the fermentation rate due to the production of stress protectants in anticipation of extreme environmental perturbations. These persistent sub-populations are beneficial to the overall population in the event of extreme stress though as they allow for the replenishment of the population once the environment becomes more hospitable or the strain becomes more tolerant to the stress (Ackermann, 2015; Avery, 2006; Holland *et al.,* 2014). This is of important consideration when

intensive brewing practices are put into place such as high or very high gravity brewing.

1.7 Aims of the thesis

A major question which needs to be answered is to what extent the insights gained about phenotypic heterogeneity in published work about wild yeast in natural environment is reflected in observations made in brewing yeast strains under brewery related stresses. Here I address this question by firstly characterising the genomic and phenotypic differences between five brewing yeast strains (two ale and three lager) before comparing their levels of phenotypic heterogeneity in non-stressed and stressed environments (ethanol, oxidative and osmotic stress). Another consideration is that of long-term stress, such as that imposed on strains undergoing serial repitching. A phenotypically heterogeneous strain would be the best candidate for forcing overall the population dynamic towards higher stress tolerance but what happens to the measurement of overall population heterogeneity once the selective pressure is removed?

A greater understanding of population dynamics will allow brewers to understand the relationship between environmental conditions and brewing yeast fermentation performance.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Yeast strains

Five strains obtained from the University of Nottingham culture collection were selected for this study (Table 2.1). The choices were based on selecting a variety of strains typically used in ale and lager fermentations.

Table 2.1. Yeast strain information

Yeast strain	Species	Description	Origin
W34/70	*Saccharomyces pastorianus ssp. carlsbergensis*	Frohberg-type lager strain	Hefebank Weihenstephan
NCYC 1332	*Saccharomyces cerevisiae*	Ale strain	National Collection of Yeast Cultures - British Brewery, 1974
M2	*Saccharomyces cerevisiae*	Ale strain	University of Nottingham culture collection
CBS 1260	*Saccharomyces pastorianus var. pastorianus*	Frohberg-type lager strain	Centraalbureau voor Schimmelcultures
CBS 1174	*Saccharomyces pastorianus*	Saaz-type lager strain	Centraalbureau voor Schimmelcultures

2.2 Yeast growth media and storage

2.2.1 Growth media composition

Yeast stocks were propagated and grown in liquid YPD media composed of 1% (w/v) yeast extract (Oxoid, USA), 2% (w/v) neutralised bacterial peptone (Oxoid, USA) and 2% (w/v) D- glucose (Fisher Scientific, UK). Solid YPD media was prepared by supplementing YPD medium with 1.2% (w/v) technical agar no. 3 (Oxoid, USA). Subsequently, the media was autoclaved at 121°C, 15psi for 15 minutes to sterilise.

2.2.2 Yeast storage

2.2.2.1 Long term cryogenic storage of yeast cultures

Stock cultures of yeast were cryogenically stored in 1.5ml cryovials (Nalgene Nunc International, UK) at -80°C for long term preservation. A liquid yeast culture was grown to stationary phase in YPD media. 500µl of this culture was added to the cryovial along with 500µl of 50% (v/v) sterile glycerol (Fisher Scientific, UK). The latter was added as a cryoprotectant to maintain cell viability during storage.

2.2.2.2 Short term cold storage of yeast cultures

YPD agar slopes were used to store short term stocks of each yeast strain at 4°C. Slopes were produced by adding 15ml of YPD agar media (Section 2.2.1) to a 30ml glass universal prior to autoclaving. Once removed from the autoclave and before the YPD agar sets upon cooling, the glass universals are laid at an angle to create a slope. Once set a sterile loop full of liquid yeast culture was

streaked onto the slope aseptically. Slopes were then incubated at 25°C in a static incubator (Sanyo, Japan) for 48 – 72 hours to allow for growth across the entire surface of the slope before being transferred to 4°C for storage.

2.3 Determination of total and viable cell counts

2.3.1 Total cell counts

Total cell counts were performed using a haemocytometer in conjunction with light microscopy (Hatfield *et al.,* 1988). Yeast cells were prepared by diluting the cells with sterile RO water to approximately $1x10^6$ – $1x10^8$ cells/ml (OD_{600} = 1). 10µl of this suspension was pipetted into a haemocytometer chamber (Neubauer counting chamber, Weber Scientific International Ltd, UK.) and examined microscopically using an Olympus BH-2 microscope (Olympus, UK) at x400 magnification. For a statistically reliable calculation, at least 200 cells were counted. Where yeast cells were budding, the daughter cell was enumerated when the bud was equal to or greater than half the size of the mother cell. The total cell number in the original sample was calculated using the following equation:

Equation 2.1 Calculation of total cells in cell suspension

Total cells/ml = Number of cells in counting area grid x 10^4 x dilution factor

(Note: 10^4 represents the $10^{-4}cm^3$ volume of the counting area)

2.3.2 Yeast viability staining

Methylene blue staining was used to assess yeast cell viability. To prepare a working solution, methylene blue powder (Sigma-Aldrich, UK) was dissolved in a 2% sodium citrate solution (w/v) (Fisher Scientific, UK) to a final concentration of 0.01 % (w/v) according to (Pierce, 1970). A cell solution of around $1x10^6$ – $1x10^8$ cells/ml (OD_{600} = 1) (Section 2.2.3.1) was added to the methylene blue solution at a 1:1 ratio and mixed via pipetting. After 5 minutes incubation at room temperature, the sample was examined microscopically on a haemocytometer (as described in Section 2.2.3.1). Cells retaining a blue colouration after this time were deemed to be dead (non-viable), while those appearing clear or white were assumed to be live (viable). Viable cells are able to break down the methylene blue enzymatically rendering them colourless (Painting and Kirsop, 1990). The percentage of viable cells was calculated using the following equation:

Equation 2.2 Calculation of percentage viable cells in cell suspension

$$\text{Yeast cell viability (\%)} = \frac{\text{(total cells)-(dead cells)}}{\text{(total cells)}} \times 100$$

2.4 Genetic characterisation of yeast strains

2.4.1 Determination of yeast species and genus using ITS PCR and RFLP

2.4.1.1 Genomic DNA extraction for ITS PCR

A rapid DNA extraction was carried out as described previously by Pham *et al.,* (2011). A yeast colony grown on YPD agar for 48 - 72 hours was transferred to

a sterile 1.5ml tube containing 50µl of yeast DNA extraction buffer; 0.002M NaOH and 0.001% (w/v) sarcosine (Fisher Scientific, UK.). Samples were then placed in a heating block set to 100°C and left to boil for 10 minutes. The crude DNA extract contained within the supernatant was collected by centrifugation, 10,000 rpm (9640 xg rcf) for 10 minutes, and placed into a fresh sterile 1.5ml tube.

2.4.1.2 ITS PCR reaction mix and conditions

All ITS PCR reactions were performed as described by (White *et al.,* 1990). The PCR reaction mixture contained 0.5µl primer ITS1 (10µM) (5' TCCGTAGGTGAACCTGCGG 3'), 0.5µl primer ITS4 (10µM) (5' TCCTCCGCTTATTGATATGC 3') (Sigma-Aldrich, UK), 25µl Quick Load Taq Master mix 2× (New England Biolabs), 3µl extracted DNA (Section 2.4.1.1) and 21µl molecular grade water (Sigma-Aldrich, UK). During preparation all components were kept in a labtop cooler (Nalgene, Nunc International, UK). The samples were placed into a thermal cycler (TC-512 Techne, UK) and run on the following program: 95°C for 15 minutes, 35 cycles of 95°C for 1 minute, 55°C for 2 minutes, 72°C for 2 minutes and finally 72°C for 10 minutes. Once the PCR reaction had completed the thermal cycler held the sample at 4°C until required.

2.4.1.3 RFLP reaction mix and conditions

ITS PCR product was digested individually with *HaeIII*, *HinfI* (New England Biolabs, UK) and *CfoI* (Promega, UK) restriction enzymes using the reaction

mixture; 8µl PCR product, 1µl restriction enzyme, 5µl 10x restriction enzyme specific buffer and 31µl molecular grade water (Sigma-Aldrich, UK). Samples were digested in a 1.5ml tube at 37°C in a water bath for 1 hour.

2.4.2.3 Visualisation of ITS PCR and RFLP amplicons using gel electrophoresis

5µl of either ITS PCR or RFLP DNA products were resolved on a 1.5% (w/v) agarose gel (Sigma-Aldrich, UK) containing 0.2 µg/ml ethidium bromide (Sigma-Aldrich, UK) in TAE buffer (comprising of 4.84 g/l TRIS, 1.14 ml/l glacial acetic acid and 0.37 g/l EDTA (Fisher Scientific, UK)). DNA resolved at 80 mV for 120 minutes. The agarose gel was visualised in the GelDoc-It®2 Imager (UVP LLC, UK) under ultra violet light. Band sizes were deduced with reference to 5µl of a 100 base pair ladder (Promega, UK).

2.4.2 Yeast DNA fingerprinting by analysis of interdelta regions

Individual yeast strains were identified using a method based on that described by Legras and Karst (2003), designed to amplify regions of the genome situated in between yeast delta region.

2.4.2.1 Genomic DNA extraction for interdelta PCR

Yeast strains were propagated in 10ml YPD media cultures, agitated using an orbital shaker (Certomat BS-1, Sartorius, USA) set at 120 rpm for 24-48hrs at 25°C. 2ml of the culture was centrifuged in a screw cap micro tube (Starsteadt, UK) at 5000 rpm (2400 xg rcf) for 5 min. 400µl of lysis buffer (Tris 10mM, pH 7.6, EDTA 1mM, NaCl 100mM, Triton X-100, 2% w/v, sodium dodecyl sulphate

(SDS) 1% w/v) (Fisher Scientific, UK), 400µl of phenol/chloroform/isoamyl alcohol (25/24/1 v/v) (Sigma-Aldrich, UK), and 1.5g of glass beads (0.45-0.55mm acid washed, Sigma-Aldrich, UK) were added to the pellet. The mixture was vortexed for 4 min. Then 200µl of Tris EDTA (pH 7.6) buffer (Sigma-Aldrich, UK) was added, and the mixture centrifuged for 5 min at 6000 rpm (3470 xg rcf). The upper phase was carefully pipetted off into a separate 1.5ml tube and 500µl of chloroform/isoamyl alcohol (98/2 v/v) (Sigma-Aldrich, UK) was added. After gentle agitation, the mixture was centrifuged at 14,000 rpm (18,890 xg rcf) for 2 min. Two volumes of ethanol were added to the aqueous phase and centrifuged at 14,000 rpm (18,890 xg rcf) for 5 min. The supernatant was discarded, and the pellet left to air dry for 15 min. The nucleic acid pellet was dissolved in 50µl 10mM TE buffer pH 8.0 (Fisher Scientific, UK). Extracted DNA was either used for PCR immediately or stored at -20°C for use at a later date.

2.4.2.2 Interdelta PCR reaction mix and conditions

The primer pair delta12 10µM (5'-TCAACAATGGAATCCCAAC-3') and delta21 10µM (5'- CATCTTAACACCGTATATGA-3') (Sigma-Aldrich, UK) described by (Legras and Karst, 2003) were used to analyse interdelta sequences within the yeast genome. PCR reactions were performed in 50µl reaction volumes containing 31µl molecular grade water, 10µl 5x Phusion HF buffer (New England Biolabs, UK), 1.5µl 50mM MgCl2, 1µl 10mM dNTPs, 0.5µl of each primer (delta12, delta21), 5µl DNA template (250ng/ml) and 0.5µl Phusion DNA polymerase (New England Biolabs, UK). DNA fragments were amplified according to the following protocol; 4 min at 95°C, 35 cycles of 60sec at 95°C,

30sec at 55°C and 120sec at 72°C with a finishing step of 10 min at 72°C. The thermal cycler was then set to hold the samples at 4°C until required.

2.4.2.3 Visualisation of interdelta PCR amplicons using gel electrophoresis

5µl of interdelta PCR DNA product was resolved on a 2% (w/v) agarose gel (Sigma-Aldrich, UK) containing 0.2 µg/ml ethidium bromide (Sigma-Aldrich, UK) in TAE buffer (comprising of 4.84 g/l TRIS, 1.14 ml/l glacial acetic acid and 0.37 g/l EDTA (Fisher Scientific, UK)). DNA resolved at 80 mV for 5 hours. The agarose gel was visualised in the GelDoc-It®2 Imager (UVP LLC, UK) under ultra violet light. Band sizes were deduced with reference to a 1kb pair ladder (Promega, UK).

2.4 Phenotypic characterisation of yeast strains

2.4.1 Growth curve analysis

Each strain exhibits its own set of kinetic growth features and reacts differently to environmental stresses. A micro-plate reader was used to produce characteristic growth curves for each strain by analysing the absorbance of aliquots of inoculated media via spectroscopy as a measure of cell number.

When required, stressors were added into each well, with the final concentrations made up with YPD media.

Starter cultures were produced by inoculating 10ml of YPD media (Section 2.2.1) with a loop full of yeast from YPD storage slopes (Section 2.2.2.2) and incubated at 25^{o}C in an orbital shaker at 120rpm (Sartorius, USA) for 48 hours. Yeast cells were harvested by centrifugation and washed twice with sterile RO

water. The cell suspension was diluted to an OD_{600} = 1 and 3µl of this suspension was added to the relevant wells (total volume of 200µl). Each test was sampled in triplicate for statistical accuracy. The 96-well plates (flat bottomed, Corning, USA) were then incubated in an automated plate reader (Infinite® 200 PRO, TECAN, UK) at 25°C for up to 96 hours. The automated plate reader analysed each well every 15 minutes at a wavelength of OD_{600} nm. Data was collected via the Magellan™ Data analysis software (TECAN, UK), exported as an excel file (Microsoft Corp, USA) and analysed on GraphPad PRISM 7 software (GraphPad Software Inc., USA).

2.4.2 Giant colony morphology via WLN assay

Giant colony morphology was determined based on the method of Hall (1971). The morphological characteristics of yeast colonies were determined by analysis of the visible appearance of colonies following cultivation on WLN agar media for an extended period of time (Fisher Scientific, UK). WLN agar (Fisher Scientific, UK) was prepared according to the manufacturer's instructions and autoclaved at 121°C, 15psi for 15 minutes before allowing to cool to 55°C for ease of pouring into petri dishes. For initial analysis, each strain was propagated in YPD media for 24 hours in an orbital shaker at 25°C and 120rpm (Sartorius, USA) until they had reached stationary growth. Yeast cells were harvested by centrifugation and washed twice with sterile RO water and enumerated using a haemocytometer (Section 2.3) then diluted to a concentration of 400 cells/ml. A 50µl aliquot of this dilution was then spread onto WLN agar and incubated aerobically at 25°C for 14 days. Colonies were examined visually to identify

differences based on size, shape, surface topography and the distribution of colour in the colony forming unit (CFU).

2.4.3 Determination of stress tolerance by spot plate analysis

Spot plate analysis was used to observe the effects of ethanol, osmotic and oxidative stress, as well as temperature on yeast growth. To achieve this, YPD agar (Section 2.2.1) was supplemented with increasing concentrations of either ethanol (Fisher Scientific, UK), sorbitol (Sigma-Aldrich, UK), or hydrogen peroxide (Fisher Scientific, UK) for analysis of stress resistance, respectively. No supplementation was required for temperature stress evaluation; this was conducted through the subsequent incubation environment.

Sorbitol was used to induce osmotic stress and hydrogen peroxide was used to induce oxidative stress (Martin *et al.,* 2008; White *et al.,* 2008). The final concentrations of ethanol utilised were 0, 5, 10, 15, 20, 25 and 30% (v/v). To counteract the effects of ethanol evaporation during the pouring of the hot agar, the ethanol was first pipetted into a sterile petri dish. Then the hot agar, which had been left to cool to a temperature of between 50-55^{o}C, was pipetted on top. The mixture was swirled, and the lid promptly replaced. The final concentrations of sorbitol utilised were 0, 10, 20, 30, 40, 50 and 60% (w/v). The final concentrations of hydrogen peroxide utilised were 0, 1, 2, 3, 4, 5 and 6mM. Serially diluted yeast cell suspensions were obtained by inoculating 10ml of YPD media (Section 2.2.1) with a loop full of yeast from YPD storage slopes (Section 2.2.2.2) and incubated at 25^{o}C in an orbital shaker at 120rpm (Sartorius, USA) for 48 hours. Yeast cells were harvested by centrifugation and washed twice

with sterile RO water. Stocks of viable cells were produced to achieve concentrations of 10^6, 10^5, 10^4, 10^3 and 10^2 cells/ml using the total and viable cell count method described in Section 2.3. A volume of 5µl from each dilution was spotted onto the YPD agar plates prepared beforehand.

All spot plates were incubated in a static incubator (Sanyo, Japan) aerobically at 25°C for 14 days. Each test was performed in triplicate and colony growth was assessed visually. It should be noted that the relative growth of each strain on the different plates gave a good indication of the range of concentrations to use in further experimentation, such as in the phenotypic heterogeneity agar plate method (Section 2.4.3).

2.4.3 Phenotypic heterogeneity determination through dose response analysis

The protocol for dose-response analysis applied for the determination of heterogeneity was adapted from methodology described previously (Holland *et al.,* 2014). Analysis was performed using YPD agar (Section 2.2.1) supplemented with increasing concentrations of stressor. In this case the stressors were ethanol, oxidative (induced by hydrogen peroxide), and osmotic (induced by sorbitol). The final concentrations of ethanol in the YPD agar plates were 0, 5, 10, 15, 20, 25 and 30% (v/v). The final concentrations of sorbitol in the plates were 0, 10, 20, 30, 40, 50 and 60% (w/v). The final concentrations of hydrogen peroxide in the plates were 0, 1, 2, 3, 4, 5 and 6mM. Yeast cells were obtained by inoculating 10ml of YPD media (Section 2.2.1) with a loop full of yeast from YPD storage slopes (Section 2.2.2.2) and incubating them at 25°C in

an orbital shaker at 120rpm (Sartorius, USA) for 48 hours. Yeast cells were harvested by centrifugation, washed twice with sterile RO water and resuspended to a concentration of 4000 viable cells/ml in sterile RO water (Section 2.3). A 50µl aliquot of this dilution was then spread onto each prepared plate to give around 200 colony forming units (CFUs). CFUs were then enumerated after 14 days aerobic incubation at 25°C in a static incubator (Sanyo, Japan). Each range of stresses was repeated for each strain in triplicate and the whole set was sampled in triplicate again independently for statistical accuracy. Percentage viability for each strain under each stress was determined with reference to the mean number of CFUs on the control plates (YPD agar with no stressor supplementation).

To model the effect of stressor concentration (x) on viability (y) a four parameter logistic regression model was applied to the data. This was done using the following equation (Equation 2.3). Equation 2.3 for each strain under each stress was then differentiated to find the slope of the curve which represents the extent of heterogeneity (Bishop *et al.,* 2007; Sumner *et al.,* 2003).

Equation 2.3

$$y = d + \frac{(a - d)}{1 + (\frac{x}{c})^b}$$

x= the independent variable (i.e increasing logarithm of stressor concentration)

y= the dependent variable (i.e percentage viability expressed as CFU's)

- a= the maximum value that can be obtained (i.e what happens with no stress applied)
- b= the minimum value that can be obtained (i.e what happens with an infinite amount of stressor applied)
- c= the point of inflection (i.e. the point on the S shaped curve halfway between a and d)
- d= Hill's slope of the curve (i.e. this is related to the steepness of the curve at point c)

This function was modified by reflecting about the y axis, such that the maximum viability occurs at *x*=0. A constant of 0.01 was also added to the stressor values since the log of 0 (no stress) is an undefined number (i.e not a real number). The shape of the distribution of resistant cells within the population determined the shape of the dose-response curve. Therefore, if the cells were normally distributed the resulting curve would be equal to the cumulative probability density function (CDF) of the normal distribution, with a slope gradient determined by the standard deviation. For this data, detailed information about the shape of the normal distribution for each strain under each stress was deficient, hence the gradient calculation was a function of the point of inflection (*c*) as a direct substitute for variance (i.e heterogeneity). All analyses were conducted in GraphPad Prism 7 (GraphPad Software Inc., USA).

2.5 Fermentation analysis

2.5.1 Wort

Hopped all-malt wort was produced at the University of Nottingham using the on-site 35 litre Briggs brewhouse. Wort produced was 13 ^{o}P (plato) - equivalent to 1.053 specific gravity (SG).

2.5.2 Wort supplementation

Malt wort provides all the nutrients required by the brewing yeast with the exception of unsaturated fatty acids, sterols and zinc (Taidi *et al.,* 2008). Oxygenation of wort negates the need to add unsaturated fatty acids and sterols. Oxygen is rapidly consumed by the brewing yeast to synthesise its own unsaturated fatty acids and sterols (Quain, 1986). Therefore, wort was sparged with oxygen for 4 hours at a flow rate of 0.5 l/min, stirred at 400 rpm and held at a temperature of 15^{o}C (lower temperature helped increase oxygen solubility) prior to use. Low levels of zinc can result in impaired yeast cell growth and fermentation performance. To ensure this was not the case, wort was supplemented with zinc ions at a rate of 0.2 mg/l through the addition of zinc sulfate heptahydrate ($ZnSO_4 \bullet 7H_2O$) (0.8 mg/l w/v) (Sigma-Aldrich, UK).

2.5.3 Yeast strain propagation

The propagation of yeast strains was performed in a manner designed to closely replicate industrial propagation processes, with the exception that YPD media (Section 2.2.1) was employed rather than wort (Section 2.5.1). This was done

for the purposes of simplicity and consistency, and ensured uniformity of yeast growth. To achieve the desired cell volume three successive cultures of increasing volume were prepared. Firstly a starter culture was prepared by inoculating 10 ml of YPD media (Section 2.2.1) with a loop full of yeast from YPD storage slopes (Section 2.2.2.2) and incubated at 25^{o}C in an orbital shaker at 120 rpm (Sartorius, USA) for 48 hours. This 10 ml culture was then transferred aseptically to a pre-sterilised 250ml conical flask containing 100 ml YPD media and incubated at 25^{o}C in an orbital shaker at 120 rpm for 48 hours. Lastly, the 100 ml of yeast cell growth was transferred aseptically to a pre-sterilised 2 l conical flask containing 900 ml YPD media and incubated at 25^{o}C in an orbital shaker at 120 rpm for 48 hours.

2.5.4 Pitching yeast

Once yeast had been propagated (Section 2.5.3), the final 1 l of culture was aliquoted into pre-weighed, sterilised centrifuge pots (Fisher Scientific, UK) and centrifuged at 4000 rpm (2830 xg rcf) for 10 minutes at 4^{o}C (Beckman, UK). The supernatant was discarded, and the pellet weighed so that an equal volume of sterile water could be added resulting in a 1:1 slurry (w/w). Total viable cell counts were performed (Section 2.3) and the required number of cells was calculated using Equation 2.4 (Stewart, 2009).

Equation 2.4

$$\text{Total number of cells required for pitching} = 1\text{x}10^{6}\ viable\ cells/ml\ \ per\ degree\ Plato$$

2.5.5 Fermentations using small scale 100ml fermenters

Small scale fermentations were carried out in 150ml glass hypo-vials (International Bottle Company, UK) in accordance to the method of (Quain *et al.,* 1985). Each glass hypo-vial contained a magnetic stirrer and was autoclaved prior to use at 121^{o}C, 15 psi for 15 minutes with a foam bung and aluminium foil sealing the aperture. 100 ml of the required fermentation media was aliquoted into each vial (YPD media (Section 2.2.1) or Wort (Section 2.5.1)), pitched with yeast (Section 2.5.4) and sealed using rubber septa and metal crimps (VWR, UK). To allow for the liberation of carbon dioxide produced during fermentation a gas outlet port (known as a Bunsen valve) was inserted into the septa. This was made from a sterile needle, a section of silicone tubing with a slit in it and a Durham tube all connected via cable ties (Figure 2.1). Once assembled the system is known as a mini fermenter. Fermentations were stirred at 250 rpm on a flatbed magnetic stirring plate in a 25^{o}C static incubator (Sanyo, Japan).

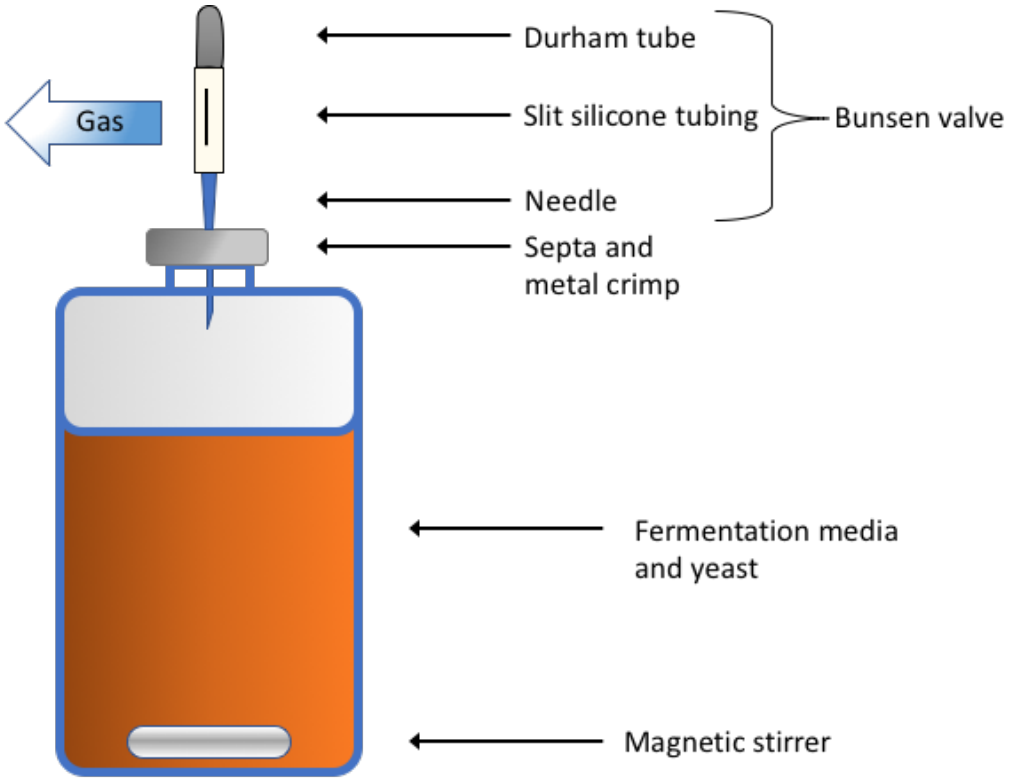


Figure 2.1 Mini fermenter set-up for 100 ml small scale analysis

2.5.6 Fermentation chemostat using 15L Infors Bioreactors

Large scale chemostat fermentations were carried out using two 15L Multifors bioreactors (Infors-HT, Bottmingen, Switzerland) each with a working volume of 10L, as shown in Figure 2.2. Vessels were autoclaved prior to use at 121°C for 15 minutes. Antifoam A (15% v/v) (Sigma-Aldrich, UK) was added to wort (1ml per 1L of wort) before inoculation with yeast to stop an overflow of foam into the filters. The wort was then supplemented with zinc and oxygenated in the vessel as described previously (Section 2.5.2). Both vessels were stirred at 200 rpm by an inclined blade impeller. Following set-up, one vessel became the fermentation vessel, and the oxygen supply was removed. The second vessel became the wort reservoir (the oxygen supply remained on) and was

connected to the fermentation vessel via silicone tubing and a peristaltic pump. The fermentation vessel was also connected to an effluent collecting receptacle via silicone tubing and a peristaltic pump. Both pumps were set to a dilution rate of 0.1 l/hr. Once connected ethanol was added to the fermentation vessel to create an initial environment of 5% ethanol (v/v). The fermenter was subsequently pitched with yeast (Section 2.5.4). The flow rate represented a cell generation time of 7 hours (based on work by Adams *et al.* (1992) meaning the cell generation time for a 24 hour would equal 3.42. The low flow rate was to ensure the chemostat maintained a steady state of cell density. The continuous chemostat was maintained at 25^{o}C for a total of 18 days. Samples were extracted via a septum in the top of the vessel using a needle attached to a 50 ml syringe.

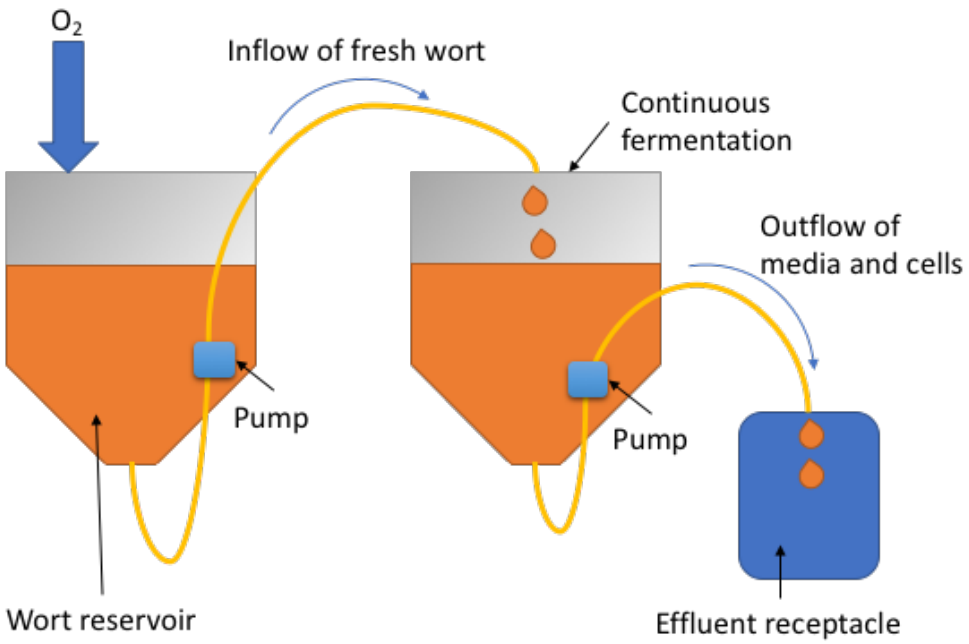


Figure 2.2 Chemostat setup

2.5.7 Gravity and alcohol determination

Fermentation samples were analysed using the DMA 4500 density meter and Alcolyzer Plus Beer system (Anton Paar Ltd, UK). Sugar content (gravity) was expressed in specific gravity (SG), which describes the ratio between the density of the sample at 20°C and the density of deionised water at 4°C (Boulton and Quain, 2006). Specific gravity was measured using an oscillating U-tube within the machine. The Alcolyzer Plus Beers system was also used to calculate the percentage alcohol content based on near infra-red (NIR) spectrometry.

2.6 Statistical analysis

The mean and standard deviation of data collected was calculated using GraphPad PRISM 7 (GraphPad Software Inc., USA). To determine any significant differences between sample groups, a range of analyses were used, dependant on the number of samples. The nature of these statistical tests is specified in the relevant Results sections; in all instances the data was evaluated using GraphPad PRISM 7.

CHAPTER 3:

GENETIC AND PHENOTYPIC CHARACTERISATION OF BREWING YEAST STRAINS

3.1 Introduction

Fermentation is an essential part of the brewing process and is typically performed using yeast belonging to the genus Saccharomyces. Brewing strains belonging to this genus are fast growing, efficient at metabolising simple sugars and, compared to naturally occurring yeast strains, are tolerant to environmental stress factors. However, despite having many characteristics in common all brewing yeast strains are different and can be broadly divided into two groups according to the type of beer they produce. Essentially, those strains belonging to the Saccharomyces cerevisiae species are used to produce ale type beer whilst Saccharomyces pastorianus strains are responsible for lager fermentations (Stewart, 2016). *S. cerevisiae* strains are evolutionary 'older' than *S. pastorianus* yeasts and are known to have been employed regularly for the production of beer-type products consumed in ancient civilizations (Legras *et al.,* 2007). Lager strains are believed to have arisen more recently as a result of a hybridisation event between an *S. cerevisiae* parent and another species, most likely a strain of the cold-tolerant S. eubayanus species (Libkind *et al.,* 2011). This type of hybridisation may have occurred more than once, giving rise to sub-groups of lager yeast known as Saaz and Frohberg types (Dunn and Sherlock, 2008). Irrespective of their source, these groups of brewing yeast became widely used during the industrialisation of beer production, and their current genetic make-up is believed to have evolved

largely via artificial selection by humans during fermentations (Gallone *et al.,* 2016).

Due to their genetic origins, ale and lager yeasts are inherently different. This variation is manifested in the nature of the fermentations which are conducted to produce beer. Fermentations using *S. cerevisiae* strains are typically performed at 18-25^{o}C, although strains typically exhibit a maximum growth temperature of approximately 37^{o}C. In contrast, *S. pastorianus* yeasts exhibit a maximum growth temperature of approximately 34^{o}C; this slightly colder limit is reflected in the fermentation temperature applied, often as low as 8-15^{o}C. In addition to temperature preferences, *S. cerevisiae* strains cannot utilise the sugar melibiose, and are known as 'top fermenters' due to the way the yeast rises to the surface of the media once nutrients become limiting. Conversely, *S. pastorianus* strains can metabolise melibiose and are known as 'bottom fermenting' due to the mechanism of flocculation which causes yeast to sink to the bottom of vessel (Briggs *et al.,* 2004; Stewart, 2016).

Although there are significant differences between ale and lager yeasts, individual strains belonging to each group also display unique characteristics. This is particularly evident for ale strains which constitute a diverse range of organisms, but is also true of lager yeasts (Pedersen, 1986; Timmins *et al.,* 1998; Smart, 2007). Previous studies have demonstrated that brewing yeasts can vary significantly in terms of their genetic composition, and consequently their phenotypic and physiological characteristics. The effects of these differences are manifested in a diverse number of ways, including variations in growth characteristics, nutritional requirements, the rate at which sugars are

assimilated, production of flavour compounds, flocculation properties, and tolerance to a wide range of stress factors. Resistance to stress is particularly important in the brewing process since all fermentations are inherently stressful (Briggs *et al.,* 2004) (Chapter 1.5.1).

While many of these characteristics are determined by the genetic make-up of a strain, it should be noted that genes do not function in isolation; the proteins and RNA which they encode for contribute to specific cellular pathways. Expression of genes also depends on many factors including temperature, nutrient availability, population density and a multitude of environmental stresses. Disruption to these conditions may require a change in activity of a single gene or a combination of many which work together (Griffiths *et al.,* 2000). With this in mind it is reasonable to suggest that the phenotype of a brewing yeast strain is built on complex genetic foundations.

In this study I aimed to identify each brewing yeast strain based on its genetic composition, and to determine their individual phenotypic characteristics specifically related to stress tolerance. Genetic identification was conducted based on analysis of specific regions of genomic DNA to identify the species and genus level. This was supported by determination of permissive growth temperature as an alternative means of differentiating between lager and ales. In addition, each strain was analysed for their capacity to withstand stress factors related to typical industrial fermentations. Specifically, the impact of oxidative stress, osmotic stress and ethanol stress on yeast cell growth was elucidated. This set of experiments was performed firstly to identify differences between each strain, but also to determine the absolute tolerance to each

stress factor. These growth plates, conducted under aerobic conditions, should also provide a trend which could be extrapolated and give an idea of how that strain would grow under similar stresses and an anaerobic environment. This is due to all of the strains being Crabtree positive meaning they will ferment in the presence of excess sugars despite an aerobic environment. The secondary analysis was also conducted to provide benchmark data essential for investigating phenotypic heterogeneity within populations as described in Chapters 4 and 5.

3.2 Results

In this study two ale strains (M2 and NCYC 1332) and three lager strains (W34/70, CBS 1174 and CBS 1260) were analysed for genetic and phenotypic characteristics. Species differentiation was performed using restriction fragment length polymorphism (RFLP) analysis of the ribosomal RNA-encoding DNA (rDNA) internal transcribed spacer region (ITS region) (Kurtzman and Robnett, 1991; White *et al.,* 1990). Further to this, amplification of the regions between delta elements (interdelta regions) was used to discriminate between strains. With respect to phenotypic differentiation, spot plate analysis was used to determine the tolerance to ethanol, osmotic and oxidative stress factors.

3.2.1 Characterisation of yeast strain brewing classification

3.2.1.1 Identification of yeast classification by growth temperature

As described in Chapter 2.4.3, ale and lager strains exhibit differences in temperature tolerances, a characteristic which also impacts on the temperature at which they are used to conduct fermentation. *S. cerevisiae* ale strains are able to grow and reproduce at temperatures of greater than 34°C, while *S. pastorianus* lager strains cannot. Based on this, a classic means of differentiating ale and lager yeast is by simple analysis of growth at 37°C. Analysis of permissive growth temperature was conducted by spot analysis as shown in Figure 3.3. None of the lager strains were able to grow at 37^{o}C whilst the ale strains (M2 and NCYC 1332) were able to thrive at this temperature (Figure 3.1).

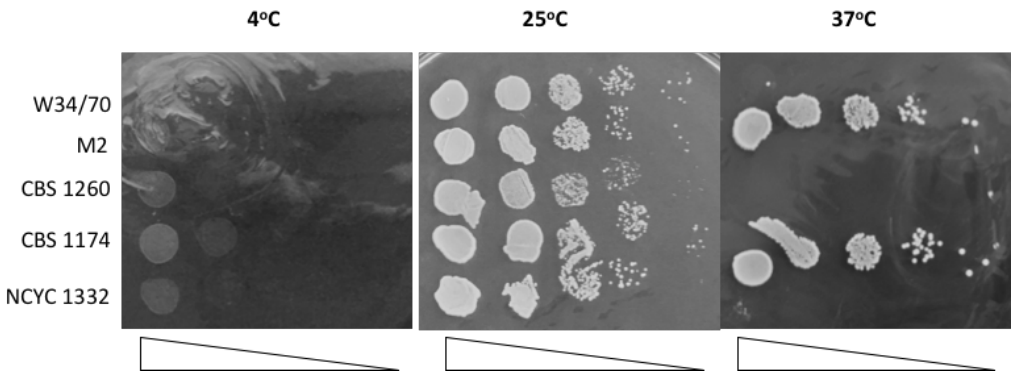


Figure 3.1 Determination of brewing classification (ale or lager) based on temperature tolerance. Decreasing cell concentrations (10^6-10^2) were spotted onto YPD agar plates and incubated at the corresponding temperatures for 24 hours (7 days in the case of 4°C) under aerobic conditions.

3.2.1.2 Identification of yeast species using ITS PCR-RFLP

The ITS region is known to be highly conserved within yeast species but can vary between these species (White *et al.,* 1990). To confirm the species of each yeast strain used in this study the ITS region was amplified and cut enzymatically using *HaeIII*, *HinfI* and *CfoI* in separate reactions (Chapter 2.3.1). For both lager and ale type strains the ITS amplicon was the same size, 880bp in length (Figure 3.2 and 3.3). This result was as expected based on previous data for *Saccharomyces* yeasts (Esteve-Zarzoso *et al.,* 1999; Guillamón and Barrio, 2017). The restriction profiles produced using *HaeIII*, *HinfI* and *CfoI* were also identical for each strain, except in one instance (CBS 1174). The enzyme *HinfI* created restriction profiles with bands at 360 and 130bp, while *CfoI* yielded DNA fragments at 340, 320 and 120bp for all strains. The enzyme HaeII was able to distinguish one strain from the others. For W34/70, CBS 1260 (both

lager strains), M2 and NCYC 1332 (both ale strains) the 4 band restriction profile produced by *HaeIII* was composed of fragments at 320, 220, 180 and 140bp, corresponding to *S. cerevisiae* (Esteve-Zarzoso *et al.,* 1999; Guillamón and Barrio, 2017) however, for CBS 1174 (also a lager strain), a 3 band profile was observed with DNA fragments of 500, 220 and 120bp in size, corresponding to *S. pastorianus* (Figure 3.2 and 3.3).

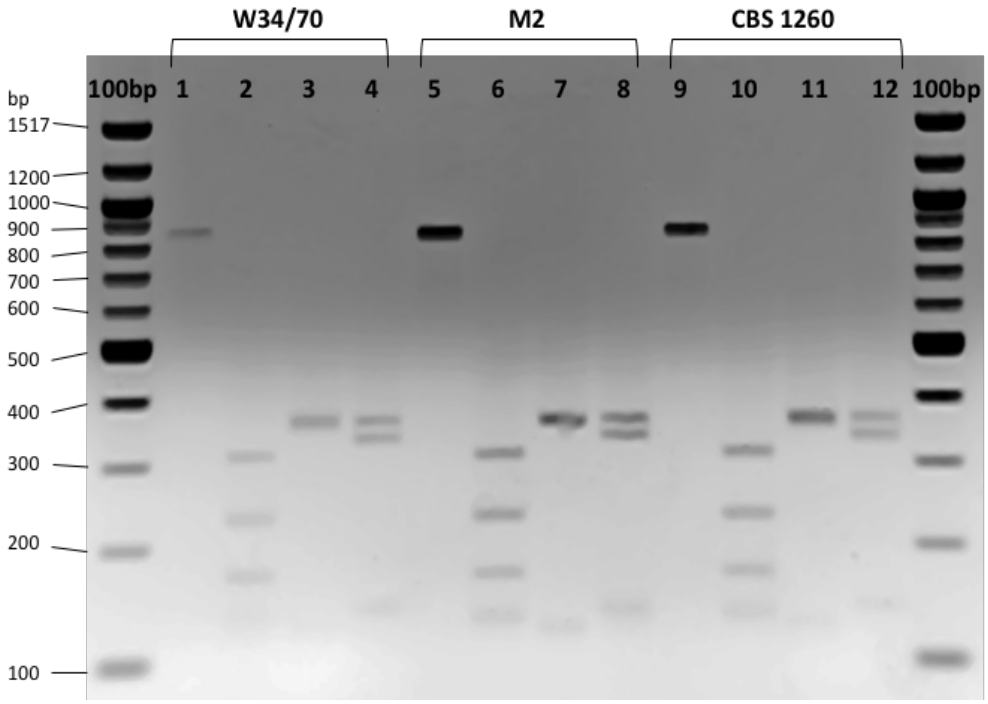


Figure 3.2 Identification of yeast species using ITS PCR-RFLP. Lanes 1, 5 and 9 reflect amplified ITS regions for each strain. Restriction fragments from *HaeIII* digests can be seen in Lanes 2, 6 and 10; digests from *HinfI* are shown in Lanes 3, 7 and 11; and digests using *CfoI* are indicated in Lanes 4, 8 and 12. 100bp ladders are shown and were used to determine fragment sizes.

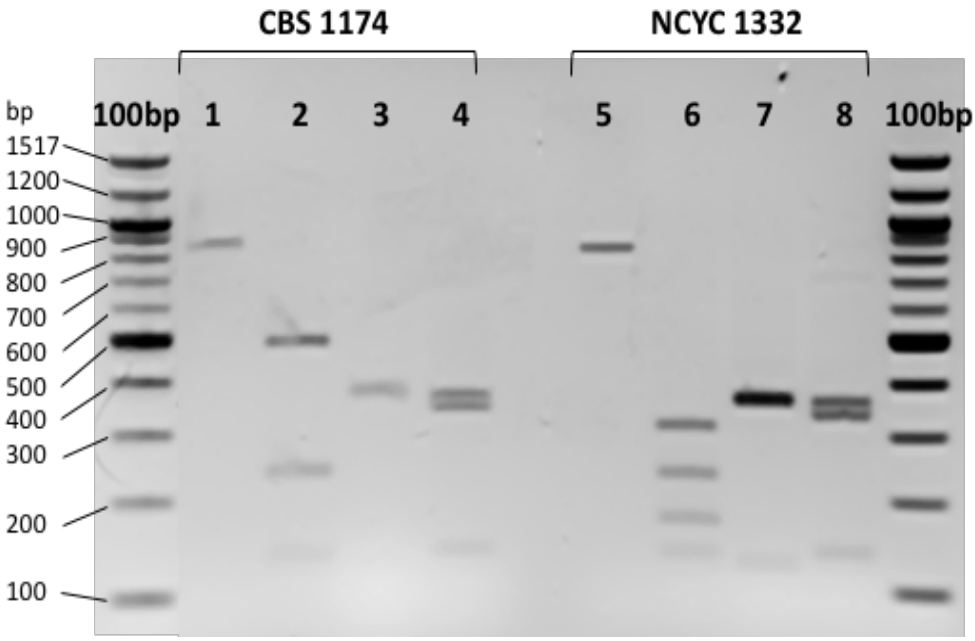


Figure 3.3 Identification of yeast species using ITS PCR-RFLP. Lanes 1 and 5 reflect amplified ITS regions for each strain. Restriction fragments from *HaeIII* digests can be seen in Lanes 2 and 6; digests from *HinfI* are shown in Lanes 3 and 7; and digests using *CfoI* are indicated in Lanes 4 and 8. 100bp ladders are shown and were used to determine fragment sizes.

This data is perhaps confusing, given that previous analysis of permissive growth temperature (Chapter 3.2.1.1) indicated that W34/70, CBS 1260 and CBS 1174 were all identified as lager strains. However, it is recognised that Frohberg type lager strains produce a 4 band profile identical to that expected for *S. cerevisiae* yeasts (Gibson *et al.,* 2013; Pham *et al.,* 2011). In contrast, Saaz type lager strains yield a 3 band profile with the *HaeIII* enzymatic digest. The reason for this difference is that these two types of lager yeast form distinct genetic groups within the *S. pastorianus* taxon, potentially arising due to two separate hybridisation events (Dunn and Sherlock, 2008). Indeed, this

difference has led to claims that the species name *S. carlsbergensis* should be reintroduced for Saaz type yeasts (Wendland, 2014). Irrespective, it is known that Frohberg yeast contain a greater proportion of DNA derived from the *S. cerevisiae* parent (reflected also in the ITS region), rather than that of the *S. eubayanus* parental strain. Interestingly this similarity may be reflected in current practice, with the majority of current lager production strains belonging to the Frohberg category. It is likely that the combination of good fermentative potential (from *S. cerevisiae*) and cold tolerance (from *S. eubayanus*) have combined to make this a highly successful hybrid organism. Related to this, it is interesting to note that Saaz type lager strains can function under a greater degree of cold tolerance than Frohberg type strains (Dunn and Sherlock, 2008), whilst neither can grow at high temperatures (37^{o}C). This data generated from analysis of the ITS region therefore corresponds with the analysis of permissive growth temperature above. Futhermore, a retrospective analysis of growth data revealed that the Saaz type lager yeast (CBS 1174) displayed improved growth at 4^{o}C compared to the other strains.

3.2.1.3 Yeast strain identification by PCR analysis of interdelta sequences

Although the ITS PCR-RFLP method can be a useful tool for yeast species identification (Chapter 3.2.1.2), it lacks the definition required to discriminate between strains and hence the power required to distinguish if a strain has undergone genetic variation or drift (Chapter 1.5.8). In order to characterise and differentiate strains, a PCR protocol was applied to amplify interdelta regions of yeast strains (Chapter 2.4.2). Delta regions are known to flank yeast

transposons and are known to be highly discriminatory in yeast (Legras and Karst, 2003); approximately 300 delta elements have been characterised in the laboratory strain S288c genome. Consequently, this method is a robust means of identifying genetic polymorphisms (including those associated with genetic variation/drift) (Legras and Karst, 2003).

PCR analysis of interdelta regions was applied to the five yeast strains of interest: W34/70, M2, CBS 1260, CBS 1174 and NCYC 1332. The electrophoretical patterns observed in Figure 3.4 show that the primer pair delta12-delta21 was able to differentiate each of the 5 strains of brewing yeast examined. Although different banding profiles existed for each strain there were some common bands present. It is likely that this is indicative of the genetic origins of strains, with similar strains yielding similar profiles. This is particularly evident for the lager strains W34/70, CBS 1260 and CBS 1174. Table 3.1 summarises the discriminatory and similar banding patterns observed for each yeast strain.

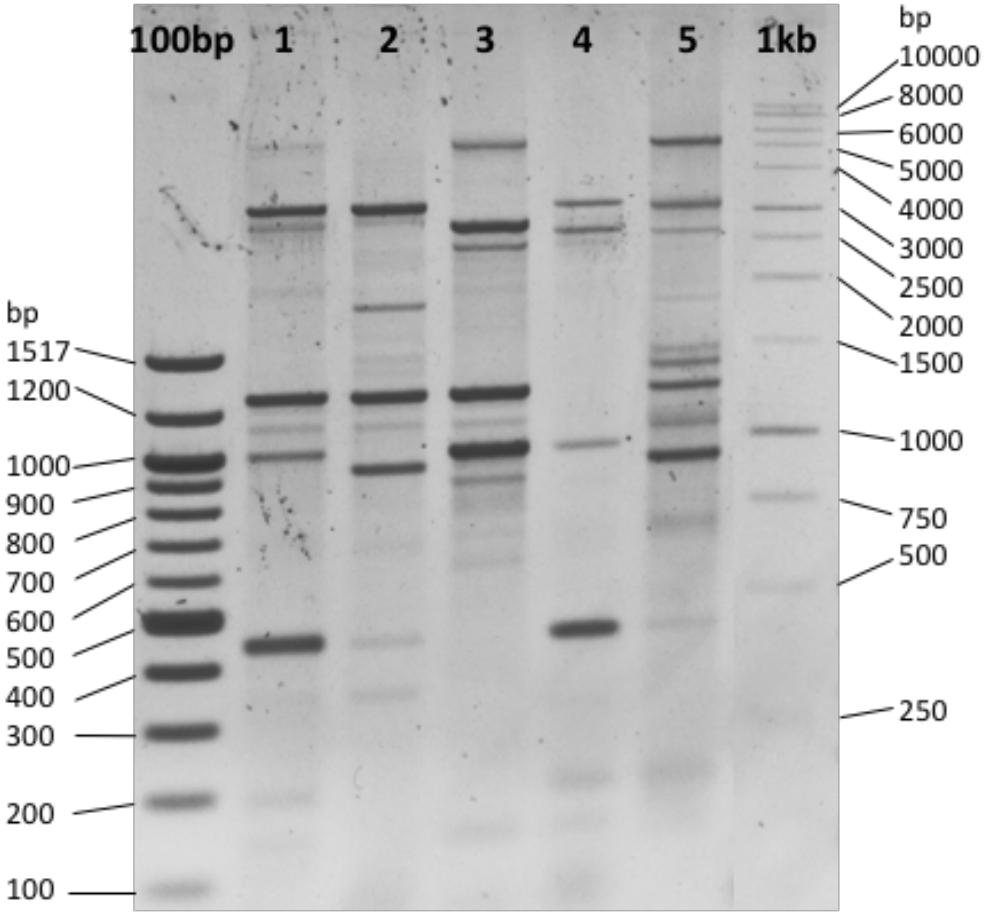


Figure 3.4 Differentiation of yeast strains by PCR amplification of inter delta sequences. The primers delta12-delta21 were used to amplify interdelta sequences giving rise to specific fingerprints. Lane 1: W34/70 (Frohberg lager), lane 2: M2 (ale), lane 3: CBS 1260 (Frohberg lager), lane 4: CBS 1174 (Saaz lager) and lane 5: NCYC 1332 (ale). 100bp and 1kb ladders are shown and were used to determine fragment sizes.

Table 3.1 Amplicons produced using interdelta PCR electrophoresis analysis of five brewing strains (banding range ~5000-320bp)

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
Band size (bp)	W34/70 (Frohberg lager)	M2 (ale)	CBS 1260 (Frohberg lager)	CBS 1174 (Saaz lager)	NCYC 1332 (ale)
~5000	X		X		X
~3000	X	X		X	X
~2800		X			
~2500	X		X	X	X
~2400			X	X	
~2000	X	X	X		
~1800		X			X
~1500		X			X
~1400		X			X
~1300	X	X	X		X
~1100	X	X	X		X
~1050					X
~1000	X		X	X	
~960		X			X
~840			X		
~750			X		
~700					X
~650					X
~600			X		
~440	X	X		X	X
~320	X	X			
Total bands	9	11	10	5	13

It can be seen from the data presented in Figure 3.4 and Table 3.1 that W34/70 and CBS 1260 show very similar banding patterns, not surprising since they are both Frohberg type lager strains. Compared to W34/70, CBS 1260 has bands missing at ~ 3000, 440 and 320 and has extra bands at ~ 2400, 840, 750 and 600. The two ale strains (M2 and NCYC 1332) also exhibited similar banding patterns. Compared to M2, NCYC 1332 has bands missing at ~ 2800, 2000 and 320 and has extra bands at ~ 5000, 2500, 105, 700 and 650. The cluster of bands

in the region of ~2000-1050 seem to be a prominent feature for these two ale strains. Strain CBS 1174 exhibited the fewest amplicons compared to the other brewing strains. This is potentially due to a difference in the number of, or the distance between, delta sequences, since smaller DNA fragments are often preferentially amplified during the PCR reaction. Differences in fingerprint may also be related to the cells ploidy; the number of homologous sets of chromosomes within the cell will dictate the number of delta regions (Hammond, 1996). Alternatively, the lack of delta regions in the Saaz yeast strain CBS 1174 may suggest that many Ty elements and solo delta elements are associated with the *S. cerevisiae* part of the genome. The cluster of bands in the region of ~2000-1050 for the ale type strains (M2 and NCYC 1332) may also further suggest that these elements are driven by genomic features more associated with ale strains than lager type strains.

3.2.2 Determination of strain kinetic growth properties in liquid media

Fundamentally, all brewing yeast have the capability to grow and undergo asymmetric division (budding) in the right environmental conditions (Chapter 1.3). However, individual strains exhibit different growth profiles from the point of inoculation into a medium due to variations in the manner by which they metabolise sugars and proliferate. This was evident by the strain differentiation seen on YPD agar growth environments (spot plates) containing rising concentrations of stressor (Chapter 3.2.3). In order to assess kinetic growth rates of each of the 5 brewing yeast strains employed in this study, growth curves were generated by inoculation of each strain into 200µl wells of

YPD media contained within a 96 well plate (Chapter 2.5.1) in triplicate and incubated at 25°C. Upon completion, raw data was collected using the Magellen™ analysis software (TEACAN, UK) and analysed using GraphPad Prism 7 software (GraphPad Software Inc., USA) (Chapter 2.5.1 and 2.7).

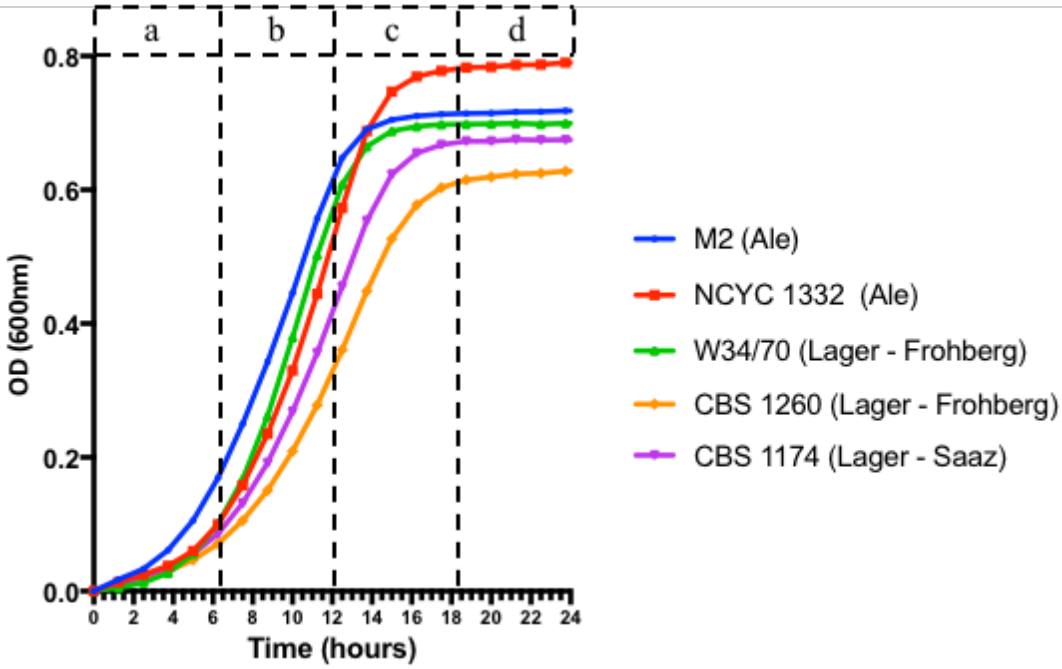


Figure 3.5 Kinetic growth curves for brewing yeast strains W34/70, M2, CBS 1260, CBS 1174 and NCYC 1332 in YPD medium at 25°C. Growth phases a, b, c and d are indicated by vertical dashed lines.

Typical, sigmoidal shaped, kinetic growth curves were produced by each strain (Figure 3.8). To highlight growth phases, data was divided into four sections, as indicated by the vertical dashed lines. The first phase, indicated by section a, was the initial lag phase. This represents a period of slow growth, due to metabolic adjustments required to adapt to the media, and ends with a rapid

and distinct increase in absorbance, indicative of upregulation in genes required for cell proliferation. It was possible to see that ale strain M2 was the first to exit the initial lag phase at approximately 4 hours, whilst the other four strains all required approximately 6.5 hours from the time of inoculation. The highlighted section b indicates the exponential phase; a period of rapid growth rate in proportion to time, due to the availability of sugars and increased cell proliferation. Analysis of this phase using the relative gradient (steepness) as a measure of the growth rate provides an indication of the replicative capacity of each yeast. In this respect, both W34/70 and NCYC 1332 exhibited the highest growth rate and CBS 1260 had the lowest, in YPD media. The third stage, indicated by section c, represents a secondary lag phase. This represents a period of slow growth due to the yeast cell population undergoing a further period of metabolic adjustment. However, at this stage this is likely to be due to a decline in the availability of fermentable sugars and a reduction in yeast cell population proliferation. Analysis of yeast population dynamics at this stage of growth revealed the greatest degree of separation in kinetic growth profile for the yeast strains. M2 and W34/70 were the first to enter the secondary lag phase at ~12 hours, followed by CBS 1174 at ~14.5 hours, NCYC 1332 at ~15 hours and lastly CBS 1260 at ~16.5 hours. The final phase, indicated by section d, denotes the stationary phase. Characterised by a stabilisation in the population, there was a cessation in the production of cell biomass, however it should be noted that yeast cells are typically viable at this stage and for an extended period of time (Vasicova *et al.,* 2015). This time frame is determined by the response of yeast strains to chronological aging (MacLean *et al.,* 2001;

Ocampo *et al.,* 2012; Powell *et al.,* 2000; Váchová *et al.,* 2012) and has importance for several reasons. Cells in stationary phase, also known as quiescent cells (Li *et al.,* 2013) depend on a store of nutrients (glygogen and trehalose) for survival (Ocampo *et al.,* 2012). The ability of brewing yeast to survive for long periods in environments of limited external nutrition is relevant for serial repitching (Chapter 1.4.3), as cells must be able to resume proliferation once placed into an environment containing fermentable sugars. Also of importance for the brewer is the extent of attenuation (Briggs *et al.,* 2004), in reference to the time taken for a yeast strain to fully convert all the sugars into biomass. Here NCYC 1332 was able to fully utilise sugars available in the YPD media to the highest extent followed by M2, W34/70, CBS 1174 and CBS 1260 respectively. A change in the capacity to utilise sugars can give rise to changes in final product characteristics and can be associated with environmental stress factors (Chapter 1.5.1) and yeast quality (Chapter 1.5).

3.2.3 Stress tolerance in ale and lager brewing yeast strains by growth on solid media

In order to determine the tolerance of yeast strains to environmental stress factors associated with brewing (Chapter 1.5.1), spot plate assays were conducted. This approach offers a simple means of screening yeast strains for tolerance to stress factors and other defined growth conditions (Carrasco *et al.,* 2001; Lewis *et al.,* 2010; Miyazaki *et al.,* 2005; Serrano *et al.,* 2006). The assay relies on the capacity of yeast to generate colony forming units on YPD agar plates containing increasing concentrations of a specific stressor (Kumar and

Snyder, 2001). In order to determine the capacity of strains to tolerate environmental stresses, agar plates were prepared to test for sensitivity to oxidative stress (Chapter 3.2.3.1), osmotic stress (Chapter 3.2.3.2) and ethanol stress (Chapter 3.2.3.3).

3.2.3.1 Oxidative stress tolerance of ale and lager strains on solid media

During the metabolism of sugars by yeast, free radicals, referred to as 'reactive oxygen species' (ROS) are released. Although primarily associated with aerobic growth, these are known to occur when yeast is metabolising both in the presence and absence of oxygen. Consequently, free radicals are produced throughout fermentation (Landolfo *et al.,* 2008) and act as a major stress factor for brewing yeast cells (Alexander Mott, Personal Communication). Common ROS generated include O_2^- (the superoxide anion), H_2O_2 (hydrogen peroxide) and $^{\bullet}OH$ (the hydroxyl radical) (Gutteridge and Halliwell, 2000; Halliwell, 2009). In order to identify differences in tolerance to oxidative stress between brewing yeasts, YPD agar plates were supplemented with H_2O_2 ranging from 0-7mM. These were spotted with 5µl of serially diluted cell suspensions (from a maximum of 10^6 cells/ml). After 14 days incubation at 25^{o}C (aerobically) the amount of growth was recorded and evaluated as described in Chapter 2.4.2.

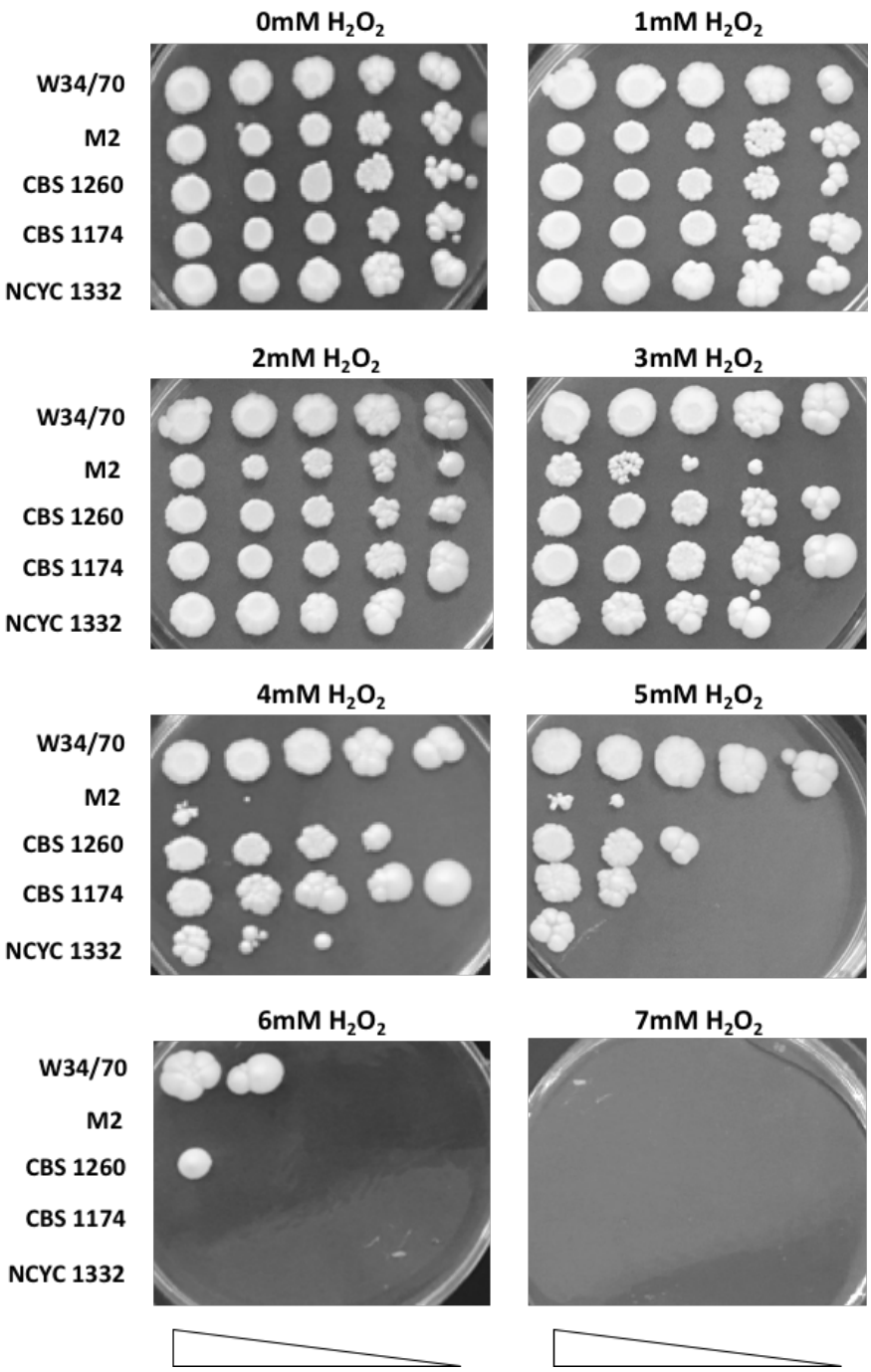


Figure 3.6 Tolerance of yeast strains to H_2O_2 induced oxidative stress. Growth of brewing yeast strains was assessed on YPD agar plates containing different concentrations of H_2O_2 as indicated. Serial dilutions (10^6 – 10^2 cells) of each strain were spotted on each plate from left to right and incubated aerobically for 14 days at 25°C.

As expected, it can be seen that there was a gradual inhibitory effect on yeast growth as the concentration of H_2O_2 was raised (Figure 3.6). However the response of each strain to oxidative stress was not the same at each concentration. Plates comprising 0, 1 and 2mM H_2O_2 showed very similar growth rates across all strains, with only the ale strain M2 showing signs of inhibition at 2mM H_2O_2. At 3mM the inhibition of M2 was much more pronounced with 10^6 and 10^5 cell spots reduced in size by around half. Similarly, the 10^4 and 10^3 spots were reduced in size even further and when 10^2 cells were applied, no CFU's were observed. At a concentration of 4mM, strains W34/70 and CBS 1174 remain unchallenged by the concentration of H_2O_2. M2 still exhibited the most notable inhibition, with only a few colonies at the two highest cell concentrations. CBS 1260 also showed signs of inhibition, with reduced growth at 10^3 and no growth at 10^2. It can be seen that NCYC 1332 also showed signs of significant inhibition, with only a few colonies produced at 10^5 and 10^4 concentrations and no growth at 10^3 and 10^2. It is apparent that a concentration of 5mM H_2O_2 exerted a considerable amount of oxidative stress on all the brewing yeast, apart from W34/70 which remained uninhibited at this level. Both ale strains showed the least amount of growth at this concentration of stress; the growth pattern of strain M2 was similar to that observed at 4mM H_2O_2, and strain NCYC 1332 only generated CFU's at the highest cell concentration (10^6). Surprisingly the rates of inhibition of CBS 1174 and CBS 1260 were different to those observed at 4mM; at this concentration CBS 1260 (with CFU's at 10^6, 10^5, and 10^4 cells) produced more growth than CBS

1174 (with CFU's at 10^6 and 10^5 cells only) in direct contrast to the lower concentration.

At 6mM H_2O_2, all strains showed complete inhibition of growth apart from the two Frohberg type lager strains W34/70 and CBS 1260 (Figure 3.5). W34/70 exhibited the most tolerance to oxidative stress generated by H_2O_2, but CFU's were only produced when concentrations of cells at 10^6 and 10^5 were used. Strain CBS 1260 only generated CFU's when 10^6 cells were added to the media. Finally, increasing the concentration of H_2O_2 to 7mM resulted in all brewing strains being unable to generate CFU's (Figure 3.6), indicating that this concentration was the limit for growth under the conditions applied here.

3.2.3.2 Osmotic stress tolerance of ale and lager strains on solid media

Osmotic stress represents one of the major environmental challenges experienced by yeast during industrial fermentations. Within the brewing sector this stress is typically associated with high gravity processes, however it is known to occur within fermentations of all types, affecting yeast physiology, viability and vitality and necessitating changes to the cell such as alterations to membrane fluidity (Zhuang *et al.,* 2017). In addition, high solute environments lead to a hyperosmotic stress response resulting in a loss of cellular water and consequently turgor (Latterich and Watson, 1993).

In order to identify the upper limits of osmotic stress on the brewing yeasts used in this study, YPD agar plates were supplemented with the sugar alcohol sorbitol at concentrations of between 0 and 60% (w/v). Sorbitol was selected since it is not assimilated by the yeast in this study and is non-toxic. This,

therefore, acts to simulate a constant level of osmotic stress much like in previous studies by Quain and Boulton, (1987), Pratt *et al.,* (2003) and Wojda *et al.,* (2003). Agar plates were then spotted with 5µl of serially diluted cell suspensions (from a maximum of 10^6 cells/ml). After 14 days incubation at 25^{o}C (aerobically) the amount of cellular growth was recorded and evaluated as described in Chapter 2.4.2.

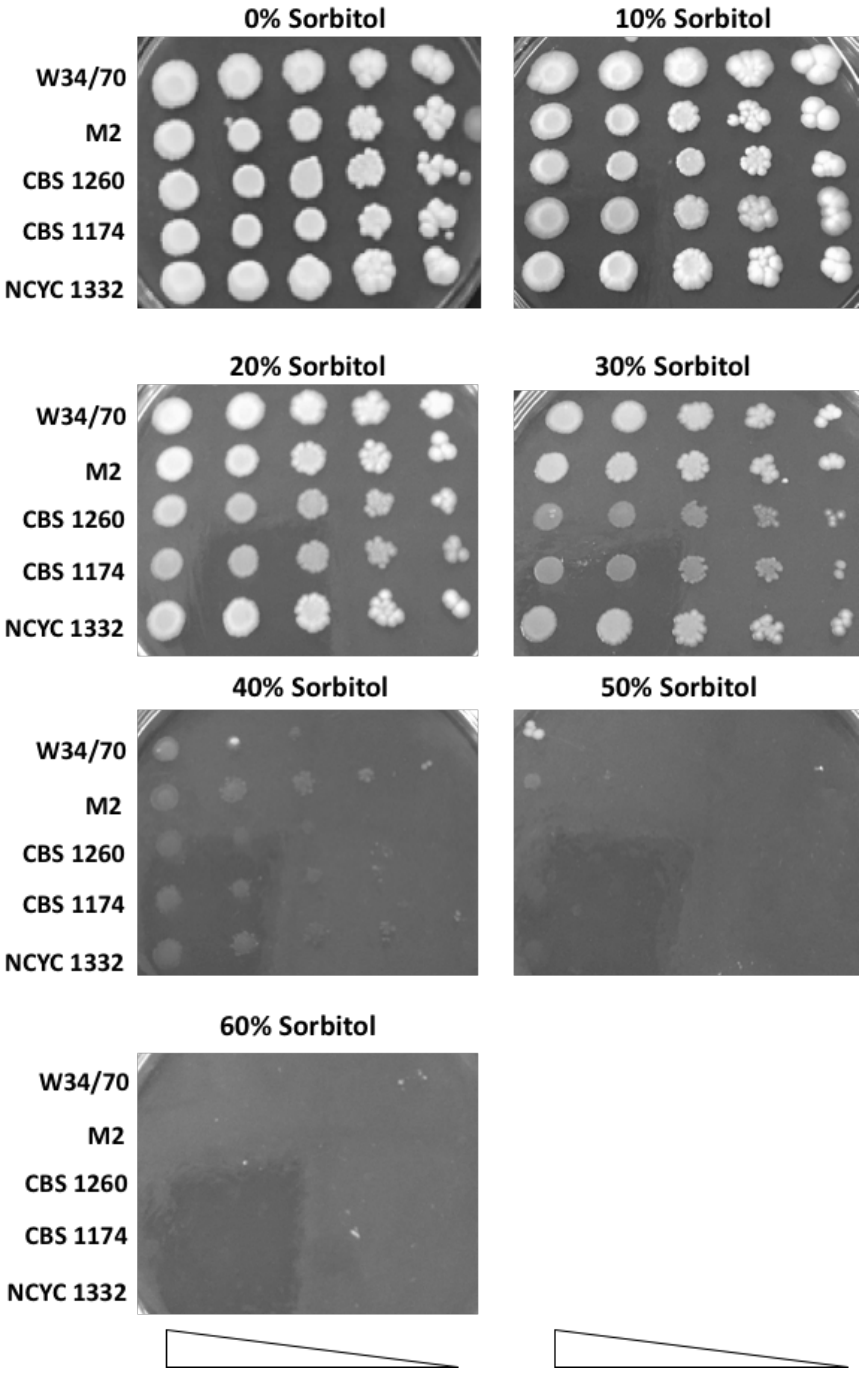


Figure 3.7 Tolerance of yeast strains to sorbitol induced osmotic stress. Growth of brewing yeast strains was assessed on YPD agar plates containing different concentrations of sorbitol as indicated. Serial dilutions (10^6 – 10^2 cells) of each strain were spotted on each plate from left to right and incubated aerobically for 14 days at 25°C.

It can be seen from Figure 3.7 that there was an overall gradual inhibitory effect of sorbitol on growth as the concentration was increased, similar to that observed for oxidative stress (Chapter 3.2.2.1). However the effect of osmotic stress was more unilateral with each increment compared to oxidative stress. Plates comprising 0 and 10% (w/v) sorbitol showed similar growth patterns for all strains; growth was observed, in all instances indicating there was no inhibitory effect at this concentration. Plates supplemented with 20% (w/v) sorbitol revealed the first signs of growth inhibition; there was less growth when low concentrations of cells (10^3 and 10^{2-} cells) were added to the media. When the sorbitol concentration was elevated to 30% (w/v), the amount of growth was further reduced for all the brewing yeasts. However, it can be seen that two lager strains (CBS 1260 and CBC 1174) were more impacted than the rest, with fewer smaller colonies produced. At 40% sorbitol (w/v) the growth of all the strains was severely affected. Only the two ale strains, M2 and NCYC 1332, produced a limited number of CFU's at the lowest cell concentration (10^2). Nevertheless, the growth of all strains was similar in that the numbers of CFU's were vastly reduced and smaller in size. At 50% sorbitol (w/v) only a very few CFU's were visible for each of the yeast strains at the highest cell concentrations, and those that were produced were all reduced in size apart from 3-4 CFU's in the 10^6 cell concentration spot for strain W34/70. Finally, an increase in sorbitol concentration to 60% (w/v) resulted in all brewing strains being unable to divide indicating that they were unable to tolerate this level of osmotic stress.

3.2.3.3 Ethanol stress tolerance of ale and lager strains on solid media

The ability of a brewing yeast to tolerate ethanol is of practical and economic importance, since ethanol is a key product of brewing fermentations. The range of ethanol produced during a fermentation can vary depending on the product, beer style, fermentation procedure, and the choice of brewing yeast. Although the majority of beers (in terms of sales volume) contain between 3.8 and 5% ethanol (v/v), with the increasing use of high and very high gravity brewing (Chapter 1.5.3) many beers are actually produced at higher alcohol concentrations. This means that brewing yeast may have to endure ethanol concentrations of 8-10% (v/v) or more is some situations (Puligundla *et al.,* 2011). This can lead to damage to cellular components, decreased cell volume, inhibition of specific growth rate, changes in metabolic pathways, and alterations to the cell wall and membrane structure. As a results, this can cause modified gene expression, reduced vitality and ultimately reduced viability of the brewing yeast (Lentini *et al.,* 2003; Stanley *et al.,* 2010) (Chapter 1.5).

In order to identify the upper limits of ethanol stress on the brewing yeasts used in this study, YPD agar plates were supplemented with ethanol between 0 and 30% (v/v). These plates were spotted with of 5µl of serially diluted cell suspensions (from an initial maximum 10^6 cells/ml). After 14 days incubation at 25^{o}C (aerobically) the amount of growth was recorded and evaluated as described in Chapter 2.4.2.

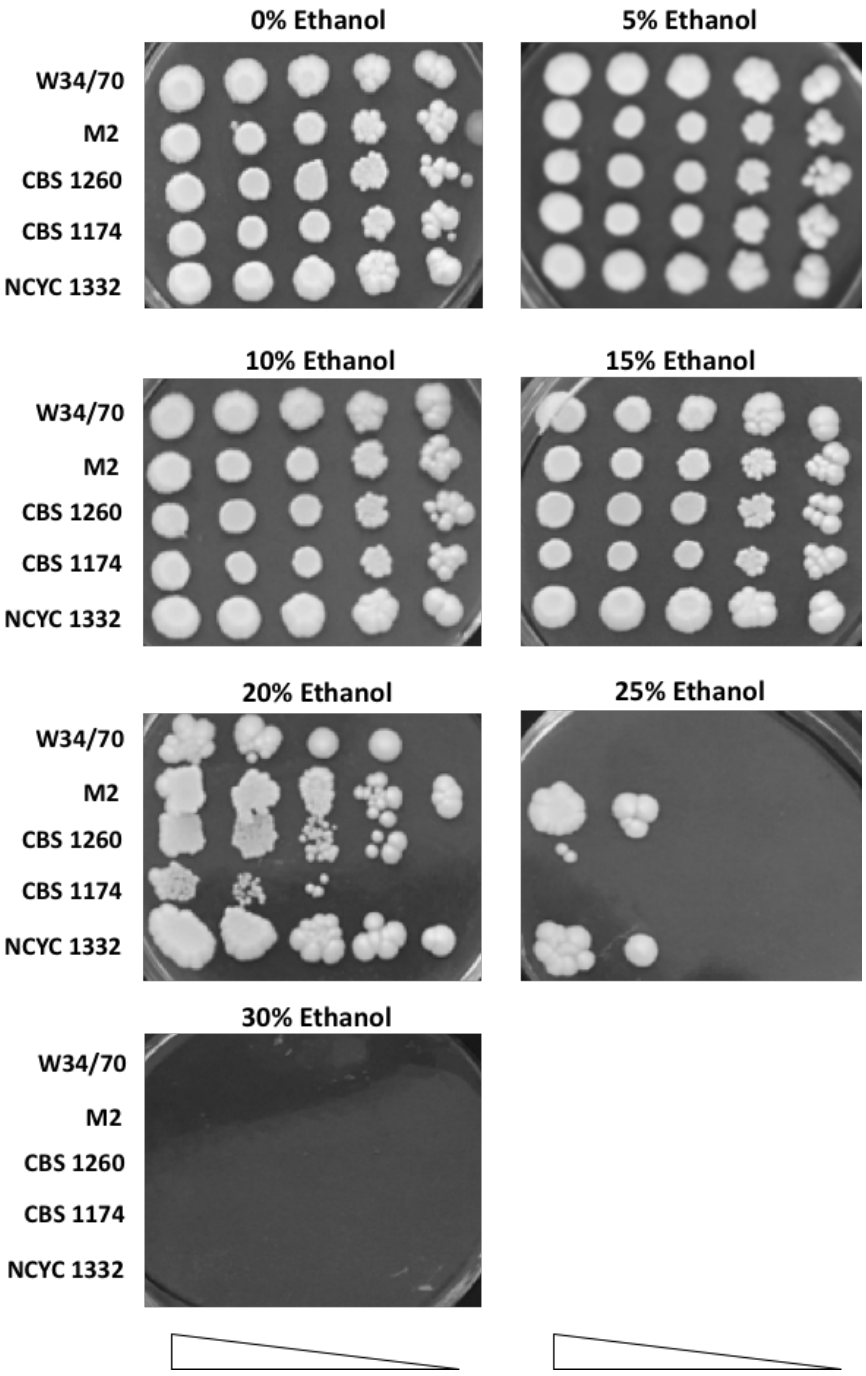


Figure 3.8 Tolerance of yeast strains to ethanol stress. Growth of brewing yeast strains was assessed on YPD agar plates containing different concentrations of ethanol as indicated. Serial dilutions (10^6 – 10^2 cells) of each strain were spotted on each plate from left to right and incubated aerobically for 14 days at 25°C.

It can be seen from Figure 3.8 that, as with the other stress factors examined above, increasing the concentration of ethanol caused growth inhibition. However, the strains employed in this study were all able to tolerate relatively high amounts of ethanol. Plates supplemented with 0 to 15% ethanol (v/v) yielded very similar growth, indicating there was limited inhibitory effects of these concentrations of ethanol on any of the strains evaluated. When 20% ethanol (v/v) was applied, the first signs of growth inhibition were observed. At this concentration and above it became more difficult to apply the initial 5µl cell suspension, due to the ethanol content within the YPD agar plate. This is likely due to the reduced surface tension created by the high alcohol concentration. This meant that when adding the cell suspension, it did not remain as a perfect circle and instead spread into irregular shapes making direct comparisons with lower ethanol concentration plates more difficult. However, it was still possible to observe that at 20% ethanol (v/v) there was some differentiation in growth between strains, due to the inhibitory effects of ethanol stress. The two ale strains, M2 and NCYC 1332, remained relatively unchallenged at this level of ethanol stress, with growth appearing similar to that observed on lower ethanol concentration plates. However, the growth of the two Frohberg type lager strains, W34/70 and CBS 1260 on 20% ethanol (v/v) showed signs of growth inhibition. This was particularly apparent at the lowest cell concentration (10^2 cells) where no growth was observed. The least tolerant strain at 20% ethanol (v/v) was the Saaz type lager strain CBS 1174. This strain showed signs of growth inhibition at all cell concentrations, with growth inhibited completely at the two lowest cell concentrations (10^3 and 10^2 cells).

Interestingly, where there were signs of growth inhibition, it was possible to see that only a few CFU's contributed to the 'spot', out of the thousands initially applied. This was particular evident for the 10^3 'spot' for strain CBS 1260, which comprised ~4 CFU and the 10^4 'spot' for strain CBS 1174 which was made up of ~3-4 CFU. Finally, increasing the concentration of ethanol to 30% (v/v) resulted in growth restriction for all of the brewing strains, indicating that they were unable to tolerate this level of stress.

3.3 Conclusion

In this chapter my aim was to investigate two ale (M2 and NCYC 1332), and three lager (W34/70, CBS 1260 and CBS 1174) yeast strains for their genetic and phenotypic characteristics. This was performed in order to gain a general understanding of these strains and a basic insight into their physiology prior to further investigations.

Based on the combination of genetic analysis using ITS PCR-RFLP and phenotypic analysis using permissive growth temperature, the yeast strains could be subdivided according to their genealogy. W34/70 and CBS 1260 were identified to be Frohberg type lager strains while CBS 1174 was designated a Saaz type lager strain. M2 and NCYC 1332 were designated as ale strains. An alternative genetic approach, interdelta PCR, was then taken to differentiate brewing yeast strains further. Amplification of the interdelta regions for each brewing yeast generated an individual fingerprint unique to each strain. This provided assurance that strains were sufficiently different such that their

physiological response to stress factors would provide a broad indication of how brewing yeast strains in general respond to environmental challenges. Furthermore, clarifying the variation in genetic make-up was important since it is known that the genetic background of yeast acts as the basis for responsive gene expression (Attfield *et al.,* 2001).

Growth rates of the five brewing yeast strains employed in this study produced typical, sigmoidal shaped, kinetic growth curves. Strain M2 was found to have the shortest lag phase, W34/70 and NCYC 1332 had the steepest exponential phases, M2 and W34/70 were the first to enter secondary lag phase and NCYC 1332 was able to fully assimilate the sugars available in the YPD media to the highest extent. The rapid and distinct changes between the growth phases were indicative of the mean cell metabolic activity in the population enabling the inference of how an individual strain reacts to the environment but masks the activity of individual cell-cell variability which would give another measure of strain 'fitness' (Avery, 2006; Brady, 2000; Sumner *et al.,* 2003; Sumner and Avery, 2002).

With the increasing use of high intensity practices such a high and very high gravity brewing (Chapter 1.5.3) stress factors inevitably become amplified in magnitude, placing brewing strains under increasing pressures (Chapters 1.5). The capacity of strains to respond to stress was evaluated by examining their ability to tolerate oxidative, osmotic and ethanol stresses. It should be noted that assessment of tolerance was based on growth capacity in this study. While it is acknowledged that there are other methods which can be applied to assess tolerance to stress (such as metabolic activity), for the purposes of this work,

and from the brewing perspective, the capacity to divide provides sufficient information. From this investigation it was possible to see that all strains were susceptible to elevated concentrations of stresses, however the way in which they reacted was both 'type' and strain dependant. The ale strains were generally less tolerant of oxidative stress than the lager strains (Chapter 3.2.2.1). All of the strains were able to tolerate high concentrations of osmotic stress, with growth observed for all strains at concentrations of up to 40% sorbitol (w/v). It should be noted that this concentration of sorbitol is known to reflect fermentations conducted with very high gravity worts and yielding approximately 10% ethanol (Zhuang *et al.,* 2017). At 40% sorbitol, the amount of osmotic stress generated caused negative impacts with CFU's becoming reduced in size. This was likely caused by hypertonic transition resulting in hypo-osmotic stress on the cells (White *et al.,* 2008); as intracellular osmolarity is increased, intracellular water loss causes a reduction in cell size.

Analysis of the response of yeast to alcohol stress indicated that the lager strains that were generally less tolerant of ethanol stress than the ale strains. This was counter to expectations since ale fermentations are often conducted at relatively low gravity with correspondingly low final alcohol concentrations. However, it should be noted that *S. cerevisiae* strains are successfully employed in related industries producing high alcohol products, including wine and spirits. In addition, many 'traditional' beers, for example, lambic beers or those employing 'natural' fermentations yield comparatively high ethanol concentrations, so perhaps these results should not be surprising.

An additional, but important, observation arising from the work conducted here is that when stresses were applied, sub-populations within each strain became evident. In the majority of instances, when stress thresholds were reached a few cells remained able to generate colonies and consequently outperform others within the population. Given that each yeast sample comprised a monoculture of vegetative cells, all of which were theoretically identical, this indicates that cell heterogeneity exists within populations. This implies that some cells within the population are able to react to stress differently, either due to certain physiological characteristics which allow them to withstand stress, or due to their capacity to regulate gene expression and thus respond to stress as a survival mechanism (Levy *et al.,* 2012). The extent to which phenotypic heterogeneity occurs will be explored within the next Chapters.

Chapter 4:

Colony variation within brewing Yeast strains

4.1 Introduction

Since Emile Christian Hansen first established pure cultures of brewing yeast in 1883 (Quain, 2017), the importance of utilising monocultures free of contamination has been widely recognised. Employing a single yeast strain for fermentation allows for close control over the process and helps ensure product consistency. However, the brewer must also consider the fine balance between wort composition, yeast growth and metabolism, and ensuring the quality of the yeast employed (Chapter 1.2, 1.3.1 and 1.5), such that the required flavour compounds and ethanol concentrations are formed within a stipulated time (Lodolo *et al.,* 2008).

The nature of brewery yeast handling inadvertently causes yeast to be subjected to a series of successive stress factors (Chapter 1.5.1), which creates a harsh and fluctuating environment for yeast. When such conditions are coupled to fermentation (especially those employing high gravity worts), the potential for the generation of variants becomes significant (Gibson *et al.,* 2007; Holland *et al.,* 2014; Powell and Diacetis, 2007). Furthermore, the practice of reusing yeast taken from a previous fermentation (serial repitching; Chapter 1.4.3) has implications for population dynamics since flocculation and sedimentation of yeast may lead to stratification during collection (Cahill *et al.,* 1999; Powell *et al.,* 2004; Rodgers *et al.,* 1999). This is relevant since it has been suggested that cropping strategies may inadvertently lead to the selection of sub-populations (Barker and Smart, 1996; Gonçalves *et al.,* 2016; Powell *et al.,* 2000; Quain *et al.,* 2001; Wendland, 2014). Furthermore, reports have indicated that brewing yeast cultures can change over time, particularly with

respect to properties important in brewing fermentations, such as flocculation (Soares, 2011), diacetyl reduction (Krogerus and Gibson, 2013; Wainwright, 1973) and flavour production (Lodolo *et al.,* 2008; Pires *et al.,* 2014; Stewart, 2016).

The changes to fermentation characteristics described above have predominantly been linked to genetic drift and the accumulation of competitive mutants. However, while this certainly drives population change, the underpinning causes remain largely unexplored. Essentially, the platform for genetic change must lie within individual cells which make up the population. Without variation at the cellular level, gross changes to population dynamic would be unlikely to occur. In organisms that reproduce vegetatively, such as yeast, it is often assumed that all individuals are identical, however it is known that significant variation can exist due to point mutations (Latterich and Watson, 1993; Ristow *et al.,* 1995; Selmecki *et al.,* 2015; Tokuriki *et al.,* 2008; Wilke and Adams, 1992), age (Gibson *et al.,* 2008; Powell *et al.,* 2003, 2000; Scheda and Yarrow, 1966; Sumner *et al.,* 2003), genetic stability (Paquin and Adams, 1983; Skoneczna *et al.,* 2015; Zhu *et al.,* 2014), gene expression (Avery, 2006; Blake *et al.,* 2006; New *et al.,* 2014) and mitochondrial genome stability (Castrejón *et al.,* 2002; Jiménez *et al.,* 1988; Lipinski *et al.,* 2010; Vevea *et al.,* 2014) all of which may contribute to population heterogeneity (Ackermann, 2015; Avery, 2006; Bishop *et al.,* 2007; Gasch and Werner-Washburne, 2002; Hewitt *et al.,* 2016; Kassen, 2002; Symmons and Raj, 2016) Section 1.7. While the majority of these studies have focused on laboratory organisms, it is likely that phenotypic individuality exists with respect to populations of

microorganisms with industrial applications (Section 1.7). However, the extent to which this occurs is currently unknown.

In addition to genetic variation, epigenetic differences (impacts that cannot be linked to genetic change Section 1.7) between cells have been observed within the microbial world and may prove to contribute towards population variation. From an industrial perspective this is significant since the manifestation of both genetic and phenotypic variations within a brewing yeast population play a crucial role in fermentation performance and in determining product quality. A caveat is that, in the majority of instances, population heterogeneity at the cellular level may go unnoticed, especially if variants are present in low numbers. This is because most conventional microbial population studies rely on data averaged across thousands or millions of cells within a sample (Avery, 2006). Irrespective, this remains a topic which has yet to be explored in brewing yeast.

In order to determine the relative extent of physiological heterogeneity between different brewing yeast strains, the propensity to form morphological variants was determined by investigating colony morphology on WLN agar plates (Section 2.4.1). Isolated phenotypic variants were analysed for visual differences and further characterised by genetically assessing interdelta region variability, recognised as a useful tool for detecting yeast mutations (Legras and Karst, 2003; Wilke and Adams, 1992) (Section 2.3.2 and 3.2.1.2). This was performed to determine the degree of phenotypic variation which could be attributed to genetic variation, and to explore the potential for epigenetic factors to influence yeast physiology.

4.2 Results

4.2.1 Yeast strain morphology analysis via WLN assay

The use of giant colony morphology analysis for the characterisation of brewing yeasts is a well-established technique (Richards, 1967). The principle of this method is that individual colonies produce a specific topography when cultivated for an extended period of time on solid media. Historically, wort or glycerol based agar were employed but at the current time WLN agar, which contains a bromocresol green dye, is preferred. This is primarily because it provides a further dimension to the analysis; the ability to reduce the green dye causes colonies to take on specific colours (Hall, 1971). The dye is also a pH indicator. Below a pH of 3.8 the dye ionises and appears yellow, above this pH the dye appears green until it deprotonates at the higher pH of 5.4 and appears blue (Sigma Aldrich; www.sigmaaldrich.com/catalog/product/sial/114359).

In this study five brewing yeast strains: W34/70, M2, CBS 1260, CBS 1174 and NCYC 1332 were assessed for giant colony morphology after growth for 14 days on WLN media as described in Chapter 2.5.2. It should be noted that the surface topography of giant yeast colonies is notoriously difficult to describe and can be subjective. For this reason, Richards (1967) devised 6 distinct groups based on observations from 80 different brewing yeast strains (Table 4.1). These broad descriptions have been implemented here.

In each instance, 20 cells of each brewing yeast strain were spread onto ten WLN agar plates for a total of 200 colony forming units per strain. After 14 days of aerobic incubation at 25^{o}C plates were examined for colony type and

morphology. The most prolific appearance observed was considered to be the usual ‘type’ morphology. Any variation from the ‘type’ was recorded using the descriptors detailed in Table 4.1. These colonies were then sub-cultured onto fresh WLN agar plates, re-incubated under identical conditions and the resulting morphologies noted.

Details of the ‘type’ giant colony morphologies observed for strains W34/70, M2, CBS 1260, CBS 1174 and NCYC 1332 can be seen summarised in Table 4.2, with intra-strain variations detailed in Tables 4.3-4.7 respectively. Analysis of giant colonies indicated that WLN agar was able to differentiate the ‘type’ morphology of each strain via a combination of distinct topography and colouration. Only W34/70 and CBS 1174 showed similar morphologies, and only the ‘shade’ of green allowed these strains to be separated based on their appearance on WLN agar (Table 4.2).

Table 4.1 Descriptions of brewing yeast giant colony topographies grouped into similar morphologies (Richards, 1967).

Group	Description
A	The greater part of the surface of this irregularly-shaped colony is occupied by a wide concavity, the whole surface having a moist appearance.
B	A central truncated pillar of yeast growth, which assumes a considerable height, occupies the greater part of the colonial surface. The colony has a smooth mat surface and possesses a serrated edge.
C	This colony-type possesses a central rounded protuberant growth of varying dimensions. The colonial surface is smooth and virtually featureless, with either an entire smooth edge or a vestigial serrated edge. Three sub-groups of this type can be recognized.
C(i)	A central cone of growth may either be well evident or virtually absent. The colony surface is perfectly smooth with a mat dull appearance, and the periphery is almost entire with only a faint suggestion of serrations.
C(ii)	This colony-type invariably possesses a smoothly raised area of growth occupying a central position on the colonial surface. The colony is round with a completely smooth edge and the colonial surface has a characteristic smooth, moist glistening appearance.
C(iii)	This differs from the previous sub group only in that the outermost periphery of the colony has delicate superficial serrations and only a very minor degree of surface marking is evident.
D	The colony possesses only a rudimentary central protuberant growth, and the whole of the colonial surface has a very rough appearance due to small surface protuberances which are randomly dispersed. The colony is irregularly shaped with the edge considerably dentated.
E	A prominent smooth conical growth occupies the centre of the colony. The greater part of the colony surface is, however rough, due to a well-developed series of concentric circular markings which usually extend to the edge of the colony. This concentricity of surface marking is diagnostic for the group. The colony is circular in shape although the edge may be serrated or lobed.
F	This is a somewhat heterogeneous group. The basic features for inclusion in this group are a smooth centre of varying dimensions, which may be either raised or flat, coupled with a rough outer portion. The markings on the outer portion of the colony usually have no regular feature, but a minor degree of concentric marking is accepted in this group.

Table 4.2 'Type' giant colony morphology of five brewing strains; W34/70, M2, CBS 1260, CBS1174 and NCYC 1332.

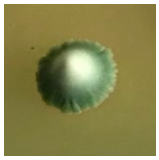
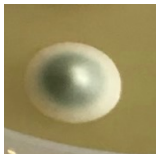
Strain	Colony	Group (Table 4.1)	Colouration
W34/70		C(i)	Mostly creamy-white in colour. One concentric circle of very pale green halfway down colony.
M2		B	Creamy-white conical centre followed by a thick concentric circle of dark green then creamy-white outer edge.
CBS 1260		C(i)	Concentric circles of thick dark green and thin creamy-white bands.
CBS 1174		C(i)	Mostly creamy-white in colour. One concentric circle of pale green halfway down colony.
NCYC 1332		D	Mostly creamy-white in colour. Some very pale green visible in concavities.

Table 4.3 Variants of W34/70 giant colony morphologies observed on WLN agar

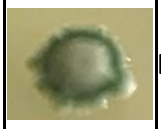
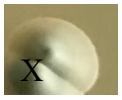
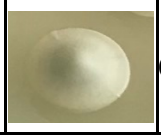
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
1A		B	1A-I		B	Retained (1)
			1A-II		C(iii)	New (2)
			1A-III		F	New (3)
1B		C(i)	1B-I		C(i)	New (4)
			1B-II		C(i)	Retained (5)
1C		C(i)	1C-I		C(i)	New (6)
2A		C(i)	2A-I		C(ii)	New (7)
			2A-II		C(i)	Type (8)
2B		C(i)	2B-I		C(i)	New (9)
			2B-II		D	New (10)
			2B-III		C(i)	Type (11)

Table 4.3 continued…

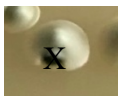
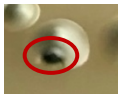
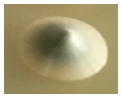
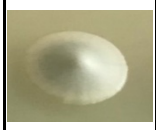
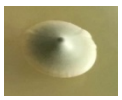
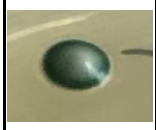
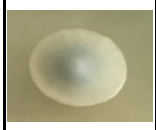
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
3A		C(i)	3A-I		C(i)	Retained (12)
3B		C(ii)	3B-I		C(ii)	New (13)
3C		C(i)	3C-I		C(i)	Type (14)
3D		C(i)	3D-I		B	New (15)
			3D-II		C(ii)	New (16)
4A		C(i)	4A-I		C(i)	Type (17)
			4A-II		C(iii)	New (18)
4B		C(i)	4B-I		C(i)	Type (19)
4C		C(i)	4C-I		C(i)	Retained (20)
4D		C(i)	4D-I		C(i)	Retained (21)
			4D-II		C(i)	New (22)

Table 4.3 continued…

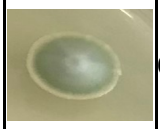
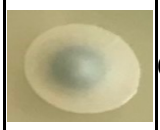
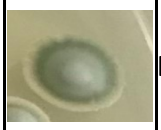
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
5A		C(i)	5A-I		C(ii)	New (23)
			5A-II		C(i)	Retained (24)
6AB		C(ii)	6AB-I		C(i)	Type (25)
			6AB-II		C(ii)	Retained (26)
7A		B	7A-I		C(i)	New (27)
			7A-II		C(i)	New (28)
			7A-III		B	Retained (29)
8A		B	8A-I		C(i)	New (30)
			8A-II		C(ii)	New (31)
8B		C(i)	8B-I		C(i)	New (32)
8C		C(i)	8C-I		C(i)	New (33)

Table 4.3 continued…

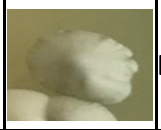
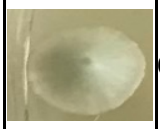
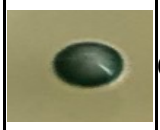
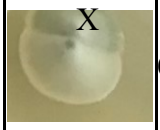
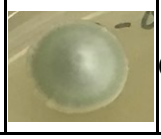
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
			8C-II		B	New (34)
8D		C(i)	8D-I		C(i)	New (35)
			8D-II		C(ii)	New (36)
			8D-III		C(i)	Retained (37)
9A	 X	C(i)	9A-I		C(ii)	New (38)
			9A-II		C(i)	New (39)
9B	 X	C(i)	9B-I		C(i)	Retained (40)
10A		C(i)	10A-I	X 	C(i)	New (41)
			10A-II		C(i)	Retained (42)

Table 4.4 Variants of M2 giant colony morphologies observed WLN agar

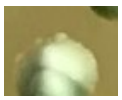
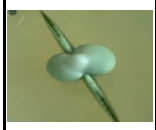
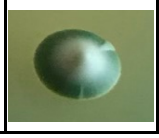
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
2A		B	2A-I		B	Retained (1)
2B		C(i)	2B-I		C(i)	New (2)
			2B-II		C(i)	New (3)
2C		B	2C-I		B	New (4)
			2C-II		B	New (5)
			2C-III		C(i)	New (6)
3A		B	3A-I		B	Retained (7)
3B		C(i)	3B-I		C(i)	New (8)
			3B-II		C(i)	New (9)
3C		B	3C-I		C(i)	New (10)
			3C-II		C(i)	New (11)

Table 4.4 continued…

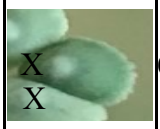
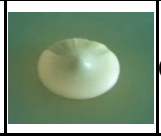
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
			3C-III		C(i)	New (12)
			3C-IV		C(i)	New (13)
			3C-V		B	New (14)
			3C-VI		C(iii)	New (15)
5A		C(i)	5A-I		C(i)	New (16)
			5A-II		C(i)	New (17)
			5A-III		C(i)	New (18)

Table 4.5 Variants of CBS 1260 giant colony morphologies observed on WLN agar

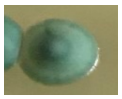
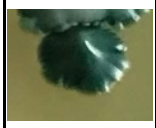
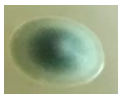
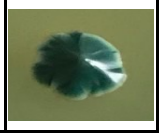
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
1A		C(ii)	1A-I		B	New (1)
1B		C(i)	1B-I		C(i)	New (2)
			1B-II		C(i)	Type (3)
1C		C(i)	1C-I		B	New (4)
			1C-II		C(i)	New (5)
2A		F	2A-I		C(iii)	New (6)
			2A-II		F	Retained (7)
2B		C(i)	2B-I		C(i)	New (8)
			2B-II		C(i)	New (9)
			2B-III		C(i)	New (10)
3A		C(ii)	3A-I		C(ii)	Retained (11)

Table 4.5 continued…

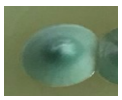
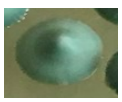
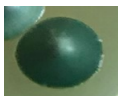
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
3B		F	3B-I		C(ii)	New (12)
			3B-II		C(i)	New (13)
			3B-III		F	Retained (14)
3C		B	3C-I		B	Retained (15)
			3C-II		C(ii)	New (16)
4A		C(i)	4A-I		C(i)	Type (17)
			4A-II		C(i)	New (18)
			4A-III		C(i)	New (19)
4B		C(i)	4B-I		C(i)	Type (20)
4C		C(i)	4C-I		C(i)	New (21)
4D		C(i)	4D-I		C(i)	Retained (22)

Table 4.5 continued…

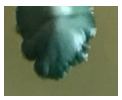
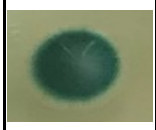
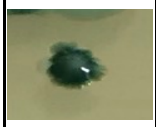
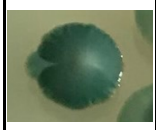
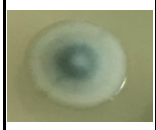
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
5A		F	5A-I		F	Retained (23)
5B		C(ii)	5B-I		C(i)	New (24)
			5B-II		C(ii)	Retained (25)
6A		C(i)	6A-I		C(i)	New (26)
			6A-II		F	New (27)
			6A-III		C(i)	Retained (28)
			6A-IV		C(ii)	New (29)
			6A-V		C(i)	New (30)
			6A-VI		C(i)	Type (31)
6B		C(ii)	6B-I		C(ii)	Retained (32)

Table 4.6 Variants of CBS 1174 giant colony morphologies observed on WLN agar

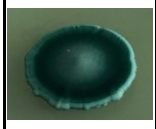
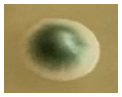
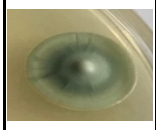
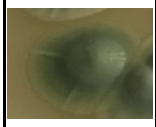
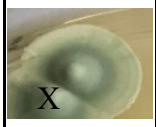
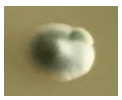
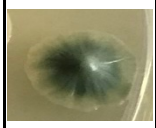
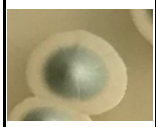
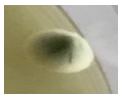
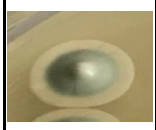
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
1B		C(i)	1B-I		D	New (1)
1C		C(i)	1C-I		D	New (2)
2A		C(i)	2A-I		D	New (3)
			2A-II		C(i)	New (4)
			2A-III		C(i)	Type (5)
2B		C(i)	2B-I		D	New (6)
			2B-II		C(i)	Type (7)
2C		C(i)	2C-I		C(i)	Retained (8)
			2C-II		C(i)	Type (9)
3A		C(i)	3A-I		C(i)	New (10)
			3A-II		C(i)	Type (11)

Table 4.6 continued…

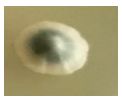
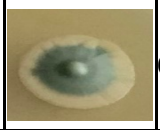
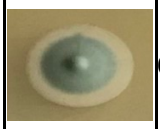
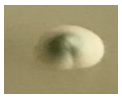
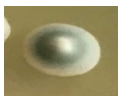
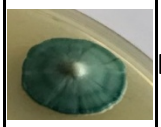
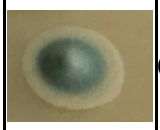
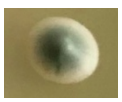
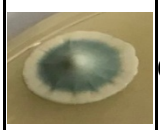
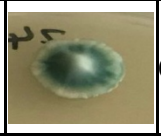
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
3B		C(i)	3B-I		C(i)	New (12)
			3B-II		C(i)	New (13)
5A		C(i)	5A-I		C(i)	New (14)
			5A-II		C(i)	New (15)
6A		C(i)	6A-I		D	New (16)
7A		C(i)	7A-I		C(i)	New (17)
			7A-II		C(i)	New (18)
			7A-III		C(i)	Type (19)
7B		C(i)	7B-I		C(i)	New (20)
			7B-II		C(i)	Retained (21)
8A		F	8A-I		C(i)	New (22)

Table 4.6 continued…

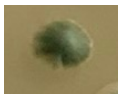
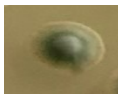
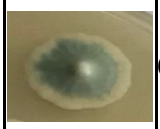
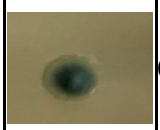
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
			8A-II		D	New (23)
			8A-III		D	New (24)
9A		C(i)	9A-I		D	New (25)
9B		C(i)	9B-I		C(i)	Retained (26)
			9B-II		C(i)	New (27)
			9B-III		C(ii)	New (28)
			9B-IV		C(i)	Type (29)
10A		B	10A-I		B	Retained (30)

Table 4.7 Variants of NCYC 1332 giant colony morphologies observed on WLN agar

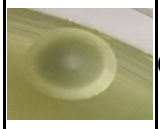
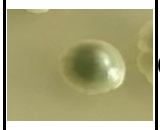
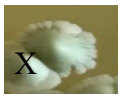
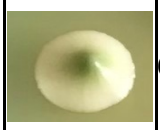
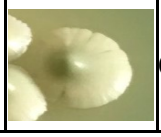
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
1AA		D	1AA-I		C(i)	New (1)
			1AA-II		B	New (2)
1AB		F	1AB-I		D	Type (3)
2A		D	2A-I		D	Type (4)
3A		D	3A-I		C(ii)	New (5)
			3A-II		C(i)	New (6)
			3A-III		C(ii)	New (7)
4A		B	4A-I		B	Retained (8)
5A		B	5A-I		C(i)	New (9)
			5A-II		C(ii)	New (10)
			5A-III		C(i)	New (11)

Table 4.7 continued…

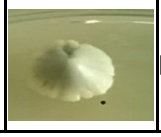
Plate 1			Plate 2 (sub-cultured from Plate 1)			
Ref.	Colony	Group (Table 4.1)	Ref.	Colony	Group (Table 4.1)	Retained, New or Type Morphology
			5A-IV		B	Retained (12)

Table 4.8 Variants from 'type' for all brewing yeast strains based on growth visualised on WLN agar (data surmised from Tables 4.3-4.7). Due to the misleading nature of the 'total variants produced from subculture plating' column the average number of variants was calculated ('total from subculture plate' divided by 'total from initial plate'). T: topography, C: colouration.

	First set of plates				Second set of plates							
										Morphology: Retained, New or switched back to Usual		
	Total variants produced	Variants due to change in 'T' only	Variants due to change in 'C' only	Variants due to change in both 'T'&'C'	Total variants produced from subculture plating	Average number of variants produced in subculture from original	Variants due to change in 'T' only	Variants due to change in 'C' only	Variants due to change in both 'T'&'C'	Number of Retained	Number of New	Number of 'Type'
W34/70	23	0	19	4	42	1.83	0	16	16	11	25	6
M2	7	3	4	0	18	2.57	0	10	6	2	16	0
CBS 1260	16	2	7	7	32	2.00	0	14	9	9	20	4
CBS 1174	15	0	13	2	30	2.00	1	16	9	4	20	6
NCYC 1332	6	0	3	3	12	2.00	1	0	9	2	8	2

Once the characteristic colony morphology of each strain had been identified, variants present in each population could be clearly identified. The data presented in Tables 4.3-4.7 and summarised in Table 4.8 highlight the phenotypic intra-strain differences in giant colony morphology. The total number of emergent variants can be seen to differ depending on strain; the lager yeasts W34/70, CBS 1260 and CBS 1174 manifested the most types of variant, with W34/70 resulting in the highest frequency of 23. Lager strains are hybrid organisms and much younger in evolutionary terms than their ale counterparts (Section 1.3.3). *S. pastorianus* brewing yeasts have extremely aneuploid genomes whereas *S. cerevisiae* have a much smaller degree of aneuploid (de Vries *et al.,* 2017). The extent of lager strain's aneuploid genome may mean they are more susceptible to morphological variation compared to the ale strains, especially if they draw upon the *S. cerevisiae* part of their genome when forming colony forming units (as it is this part, rather than that from the other parental strain, which contains the most genomic variation (Section 1.3.3) (Dunn and Sherlock, 2008)). In order to cope with the aneuploid nature of their genome, *S. pastorianus* have a massively diverse range of chromosome copy numbers across their strains (Frohberg strains contain between 42 – 84 and Saaz strains contain between 45 – 52) (Van den Broek *et al.,* 2015). However, chromosome copy number can also be linked to phenotypic traits. Van der Broek *et al.,* (2015) found this to be the case for two brewing related traits; diacetyl production and flocculation. In both cases different diacetyl production and flocculation profiles directly correlated with the copy numbers of the structural genes involved in their pathways. The

hybridisation event which lead to *S. pastorianus* meant that hybrid genes which are truly unique to the species had now emerged (Hewitt *et al*., 2014). This unique set of hybrid genes results in hybrid alleles and therefore has interesting cellular and molecular consequences for the yeasts (Monerawela and Bond, 2017). First of all, as well the hybrid alleles, the cells consist of allelic variants encoded by both the parental *S. cerevisiae* and *S. eubayanus* chromosomes. In addition to this, the copy number and ratio of the allelic variants can vary depending on the chromosome copy number making expression strain dependant and creating a metabolic landscape unique to *S. pastorianus* (Monerawela and Bond, 2017).

The reason lager strains present more genomic variation could simply be down to them being in an early part of their existence, an adolescent phase, before their genome 'works itself out' like ale strains must have done after their first whole-genome duplication event thousands of years ago (Section 1.3.3, Byrnes *et al.,* 2006; Dunn and Sherlock, 2008; Scannell *et al.,* 2006; Wolfe and Shields, 1997). For all strains, most of these variations were due to a change in colouration rather than topography, highlighting the importance of utilising the bromocresol dye in WLN agar as an indicator of phenotype. NCYC 1332 manifested the least types of variant, with the majority of the colonies produced on initial plates presenting the 'type' giant colony morphology for that strain.

Once initial colony types had been observed, samples were sub-cultured onto fresh media. This was performed to further investigate the variants for each strain: to determine whether each colony remained the same, continued to

produce more variants, or if they were able to revert to the original type. The column representing the total number of types of variant, based on growth from subcultured variant colonies, may be misleading since data were based on the number isolated from initial plates. However, the relative frequency of mutation can be calculated by dividing the number of variants from subculture by the initial number of variants; this revealed that in each instance the original variant produced approximately further 2 variations irrespective of yeast strain (Table 4.8). These new morphologies were mostly characterised by changes in colouration (apart from NCYC 1332, which exhibited differences in both colouration and topography), and most were new morphologies rather than a retention of variant morphology from the first set of plates, or of reverting back to the 'type' morphology for a particular strain. The number of variants produced was surprising, particularly given that they were all grown on a nutrient rich agar under non-limiting conditions and derived from fresh stocks propagated in YPD media. Levy *et al.* (2012) noted a similar phenomenon when cells were cultivated in an optimum environment, although in this instance a reduced growth rate was observed rather than colony morphology. Colonies which are out of specification for a particular strain have previously been considered to be either contaminants or mutants with aberrant DNA. However, Slutsky *et al.,* (1985) observed at least seven morphological phenotypes for the yeast *Candida albicans*, a yeast which has been described as containing subpopulations of "persister cells" (LaFleur *et al.,* 2006). These isolates were identified as phenotypic variants, able to survive the stress of antifungal treatments. Even in recent years the diversity in yeast colony morphologies had

been described as “puzzling, intriguing and interesting but an enigma” (Soll, 2014; Voordeckers *et al.,* 2012) which may give some indication to the complexity behind the mechanisms involved. It has been found that yeast colonies are able to form multicellular communities resembling that of higher multicellular organisms due to their differential gene expression and morphology (Honigberg, 2011). Furthermore, higher specialisation and communication during growth on solid media has been observed in yeast colonies due to differential gene expression, changes in metabolism, as well as intracellular signalling and spatial organisation (Mináriková *et al.,* 2001; Palková *et al.,* 1997; Scherz *et al.,* 2001; Váchová *et al.,* 2009; Varon and Choder, 2000; Voordeckers *et al.,* 2012). One gene in particular which has been identified as a principle component in colony development, adhesion and biofilm formation is *FLO11* which encodes for a large cell-surface protein (Reynolds and Fink, 2001; Váchová *et al.,* 2011). *FLO11* is not directly involved in cell - cell adhesion like other genes within the *FLO* family, this is believed to be due to the fact that the N-terminal domain lacks a lectin-like binding structure to bind specific sugar residues together on a selective basis (Goossens *et al.,* 2011; Verstrepen and Klis, 2006). Importantly, differences in overall *FLO11* expression levels have also been found to be directly linked to differences in colony morphology (Voordeckers *et al.,* 2012).

Although this observation supports the hypothesis that cell – cell heterogeneity exists, without further investigation it was not possible to confirm whether this was the cause of the differences in morphology observed. In order to establish whether the physical variant manifestations were a consequence of phenotype

or rooted in genotype, each colony was analysed by PCR fingerprinting as described below.

4.2.2 Assessment of the genomic fingerprints produced by morphological variants

In the previous section, the variable nature of the brewing yeast strains used in this study was highlighted. Without the application of any selective pressure or external stress, each strain was observed to produce giant colonies with morphological variations considerably different to their 'type' morphology. It should be noted that issues with the use of WLN agar for differentiation have been raised; observations can be subjective based on the operator (even with the guidance of described and tabulated morphological groups, Table 4.1), the presence of contaminants may confuse results, and ultimately the data was purely observational and does not consider any underpinning genetic variability. To address the latter, molecular analysis of the strains capable of producing the most (W34/70) and the least (NCYC 1332) number of variants was performed using interdelta PCR (Section 2.3.2 and Section 3.2.1.3). This was conducted with the aim of verifying the presence or absence of genetic variation and providing an indication of the extent of this in determining phenotypic heterogeneity in yeast strain morphology.

The DNA fingerprint patterns observed in Figures 4.2, 4.3 and 4.4 show that there was very little variation in the interdelta banding patterns between each sample and the control profile for lager strain W34/70 (Figure 4.1). It should be noted that careful consideration was required when making comparisons

between profiles. It is known that the relative intensity of amplicons between samples can occur due to differences in amplification of the DNA during the PCR reaction. Since PCR involves the logarithmic amplification of template DNA, even minor differences in the concentration of DNA sequences at the beginning of the reaction can present large differences in band intensity. Hence only variations in size fragment number were used as indicators of genetic variation. As described in Chapter 3.2.1.3, amplicons present between ~6000 and ~300bp in size were selected to be the focus of each fingerprint. The interdelta amplifications for the 42 W34/70 colony morphology variants produced three banding patterns outlined in Table 4.9.

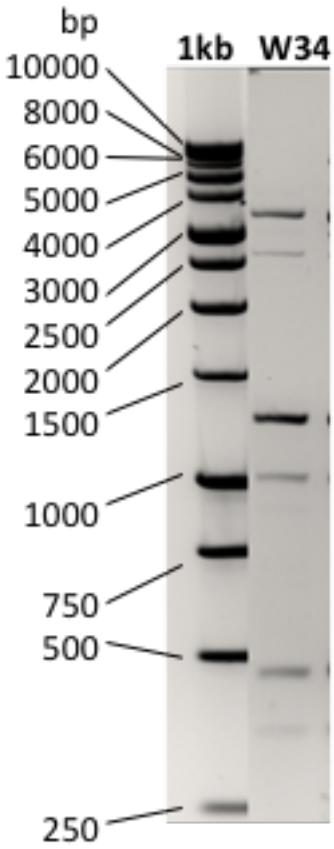


Figure 4.1 Interdelta PCR fingerprint analysis of W34/70. A 1kb ladder used to determine fragment sizes is also shown.

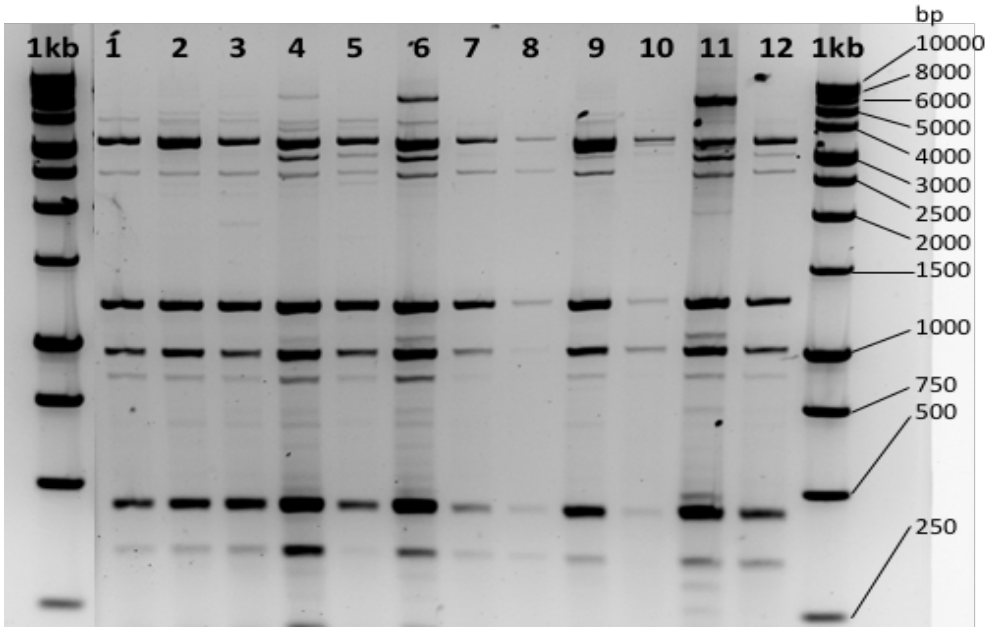


Figure 4.2 Interdelta PCR fingerprint analysis of second-generation morphological variants produced on WLN agar. Lanes 1-12 refer to samples 1-12 of the variants extracted from the sub-cultured second round of giant colony morphology analysis for W34/70 (Table 4.3). 1kb ladders used to determine fragment sizes are also shown.

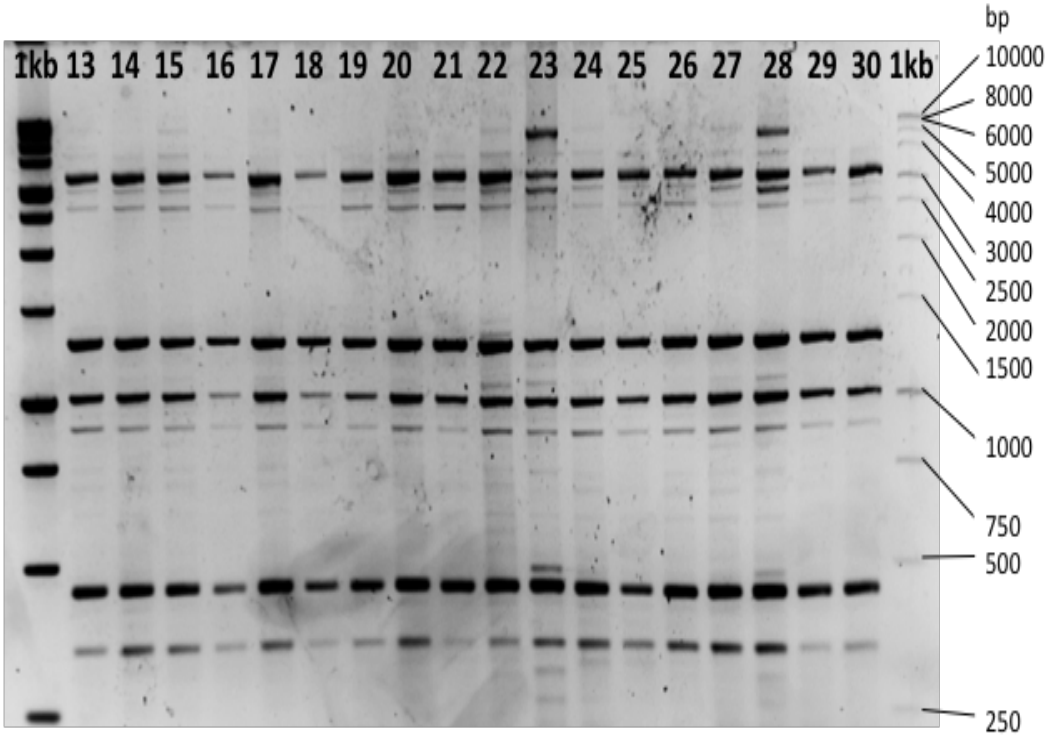


Figure 4.3 Interdelta PCR fingerprint analysis of second-generation morphological variants produced on WLN agar. Lanes 13-30 refer to samples 13-30 of the variants extracted from the sub-cultured second round of giant colony morphology analysis for W34/70 (Table 4.3). 1kb ladders used to determine fragment sizes are also shown.

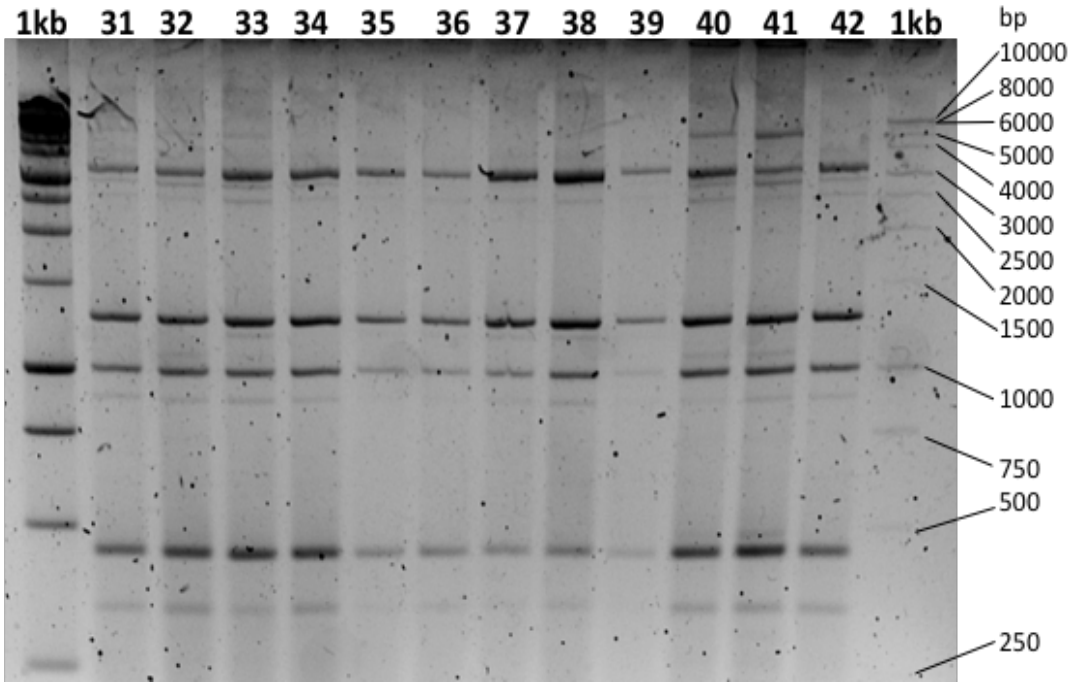


Figure 4.4 Interdelta PCR fingerprint analysis of second-generation morphological variants produced on WLN agar. Lanes 31-42 refer to samples 31-42 of the variants extracted from the sub-cultured second round of giant colony morphology analysis for W34/70 (Table 4.3). 1kb ladders used to determine fragment sizes are also shown.

Table 4.9 Amplicons produced using interdelta PCR electrophoresis analysis for W34/70 colony morphology variants (banding range ~6000-300bp)

Amplicon band size (bp)	Number of amplicons	Observed samples
4000, 3000, 2500, 1300, 1000, 960, 440, 320	8	Control, 1, 2, 3, 7, 8, 10, 35, 36, 37, 38, 39
5000, 4000, 3500, 3000, 2750, 2500, 1300, 1100, 1000, 960, 500, 440, 320	13	4, 6, 11, 23, 28, 40, 41
4000, 3500, 3000, 2750, 2500, 1000, 960, 440, 320	9	5, 9, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24, 25,26, 27, 29, 30, 31, 32, 33, 34, 42

The movement of transposable elements has been linked to the formation of spontaneous mutations (Kunz *et al.,* 1998). Interdelta PCR was chosen as a powerful differentiation tool which could examine the genetic basis upon which spontaneous morphology variants were occurring. Table 4.9 indicates that the morphological variants produced by W34/70 were split into only three banding pattern clusters. Samples 1, 2, 3, 7, 8, 10, 35, 36, 37, 38 and 39 were identical to the control sample. Samples 5, 9, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24, 25,26, 27, 29, 30, 31, 32, 33, 34 and 42 differed slightly with extra bands at 3500 and 2750 bp a missing band at 1300 bp. The largest variation from the control came from the cluster containing samples 4, 6, 11, 23, 28, 40

and 41. This cluster contained 5 extra bands at 5000, 3500, 2750, 1100 and 500 bp. On comparison of these clusters with the morphological phenotype (Table 4.3) there was no discernible link between the genomic clustering and the visual representation of the colony. Moreover, it was surprising there was not more genetic variation considering the amount and extent of the change in colony morphology. While it is acknowledged that the technique applied may not have been able to detect certain changes within the genome, this data does suggest that there was some capacity for phenotypic change in colony morphology, which was not controlled by changes within the genomic DNA. To confirm that there are no copy number polymorphisms within the population (duplications or deletions ranging from 50 basepairs to whole chromosomes) a technique such as microarray karyotyping would have to be employed to visulaise copy number changes (Dunn and Sherlock, 2008).

For the ale strain NCYC 1332, the interdelta amplifications for the 12 colony morphology variants produced three banding patterns outlined in Table 4.10. Table 4.10 indicates that the morphological variants produced by NCYC1332 were split into only one banding pattern cluster with 3 other isolates. Samples 2, 3, 4, 6, 7, 8, 9, 11 and 12 were identical to the control sample. Sample 1 differed the most with 5 extra bands at 5000, 4000, 2750, 2000 and 850 bp and 12 missing bands at 6000, 3100, 2500, 2400, 1900, 1500, 1100, 900, 750, 700, 400 and 300 bp. Sample 5 differed slightly with only 4 band missing at 6000, 750, 700 and 600 bp. Sample 10 differed with one extra band at 1700 bp and 6 missing bands at 2500, 2400, 1900, 600, 450 and 400. On comparison of these clusters with the morphological phenotype (Table 4.7) Samples 1, 5 and 10

stand out from the rest of the variants with sample 1 being the most unique. However, although sample 7 looked very similar to 5 and 10, this colony type did not exhibit any genetic similarity.

From the collective results of the interdelta PCR analysis, it can be seen that all of the variants within a strain showed a high degree of homology, both to each other and to the original 'type' genetic fingerprint. This was encouraging since it effectively rules out the possibility of contamination (however unlikely), from affecting the results. Therefore, I can be confident that morphologies were likely macro-morphological variants of the same strain, and that variation was a true phenotypic trait.

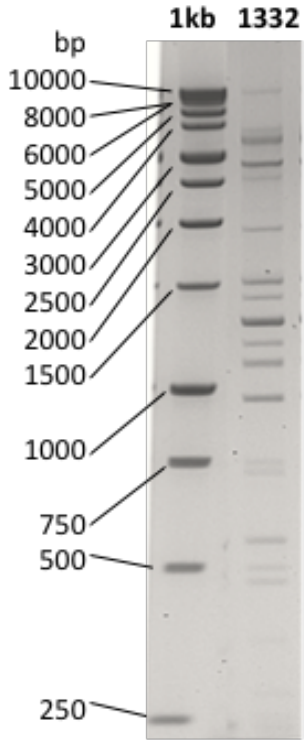


Figure 4.5 Interdelta PCR fingerprint analysis of NCYC 1332 taken from stock. 1kb ladder shown and was used to determine fragment sizes.

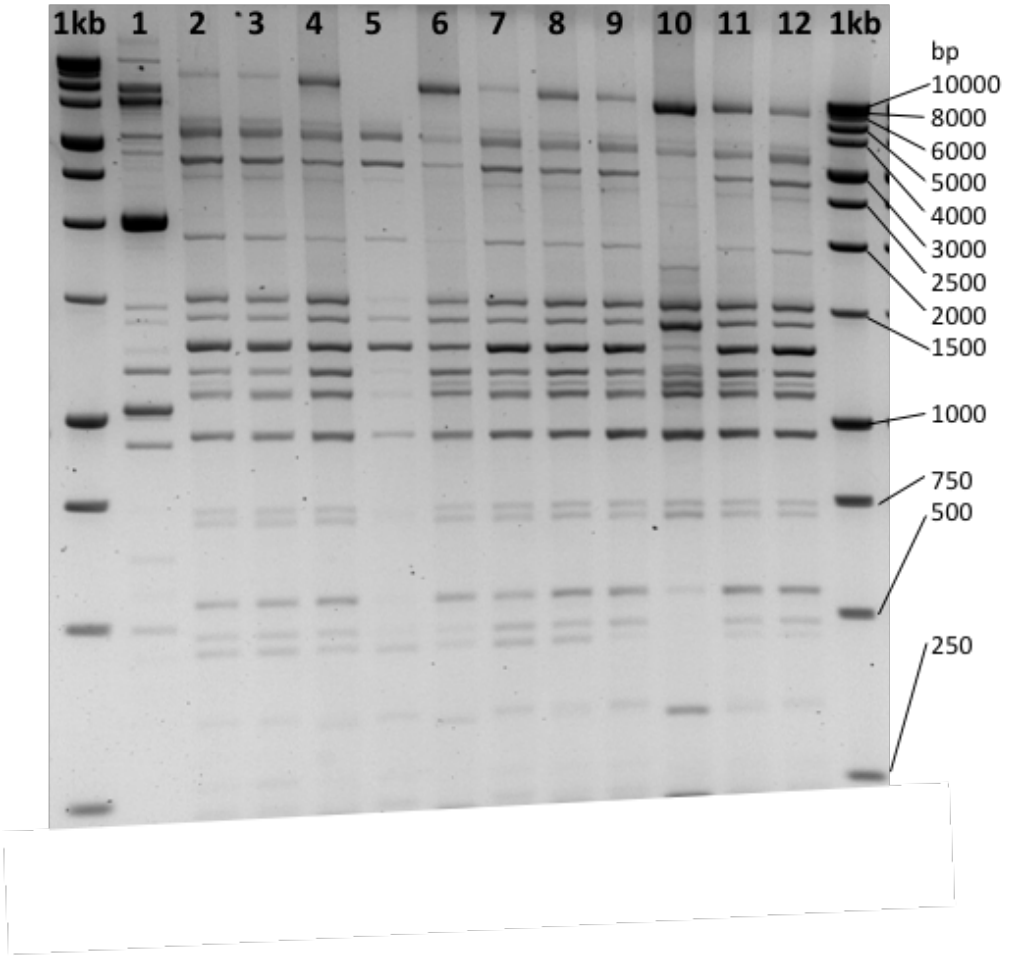


Figure 4.6 Interdelta PCR fingerprint analysis of second-generation morphological variants produced on WLN agar. Lanes 1-12 refer to samples 1-12 of the variants extracted from the sub-cultured second round of giant colony morphology analysis for NCYC 1332 (Table 4.7). 1kb ladders are shown and were used to determine fragment sizes.

Table 4.10 Amplicons produced using interdelta PCR electrophoresis analysis for NCYC 1332 colony morphology variants (banding range ~6000-300bp)

Amplicon band size (bp)	Observed samples
6000, 3100, 3000, 2500, 2400, 1900, 1500, 1400, 1300, 1200, 1150, 1100, 900, 750, 700, 600, 450, 400, 300	Control, 2, 3, 4, 6, 7, 8, 9, 11, 12
5000, 4000, 3000, 2750, 2000, 1400, 1300, 1200, 1050, 850, 600, 450	1
3100, 3000, 2500, 2400, 1900, 1500, 1400, 1300, 1200, 1150, 1100, 900, 450, 400, 300	5
6000, 3100, 3000, 1700, 1500, 1400, 1300, 1200, 1150, 1100, 900, 750, 700, 600, 300	10

4.2.3 Effect of increasing stressor dose on growth of strains capable of producing the most (W34/70) and the least (NCYC 1332) number of morphological variants

From the previous data (Chapter 4.2.1) it was evident that the five individual brewing yeast strains were able to produce variations in their colony morphology. Another aspect to those spontaneous morphology changes was the number of types of variant produced by a strain. W34/70 was found to produce the most variants after subculturing on WLN agar plates (42), 3.5 times

as many as NCYC 1332 which produced the least amount of different types of variant colony (12). These phenotypes were part of the strains fundamental capacity to grow, metabolise sugars from the solid media, metabolise the bromocresol green dye and specific divisional rates. In order to investigate potential reasons for the difference in the propensity to produce variants, each strain was examined for kinetic growth characteristics under stress conditions. This was performed to determine if the capacity to perform under stress was related to the extent to which variants were produced. It was anticipated that this would provide an alternative and more relevant metric than simple spot plate analysis as presented in Chapter 3. Consequently, population (growth) kinetics were examined under ethanol (0-25% v/v), oxidative (via H_2O_2 (0-5mM)) and osmotic (via sorbitol (0-50% w/v)) stress in YPD media as a base (Chapter 2.2.1).

Data presented in Figures 4.5, 4.6 and 4.7 revealed both W34/70 and NCYC 1332 showed typical sensitivity to gradual increases in stressor concentration during growth in a nutrient rich media, indicated by the increase in time to exit lag phase, reduction in steepness of exponential phase, later secondary lag phase and reduced total biomass in the stationary phase.

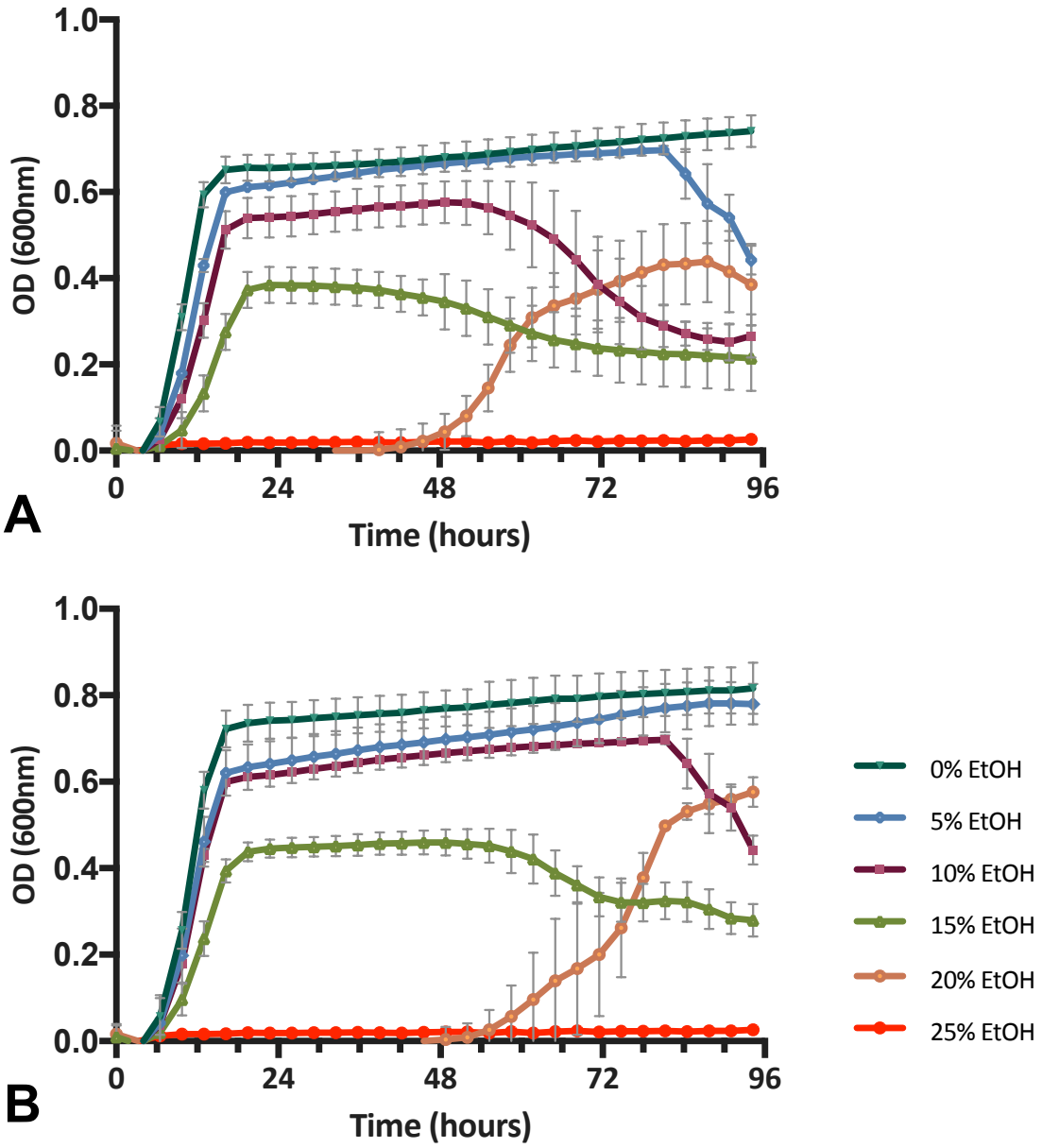


Figure 4.7 Kinetic growth curves produced under ethanol stress by brewing strains W34/70 (A) and NCYC 1332 (B) over 96 hours. Each point represents the mean of triplicates with error bars representing the standard error of the mean.

Analysis of the response of W34/70 and NCYC 1332 to ethanol indicated that both strains exhibited very similar growth profile patterns under increasing

concentrations (Figure 4.7). The main differences were that NCYC 1332 was able to attenuate the YPD media to a larger extent than W34/70 at every ethanol concentration which was evident by the maximum absorbance achieved; 0.817 compared to 0.743 at 0%, 0.782 compared to 0.697 at 5%, 0.687 compared to 0.577 at 10%, 0.460 compared to 0.386 at 15% and 0.580 compared to 0.455 at 20% ethanol (v/v). In both cases there was no increase in cell biomass at 25% ethanol (v/v) suggesting the cells were non-viable. At 20% ethanol (v/v) the end of the lag phase was reached earlier by W34/70 populations (~48hours) than for NCYC 1332 (~55hours) which was surprising considering that NCYC 1332 'outperformed' W34/70 on other aspects of growth. Another defining feature, which was not evident in the kinetic growth curve data presented in Chapter 3.2.2, was a phase in declining biomass. Although this could have been caused by cell shrinkage, a result of death due to accumulated stress, or alternatively due to autophagy (Mizushima and Klionsky, 2007). Autophagy in brewing yeast is often associated with nutritional starvation (Reggiori and Klionsky, 2013) and is a complex series of events triggered to allow the culture to maintain viability during nutrient depletion through a process of 'self digestion' (Boulton and Quain, 2006). Another possible explanation could have been a stress induced switch from planktonic (in suspension) to sessile cells (adherent/biofilm). If cells formed a biofilm on the side of the well the overall result would be a decline in the absorbance. For W34/70 a decline in absorbance was observed in response to 5, 10, 15 and 20% ethanol (v/v) and at 10 and 15% ethanol (v/v) for NCYC 1332. At 5% ethanol (v/v) W34/70 declined after 79 hours whereas NCYC 1332 remained in

stationary phase throughout the 96 hours. At 10% ethanol (v/v) the W34/70 population started to decline after 50 hours compared to NCYC 1332 at 80 hours. At 15% ethanol W34/70 this occurred after 24 hours compared to NCYC 1332 at 55 hours. At 20% ethanol (v/v) W34/70 cells began to decline after 90 hours whilst at this stage NCYC 1332 cells appeared to be in a period of slow growth.

The data indicates that under increasing ethanol stress and nutrient limitation, NCYC 1332 was able to maintain a constant biomass of stationary cells for longer time periods than W34/70.

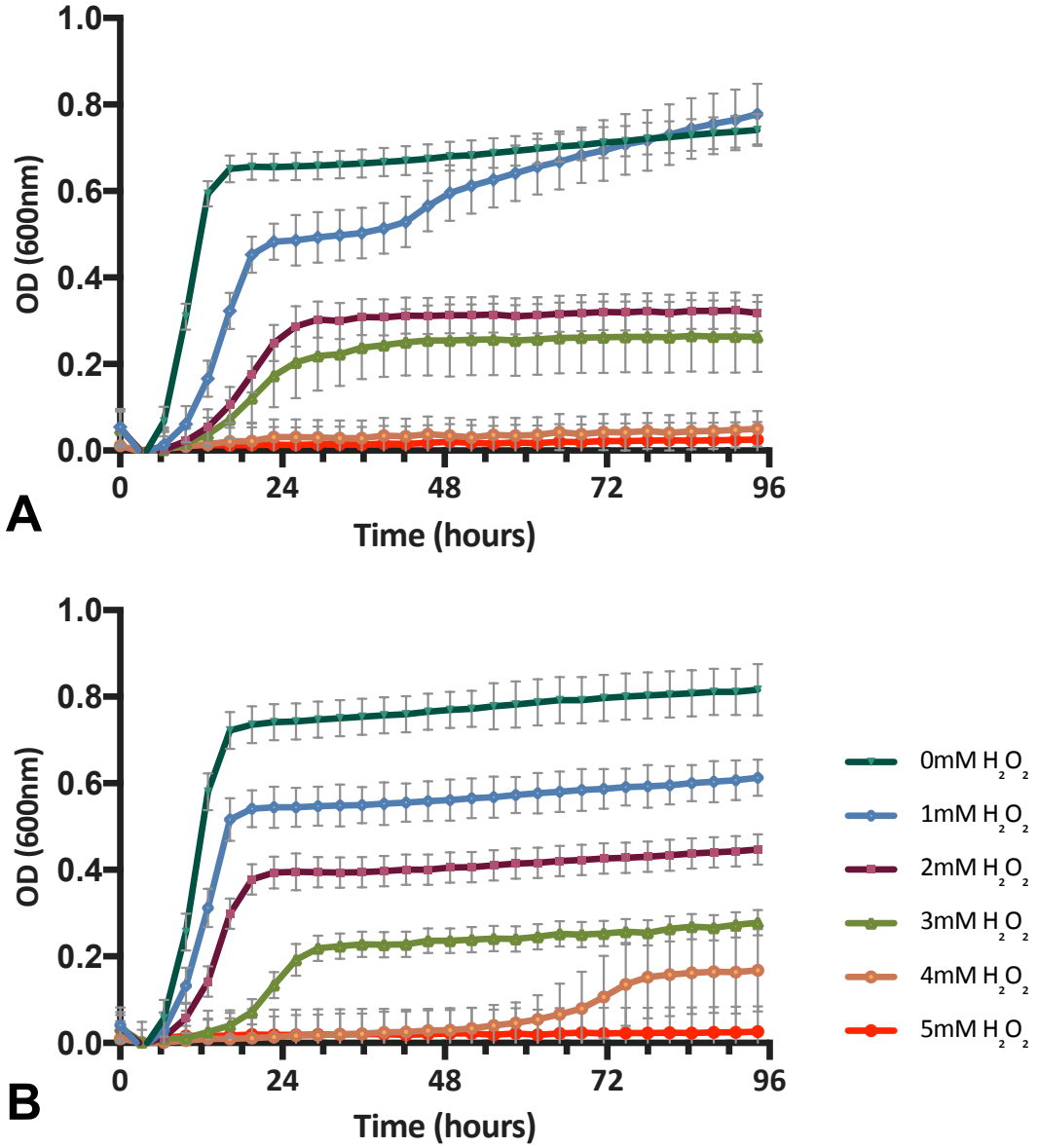


Figure 4.8 Kinetic growth curves produced under H_2O_2 induced oxidative stress by brewing strains W34/70 (A) and NCYC 1332 (B) over 96 hours. Each point represents the mean of triplicates with error bars representing the standard error of the mean.

Analysis of the response of W34/70 and NCYC 1332 to H_2O_2 indicated that both strains exhibited very similar growth profile patterns under increasing

concentrations (Figure 4.8). The main differences were that NCYC 1332 was able to attenuate the YPD media to a greater extent than W34/70 at most H_2O_2 concentrations, evident from the maximum absorbance achieved: 0.817 compared to 0.743 at 0mM, 0.450 compared to 0.325 at 2mM, 0.281 compared to 0.268 at 3mM, and 0.168 compared to no growth at 4mM H_2O_2. In both cases there was no increase in cell biomass at 5mM H_2O_2 suggesting that cells were non-viable at this high concentration. Interestingly, at 1mM H_2O_2, strain W34/70 appears to go through a secondary, nonetheless much slower, post secondary lag growth phase which began at around 44 hours and resulted in the maximum growth exceeding that of the control (at an OD_{600} = 0.785). There are two potential possibilities for the cause of this pattern of growth. Either there has been a period of metabolic adjustment causing W34/70 to cope with the stress and resume growth or alternatively a small, slow growing sub-population of H_2O_2 resistant cells may have had time to replicate by this point, adding to the overall biomass at 44 hours. For either situation there would have had to be sugars and other nutrients available which would be the case as no growth had been detected up until this point. Differences between strains are likely to be a consequence of strain dependent oxidative tolerance; it is known that H_2O_2 stress is strain and phase growth dependant, primarily due to varying levels of catalase activity and glutathione content within cells (Martin *et al.,* 2008).

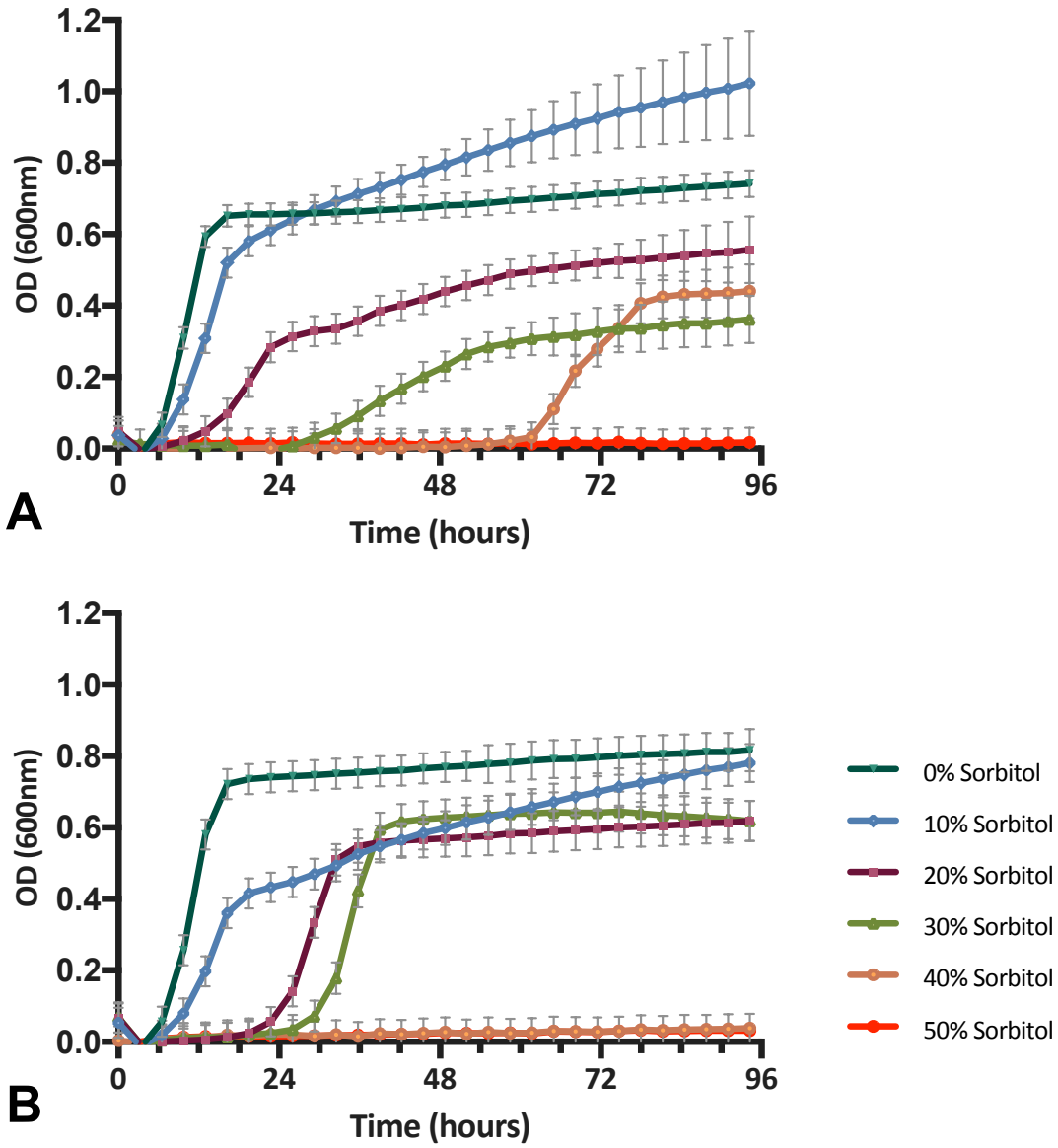


Figure 4.9 Kinetic growth curves produced under sorbitol induced osmotic stress by brewing strains W34/70 (A) and NCYC 1332 (B) over 96 hours. Each point represents the mean of triplicates with error bars representing the standard error of the mean.

Analysis of the response of W34/70 and NCYC 1332 to osmotic stress induced by sorbitol indicated that strains exhibited variable growth patterns (Figure

4.9). The primary differences were that NCYC 1332 was able to attenuate the YPD media to a greater extent than W34/70 at most sorbitol concentrations, evident from the maximum absorbance achieved; 0.817 compared to 0.743 at 0%, 0.620 compared to 0.559 at 20% and 0.644 compared to 0.368 at 30% sorbitol (w/v). In both cases there was no increase in cell biomass at 50% sorbitol (w/v) indicating that cells were non-viable at this concentration. Only W34/70 was able to grow under 40% sorbitol (w/v) stress after an extended lag phase of 65 hours after inoculation.

Interestingly, at 10% sorbitol (w/v) both W34/70 and NCYC 1332 appeared to enter a secondary lag phase which began at around 17 hours such that the maximum growth exceeded that of the control (Figure 4.7). This growth pattern was replicated by W34/70 under 20% sorbitol (w/v) except with a reduced total biomass production. It was interesting that NCYC 1332 had a reduced exponential phase followed by respiratory growth at 10 sorbitol (w/v), however at both 20 and 30% (w/v) the growth curves returned to a more typical sigmoidal profile. This may be due to the extended lag phase giving the population enough time to properly adjust metabolically to the osmotic stress before entering into exponential growth. Despite NCYC 1332's ability to attenuate the sugars in YPD media to a greater extent than W34/70 at lower concentrations of sorbitol, at 40% (w/v) this strain did not acquire any increase in biomass, whereas W34/70 did after a lag phase of 65 hours. The differences observed are likely to be due to the initial response to osmotic stress, which is characterised by accumulation of osmoprotectants (such as trehalose), which stabilise membranes, enzymes, and other proteins (Wiemken, 1990). During

sustained exposure, an alternate strategy may be used by yeast cells, involving the production of osmotica (molecules which moderate intracellular osmotic potential) such as glycerol which acts as a compatible solute (White *et al.,* 2008).

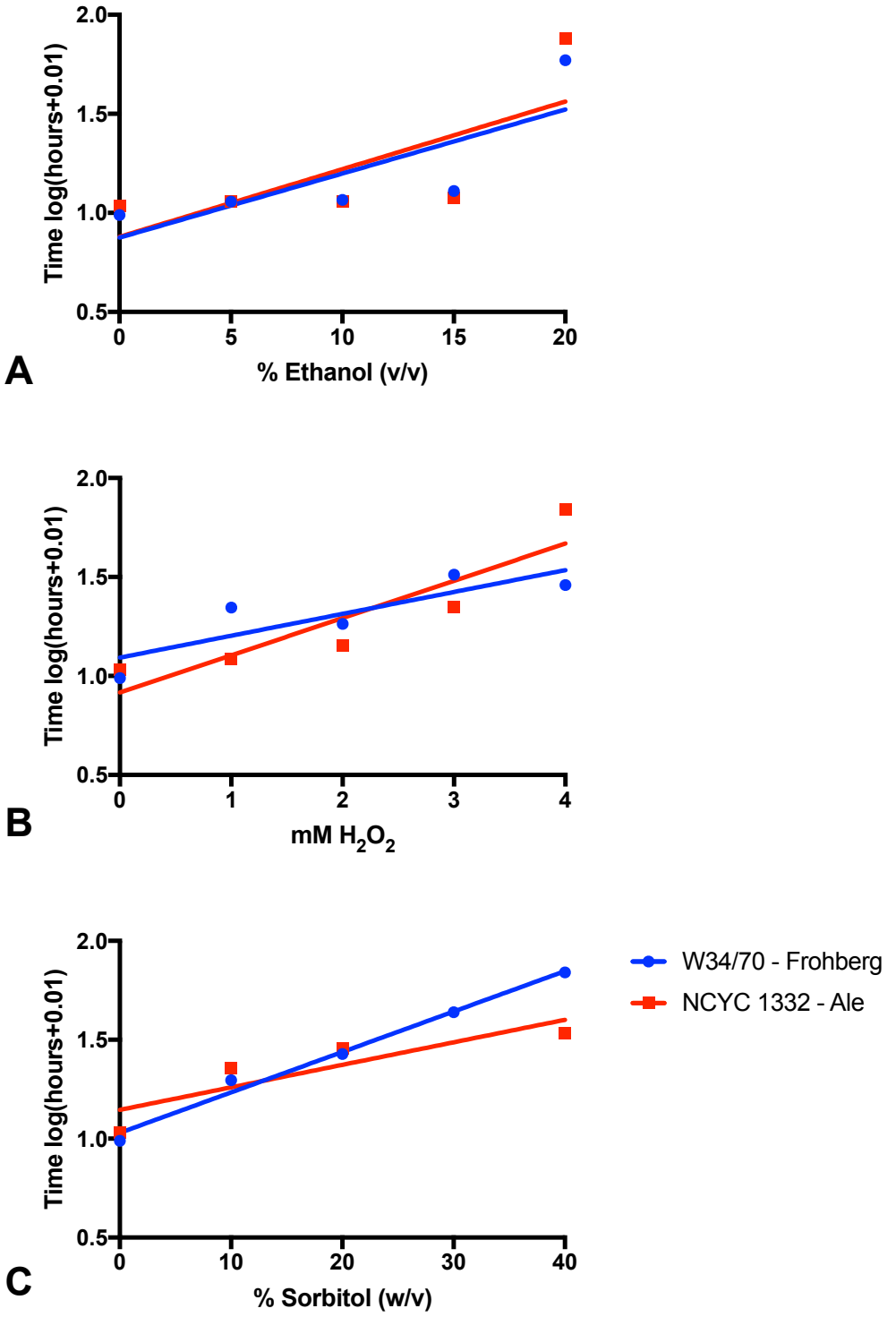


Figure 4.10 The relationship between 50% of maximal growth and stress concentration. A: ethanol stress, B: oxidative stress, C: osmotic stress.

To model the relationship between stressor and yeast cell biomass production, the data was first transformed against the log of time to normalise the data. Following this a sigmoidal four parameter model was applied to the data using the GraphPad PRISM 7 (GraphPad Software Inc., USA) (Chapter 2.7). From this, 50% of the total biomass was plotted against stressor concentration and modelled for linear regression (Figure 4.10). For W34/70 and NCYC 1332 there was a range of correlations observed. For ethanol stress (A) the relationship was both similar and positive for both strains (slope value of 0.03234 for W34/70 and 0.03416 for NCYC 1332). For oxidative stress induced by H_2O_2 (B) both strains exhibited a positive response, however NCYC 1332 yielded a significantly greater positive response, with a slope of 0.1881 compared to 0.1105 for W34/70. For osmotic stress induced by sorbitol (C) both strains exhibited a positive response, however W34/70 was more positive with a slope of 0.02044 compared to 0.01138 for NCYC 1332.

When linking the growth dynamics of W34/70 and NCYC 1332 and their overall tolerance to ethanol, osmotic and oxidative stress in liquid media to the strains ability to produce variant colony morphologies on WLN agar plates, there does not appear to be a clear link. Firstly, it was evident from the kinetic data in Figures 4.5-7 that stress responses in different yeast strains have different impacts depending on the stress applied. Figure 4.8 helps to summarise this relationship as ethanol having a similar effect, oxidative stress having a more positive correlation with NCYC 1332 and osmotic stress having a more positive correlation with W34/70. Also, both strains exhibit the ability to initiate growth

under extreme stress and both exhibit a kinetic reduction under ethanol stress. Therefore, the frequency in colony variance was not an indicator of stress tolerance or growth dynamics.

4.3 Conclusion

It is important to consider genetic variation since previous work has shown that monoclonal brewing yeast cultures which undergo serial repitching into subsequent fermentations can undergo a genetic change (Paquin and Adams, 1983). This phenomenon was likely to be driven by the yeast cell trying to adapt to its environment via transposition (the process of a transposable element, such as a non-structural gene, being duplicated and/or removed from one site and inserted into another) (Cameron *et al.,* 1979). Duplication can provide an increased gene dosage and movement of a transposable element (TE) can create novel joints which influence gene expression. These directed rearrangements of TE's have been used to describe faster evolution under stressful conditions compared to that possible with single base changes in structural genes (Cameron *et al.,* 1979). During extended serial repitching the possibility of accumulating viable variants becomes increased causing certain characteristics to passed on to the next generation of brewing yeast cells and then can outcompete the original culture (Powell and Diacetis, 2007) (Section 1.4.3). However, from the collective results of the interdelta PCR analysis of a strain producing a high number of morphological variants (W34/70) and a strain producing a low number of morphological variants (NCYC 1332), it can

be seen that all of the variants within a strain showed a high degree of homology, both to each other and to the original 'type' genetic fingerprint. This rules out the possibility of contamination and I can be confident that morphologies were likely macro-morphological variants of the same strain making that variation a true phenotypic trait.

Morphological variation on WLN agar represents spontaneous variation whilst under no stress. The reason for genetically identical cells grown in the same, benign environment displaying heterogeneity in their morphology was likely to be an underlying bet-hedging strategy (Levy *et al.,* 2012). The frequency of morphological variants appears to be strain dependant based on this subset of brewing strains tested. The lager stains produce more morphological variants when compared to the ale strains. This may be due to the hybrid, aneuploid nature of lager strains making them more susceptible to phenotypic variation. Aneuploid-induced genomic instability could result from imbalances in particular genes and/or from proteotoxic stress (damage caused by misfolded proteins) (Sheltzer *et al.,* 2011). Another hypothesis for this difference between lager and ale strains may lie in the history of the strains (Section 1.3.3). Ale strains have had thousands of years to stabilise their genome whilst lager strains presenting more genomic variation could simply be down to them being in an early part of their existence (Section 1.3.3, Byrnes *et al.,* 2006; Dunn and Sherlock, 2008; Scannell *et al.,* 2006). While lager strains 'work themselves out' it makes sense that they would be more well equipped to exploit new environmental niches. A sign of a domesticated genome is the upregulation of

traits desirable for humans but are a burden for the organism in a wild environment. A key example this is the ability brewing yeast has to ferment maltotriose, the most abundant carbon source in wort, but not commonly found in high concentrations in wild environments (Gallone *et al.,* 2018). An ability to efficiently metabolise maltotriose therefore imposes a selective advantage in a brewing environment (Gallone *et al.,* 2018). Yeast displaying changes in ploidy, segmental duplication and copy number variations have been found to be more adept at adapting to specific niches (Selmecki *et al.,* 2015; Voordeckers *et al.,* 2015).

This may be why increased colony morphology in lager strains was observed as they were the product of cell – surface and cell – cell interactions during colony development. Previous studies have found specialisation of yeast growing on solid media (Mináriková *et al.,* 2001; Palková *et al.,* 1997; Scherz *et al.,* 2001; Váchová *et al.,* 2009; Varon and Choder, 2000; Voordeckers *et al.,* 2012). This is of importance to giant colonies as age stratification will become apparent as the lower, centralised cells within the colony will cease dividing, enter the stationary phase and then lose viability (Váchová *et al.,* 2012, 2009). Colonies formed by yeast pass though developmental phases characterised by pH changes and ammonia production (which functions as a universal interspecies yeast signalling molecule) (Palková *et al.,* 1997; Váchová *et al.,* 2012, 2009). This was likely to be the reason for the radial patterns of colouration (creamy white to dark green) in the colonies grown on WLN agar due to the uptake of the bromocresol dye acting as a pH indicator. The programmed apoptotic-like

cell death of the central portion of cells is important for the continuation of the colony as they provide nutrients to the young, budding cells on the upper layer located away from the nutrient agar (Váchová *et al.,* 2009; Váchová and Palková, 2005). Nutrient poor conditions are known to activate the gene *FLO11* (Voordeckers *et al.,* 2012) which plays an important role in the generation of phenotypic variation at the cell surface by epigenetic silencing or expression and occurs primarily in the late-exponential and stationary phase cultures (Halme *et al.,* 2004; Teunissen and Steensma, 1995). The ability for *S. cerevisiae* to form colony mat structures, radial symmetry and spoke like structures have all been linked to the Flo11p protein produced by *FLO11* (Galitski *et al.,* 1999; Halme *et al.,* 2004; Reynolds and Fink, 2001). The mating type of isogenic strains was also found to influence colony morphology due to the amount of *FLO11* they are able to express; reduced amount in diploids compared to haploids meaning *FLO11* transcription decreases with increased ploidy (Galitski *et al.,* 1999). This also supports the idea that ale strains would produce less proteins which induce colony variation. Flo11p on the cell surface makes cells more hydrophobic meaning patterns in the colony morphology can arise from both frictional forces and cell – cell interactions (Reynolds and Fink, 2001). *FLO11* expression is dependent on many central signalling pathways and interestingly by acidic pH ranges (3.9 - 5.5) (Bayly *et al.,* 2005) which may also help to justify radial patterns in the topography as the growth stages shift from acidic, to alkali and back to acidic (Váchová *et al.,* 2012, 2009).

The kinetic growth data of the highest variant producing strain (W34/70) and the lowest variant producing strain (NCYC 1332) against brewery related

stresses (ethanol, osmotic and oxidative stress) did not reveal a link between a strains propensity to produce morphological variants and stress tolerance. Previous work by Voordeckers *et al.* (2012) also found no link between colony morphology (wild wrinkled type vs smooth knockout type) and stress tolerance (heat and desiccation). Instead a screen comparing gene expression between the two strains revealed the differences to be associated with central processes such as ion homeostasis, cell – cell adhesion, electron transport chain and oxidation-reduction. Meaning that variations in colony morphology are more likely to be influenced by nutrient availability and osmotic pressure. The relationship between 50% of maximal growth and the concentration of sorbitol (Figure 4.10C) shows that W34/70 was less tolerant to osmotic stress than NCYC 1332, which relates to the hypothesis that increased variation in cell morphology is linked to osmotic pressure. However, further analysis of strains exhibiting vastly different frequencies of colony morphology variation need to be screened against osmotic stress and nutrient limitation to confirm this hypothesis in brewing yeast cultures.

The reasons for changes in cell hydrophobicity in colony formation may give cells a competitive advantage during nutrient limitation (Avery, 2006) meaning that a sub-set of phenotypically diverse cells are able to maintain viability until they next come into contact with a nutrient rich environment.

CHAPTER 5:

HETEROGENEITY IN THE STRESS RESPONSE OF BREWING YEAST CULTURES

5.1 Introduction

Traditionally, inconsistency in brewing yeast slurries, giving rise to poor fermentation profiles or a final product that fails to meet specification, would be attributed to the quality of the yeast population (viability or vitality), or occasionally to genetic drift. However, the results described in Chapter 4 suggest that phenotypic heterogeneity may also play a significant role in performance. Indeed, (Levy *et al.,* 2012) demonstrated that individual cells from monoclonal populations of yeast grown in nutrient rich and non-limiting environments displayed a range of growth rates spread across a wide and continuous distribution. This suggests that, within isogenic populations, cells exist which are effectively on 'standby' mode, since even though the environment is optimal for growth, they present sub-optimum metabolism (Fischer and Sauer, 2005). However, it can be argued that slow somatic growth under stress conditions may actually serve a population better in terms of a survival strategy. This is because it would take a substantial investment in metabolic resources to maintain a complex bet-hedging mechanism which distributes risk among individuals in the anticipation of a changing environment (Fischer and Sauer, 2005; Levy *et al.,* 2012). Conversely, phenotypic heterogeneity has also been shown to be beneficial in fluctuating environments since it increases the chances of individual cells surviving, allowing for population survival (Beaumont *et al.,* 2009). Consequently, although both strategies may appear viable, it can be argued that strains with phenotypically diverse subpopulations may be more suitable to cope with environments comprising successive stress factors, such as those encountered during

brewery yeast handling and fermentation (Chapter 1.5). Thus, brewing strains with the ability to upregulate or downregulate certain genes in response to stress would enhance the populations long-term fitness (Carlquist *et al.,* 2012; Ryall *et al.,* 2012; Symmons and Raj, 2016). Inevitably, the degree of phenotypic heterogeneity is likely to vary between different strains of the same species, and the extent of intra-species variation is likely to be derived from adaptive experiences encountered over many years. In the natural environment this provides an opportunity for new strains to become established via natural selection (Hewitt *et al.,* 2016; Holland *et al.,* 2014). However, the majority of brewing strains are now only encountered within industrial environments and have essentially arisen due to 'artificial selection' at the hands of brewers. This suggests that variation is innate since it is logical to assume that current strains have been selected based on their individual fermentation and handling characteristics. Despite this, the extent of brewing yeasts' phenotypic heterogeneity has not yet been fully explored.

In order to determine the relative degree of phenotypic heterogeneity between different brewing yeast strains, the distribution of viable persisters (i.e cells which remain viable under extreme environmental stress) was determined by investigating population distribution across a range of different stress challenges. A quantitative measurement of heterogeneity in response to ethanol, oxidative and osmotic stress was achieved by producing a gradient dose-response curve for each of the 5 brewing yeast strains under investigation.

5.2 Results

5.2.1 Yeast stress tolerance via dose-response assay

To directly study phenotypic heterogeneity under stresses associated with industrial fermentations (ethanol, oxidative and osmotic stress), a technique to purify divergent phenotypic sub-populations was implemented according to Chapter 2.4.3. S-shaped or sigmoidal dose-response survival curves were produced using progressively increasing concentrations of stressor, until cells were no longer able to survive on agar plates. In each instance, the upper limits for a stressor was deduced from the spot plate analysis described in Chapter 3.2.2. These dose-response survival curves allow the presence of sub-populations (which may include slow-growing persister cells) to be noted, where they would often be masked using conventional microbiological techniques. Heterogeneity was determined by a comparative analysis of the gradients of each slope (Chapter 2.4.3) and strains were characterised based on the shape of each curve; those displaying relatively shallow hillslope gradients were designated as having a high degree of phenotypic heterogeneity, whilst those with a steep hillslope gradient were categorised as having a low phenotypic heterogeneity. A visual representation of this concept can be seen in Figure 5.1.

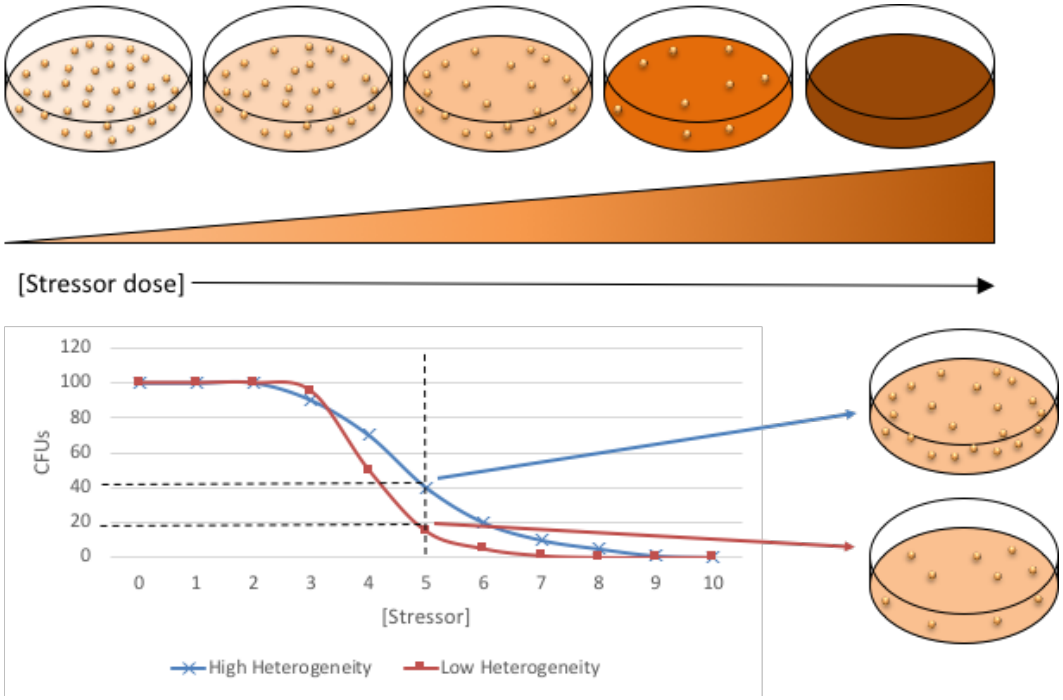


Figure 5.1 Visual representation of dose-response analysis used to quantify phenotypic heterogeneity. A series of YPD agar plates containing an increasing concentration of stressor acts as the basis for a decline in colony forming units (CFUs). The resulting dose-response curves indicate the level of heterogeneity; relatively shallow gradients represent high heterogeneity and relatively steep gradients represent low heterogeneity (adapted from Hewitt *et al.,* 2016)

In order to determine heterogeneity in the stress response of brewing yeast cultures, each strain was exposed to ethanol, osmotic and oxidative stress in separate reactions. For statistical accuracy, tests were repeated on three independent days, with plating in triplicate for each strain and condition on each day. This meant that each strain was propagated from stock cultures on three separate occasions (Holland *et al.,* 2014). The percentage viability within each experiment was determined with reference to mean CFUs on control plates produced with stressor absent. The four-parameter logistic equation (Chapter 2.4.3) used to model the data created a sigmoidal dose-response curve with a point of inflection which separated the graph into two equal regions of opposite concavity. It was also at this point that the hill slope was measured. As the steepness of the slope changes at this point, so does the extent of the sigmoidal shape. Steeper slopes were associated with the upper and lower asymptotes (the description of the straight line approached by, or departing a given curve as one of the variables in the equation of the curve approaches infinity but does not touch it) being more angular and closer to the point of inflection on the logarithmic scale whilst less steep (or more shallow) slopes were associated with the upper and lower asymptotes being smoother and elongated and further from the point of inflection on the logarithmic scale. The change in the extent of the sigmoidal shape around the point of inflection is represented in Figure 5.2.

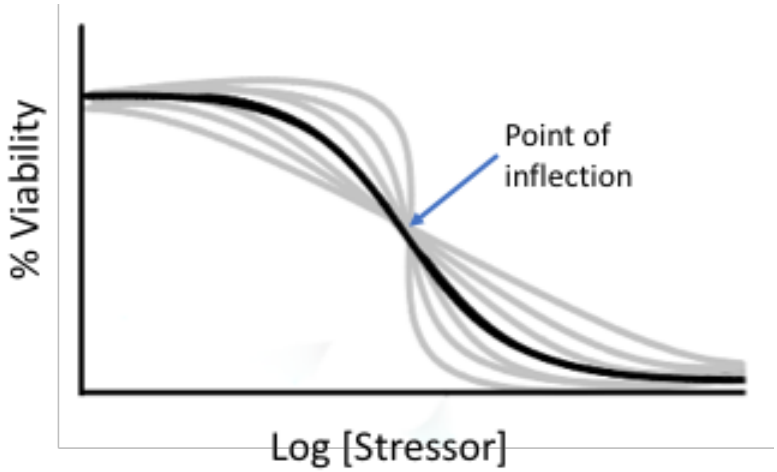


Figure 5.2 Visual representation of the possible sigmoidal shapes produced by the four-parameter logistic equation used to model the data. A change in steepness around the point of inflection is associated with changes in the curvature of the upper and lower asymptotes and their relation to their position on the logarithmic scale (x-axis) Adapted from Veroli *et al.* (2015).

A variation in the IC50 value, the point at which viability was reduced to 50% under an individual stress, also causes the sigmoidal curve to shift within the logarithmic space. A lower IC50 value representing a measurement of lower stress tolerance due to 50% of the viability being lost at a lower stressor concentration caused the curve to shift in a negative direction (towards the y-axis). Conversely, a higher IC50 value representing a measurement of higher stress tolerance due to 50% of the viability being lost at a higher stressor

concentration caused the curve to shift in a positive direction (away from the y-axis).

All strains and stressors were compared to a control of 0% stress application however, 0 cannot be subjected to a log transformation, therefore the stressor concentrations all have a value of 0.01 added, prior to being converted to a logarithmic scale (Chapter 2.4.3). The log (0.01) equals -2.00 and has been omitted from the graph area due to the sigmoidal curve being the artefact of interest. A decrease in viability between the control and the first stressor concentration resulted in the graph beginning at <100% viability when x=0.

5.2.1.1 Population heterogeneity in response to ethanol stress

In order to determine the yeast population response to ethanol stress, cells were subjected to 0 – 30% ethanol on agar plates incubated aerobically for 14 days at 25°C, as described in Chapter 2.4.3. Incubation time was purposely long since it is known that stress resistant subpopulations can have reduced growth rates compared to less resistant subpopulations (Levy *et al.,* 2012; Stratford *et al.,* 2014). The response of brewing strains to ethanol exposure can be seen in Figure 5.3. Based on viable CFU counts, a sigmoidal curve was fitted to model the effect of ethanol concentration (x) against viability (y) as described in Chapter 2.4.3.

By overlapping the descending sigmoidal curves produced by the dose-response analysis for each yeast it was possible to observer a similar shape, suggesting the dynamics of ethanol toxicity were similar between strains. However, the extent of the asymptotes of the curves showed differences

between the strains, with W34/70 exhibiting the smoothest curvature, and CBS 1174 exhibiting the most angular curvature.

The end point of each maximum asymptote can be seen to indicate the point at which cells begin to lose viability due to ethanol toxicity (Figure 5.3). Therefore, those maximum asymptotes which are positioned further along the x-axis (higher ethanol concentrations) indicate an overall stronger tolerance to ethanol stress. Subsequently, the position of the slope along the x-axis was related to the IC50 values represented in Figure 5.4. IC50 is a general measurement of tolerance due to the amount of a stressor it requires to reduce population viability by 50% (i.e half the maximal response). Under ethanol stress the IC50 values for each strain indicated that the ale strain NCYC 1332 (IC50 15.5% ethanol v/v) was most tolerant and the lager strain W34/70 (IC50 12% ethanol v/v) was the least tolerant. This partially corroborates the findings from the spot plate data in Chapter 3 (Chapter 3.2.3.3) with NCYC 1332 being one of the most tolerant strains (along with the other ale strain M2), however W34/70 was not the least tolerant strain shown under these conditions (this was the lager strain CBS 1174). Investigating the steepness of the slope at the point of inflection gives a greater insight into the stress resistance of a population since it provides information on the presence of stress tolerant subpopulations which could generate biomass when sub-cultured. From a practical perspective, the steepness of the slope is also important to consider since a rapid decrease in strain viability could have implications in certain circumstances. For example, in this instance, a homogeneous population used for high ethanol fermentations could lead to a sudden and dramatic reduction

in cell viability, potentially leading to stuck fermentations or flavour inconsistencies. Based on the gradient of the slope, the lager strain CBS 1174 showed the least tolerance to ethanol and W34/70 showed the capacity to be the most resistant (Figure 5.3). However the IC50 values in Figure 5.4 indicate that for both of these strains, the majority of cells were highly sensitive to ethanol. This shows that IC50 values are not necessarily indicative of strains absolute tolerance to a stressor, and that both metrics are useful to determine phenotypic heterogeneity as well as limits of tolerance within a population.

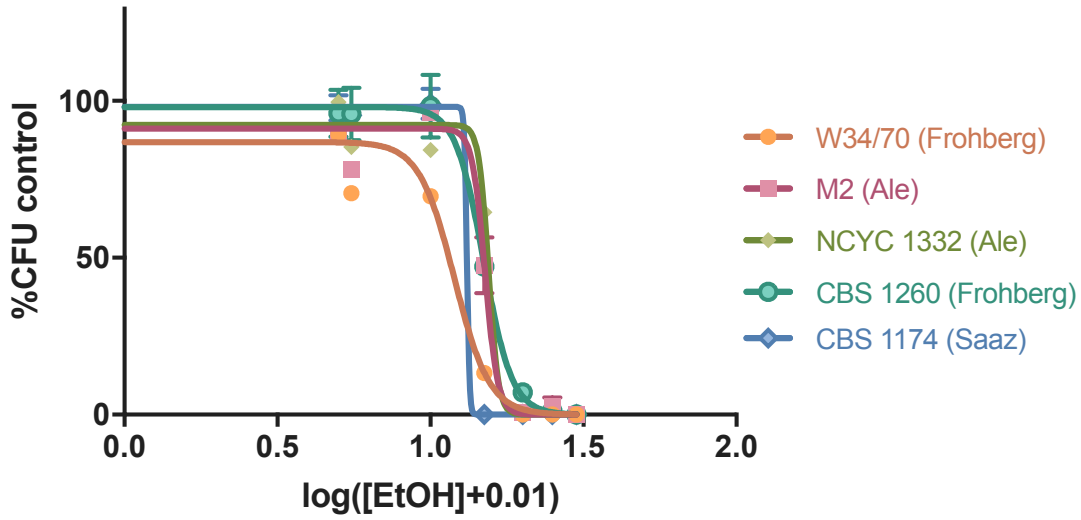


Figure 5.3 Heterogeneity analysis of brewing strains W34/70, M2, NCYC 1332, CBS 1260 and CBS 1174 in response to ethanol stress. Ethanol concentrations include 0, 5, 10, 15, 20, 25 and 30% (v/v). Data indicates triplicate independent experiments at each point. Error bars representative of standard deviation.

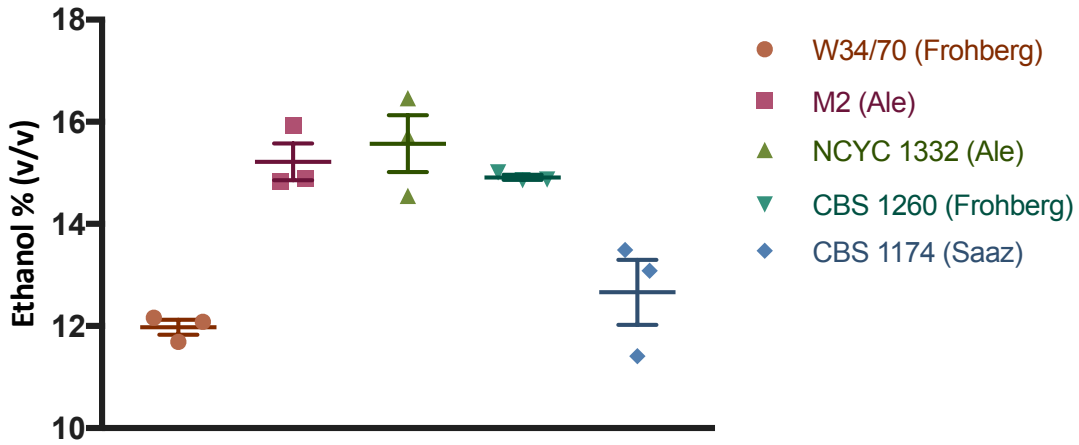


Figure 5.4 IC50 values extrapolated from heterogeneity analysis of brewing strains W34/70, M2, NCYC 1332, CBS 1260 and CBS 1174 in response to ethanol stress. Mean of the three independent data points represented with a line. Error bars representative of standard deviation.

5.2.1.2 Population heterogeneity in response to oxidative stress

In order to determine the yeast population response to oxidative stress, cells were subjected to 0 – 7mM H_2O_2 on agar plates incubated aerobically for 14 days at 25°C, as described in Chapter 2.4.3. Figure 5.3 depicts the response of brewing strains to H_2O_2 induced oxidative stress. Based on viable CFU counts, a sigmoidal curve was fitted to model the effect of H_2O_2 concentration (x) against viability (y) as described in Chapter 2.4.3.

By overlapping the descending sigmoidal curves produced by the dose-response analysis for all the strains it was possible to see a similar shape,

suggesting the dynamics of oxidative toxicity were similar between yeast (Figure 5.5). However, in response to oxidative stress the extent of the asymptotes of the curves showed differences between strains with W34/70 exhibiting the smoothest curvature of the upper and lower asymptotes and most gradual slope. CBS 1174 exhibited the sharpest decrease in viability as shown by the more angular asymptotes and steeper slope.

The end point of each maximum asymptote can be seen to indicate the point at which cells begin to lose viability due to H_2O_2 induced oxidative toxicity (Figure 5.5). Therefore, those maximum asymptotes which are positioned further along the x-axis (higher stressor concentrations) indicate an overall stronger tolerance to H_2O_2. Subsequently, the position of the slope along the x-axis was related to the IC50 values represented in Figure 5.6. The IC50 values show that W34/70 (IC50 4.3mM H_2O_2) was most tolerant to oxidative stress and M2 (IC50 3.2mM H_2O_2) was the least tolerant. This corroborates previous findings based on spot plate analysis (Chapter 3.2.3.1) which also demonstrated that W34/70 was the most tolerant strain and that M2 was particularly susceptible to the oxidative stress produced by H_2O_2. For each strain analysed, the rate at which viability was lost due to increasing concentrations of H_2O_2 was observed to change the steepness of slope. However, in contrast to the response to ethanol, the IC50 values appeared to correspond directly to the slope steepness (i.e. the curve with an asymptote furthest along the x-axis also has the highest IC50 value etc..), indicating that resistant sub-populations were not present to the same extent in this instance.

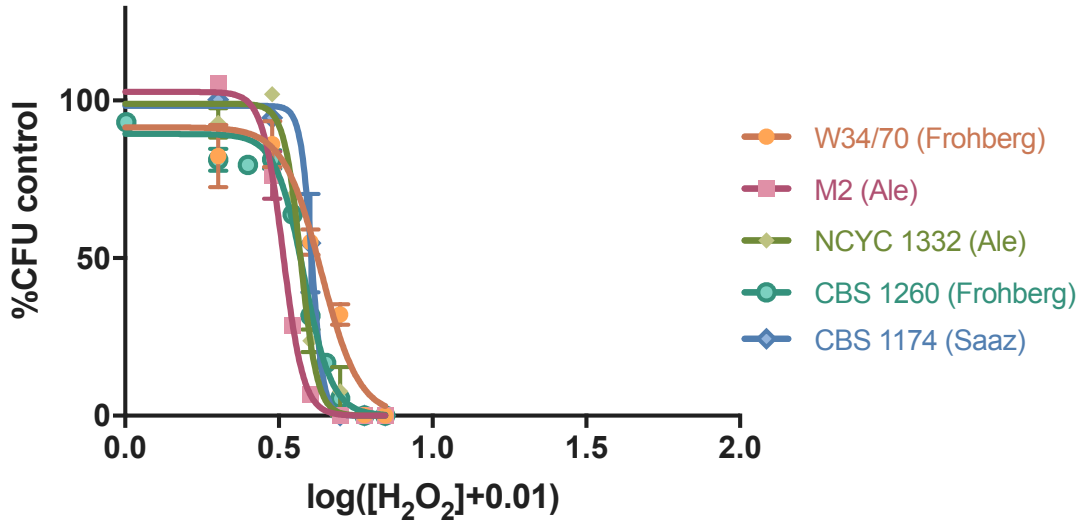


Figure 5.5 Heterogeneity analysis of brewing strains W34/70, M2, NCYC 1332, CBS 1260 and CBS 1174 in response to H_2O_2 induced oxidative stress. H_2O_2 concentrations include 0, 1, 2, 3, 4, 5, 6 and 7mM. Data indicates triplicate independent experiments at each point. Error bars representative of standard deviation.

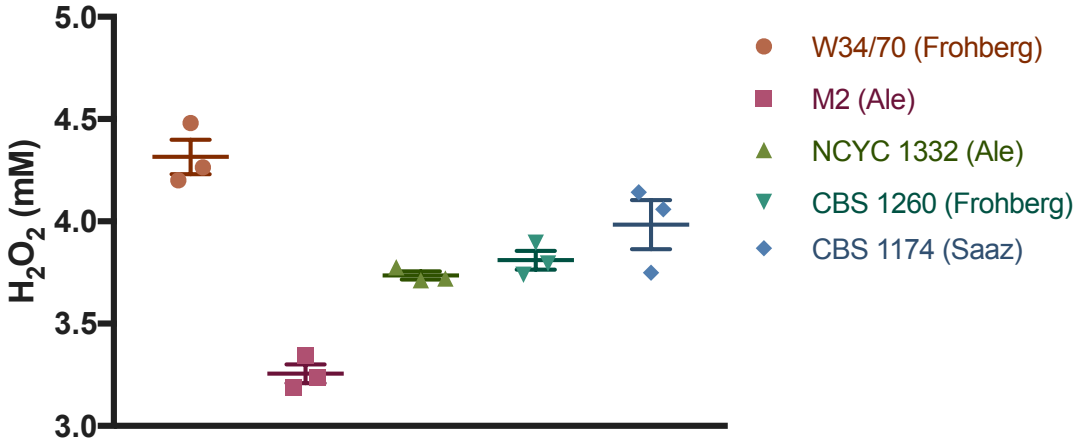


Figure 5.6 IC50 values extrapolated from heterogeneity analysis of brewing strains W34/70, M2, NCYC 1332, CBS 1260 and CBS 1174 in response to H_2O_2 induced oxidative stress. Mean of the three independent data points represented with a line. Error bars representative of standard deviation.

5.2.1.3 Population heterogeneity in response to osmotic stress

In order to determine the yeast population response to osmotic stress, cells were subjected to 0 – 60% sorbitol on agar plates incubated aerobically for 14 days at 25°C, as described in Chapter 2.4.3. Figure 5.7 depicts the response of each of the brewing strains employed in this study to sorbitol induced osmotic stress. Based on viable CFU counts, a sigmoidal curve was fitted to model the effect of sorbitol concentration (x) against viability (y) as described in Chapter 2.4.3.

By overlapping the descending sigmoidal curves produced by the dose-response analysis for each yeast it was possible to observe a similar trend for all of the strains, indicating that the dynamics of osmotic toxicity were similar.

However, the extent of the asymptotes of the curves revealed differences between strains. Strain CBS 1260 exhibited the smoothest curvature of the upper and lower asymptotes, and the most gradual slope. From the data obtained ((Figure 5.7), the sharpest decrease in viability, as shown by the more angular asymptotes and steeper slope, was harder to discern than previously, due to the overlapping of the strain responses. Irrespective, the end point of each maximum asymptote could still be used to indicate the stage at which cells began to lose viability due to sorbitol induced osmotic toxicity. Subsequently, the position of the slope along the x-axis was related to the IC50 values in Figure 5.8. These IC50 values indicated that M2 (IC50 33.3% sorbitol w/v) was most tolerant and CBS 1260 (IC50 24.8% sorbitol w/v) was the least tolerant to osmotic stress. However, despite this observation, the IC50 values for the other strains analysed were similar to the IC50 value of the maximal strain M2 (IC50 W34/70 32.8%, NCYC 1332 32.5%, CBS 1174 33% sorbitol w/v). This partially corroborates the previous findings from analysis of stress tolerance via spot plate analysis (Chapter 3.2.3.2). This data indicated that all of the stains exhibited a similar response to sorbitol induced osmotic stress, with the ale strains (M2 and NCYC 1332) showing slightly more tolerance overall and W34/70 producing a few larger colonies at 50% sorbitol (w/v) (a point at which all other strains showed diminished growth).

Further analysis of the stress dose-response curves indicated that the rate at which viability was lost due to increasing concentrations of sorbitol changed the steepness of slope giving an alternative measure of strain tolerance, taking strain heterogeneity into account. In this case the IC50 values appear to

correspond with slope steepness, indicating that CBS 1260 exhibited the highest degree of osmotic tolerance. However neither the IC50 value or the graph of the modelled slopes could distinguish between the rest of the strains without further mathematical extrapolation.

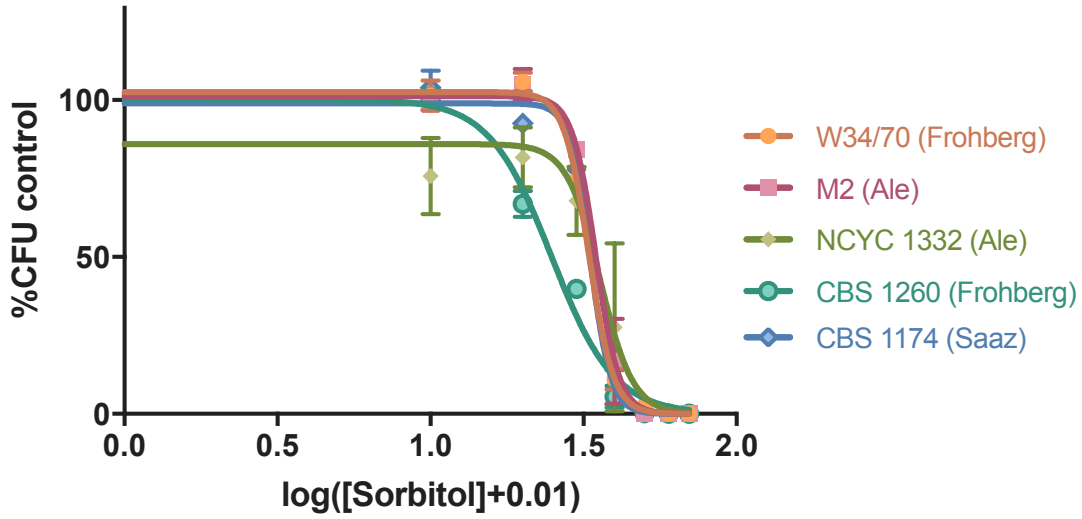


Figure 5.7 Heterogeneity analysis of brewing strains W34/70, M2, NCYC 1332, CBS 1260 and CBS 1174 in response to sorbitol induced osmotic stress. Sorbitol concentrations include 0, 10, 20, 30, 40, 50 and 60% (w/v). Data indicates triplicate independent experiments at each point. Error bars representative of standard deviation.

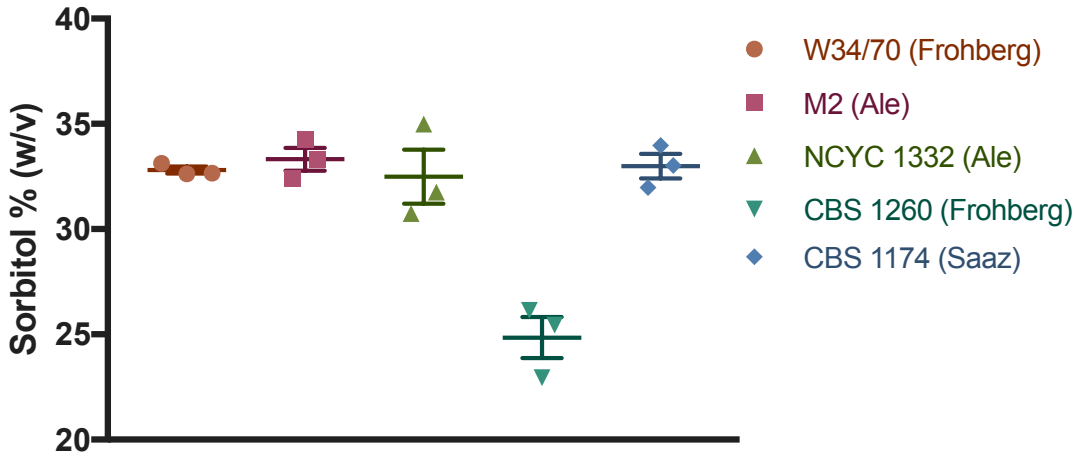


Figure 5.8 IC50 values extrapolated from heterogeneity analysis of brewing strains W34/70, M2, NCYC 1332, CBS 1260 and CBS 1174 in response to sorbitol induced osmotic stress. Mean of the three independent data points represented with a line. Error bars representative of standard deviation.

5.2.1.4 Comparative analysis of yeast population heterogeneity

Analysis of inflection points, as described above, provides some indication of the tolerance of a strain to environmental challenges. However, the slope of the curve is of arguably greater significance when considering phenotypic heterogeneity since it reflects the extent of variation within the population (Figure 5.1). It can be seen from Figures 5.3, 5.5 and 5.7 that fundamentally all brewing yeast populations declined in viability with an increase in stress concentration, as expected. However, the rate at which cell death occurred was variable between strains. This is significant since it reflects the heterogeneous nature of the population and, from a brewing perspective, may be important for several reasons. Populations which are highly heterogenous will by nature contain a greater number of 'weaker' cells within a population, which may impact on viability loss during fermentation (Chapter 1.4.3). Conversely, populations which are highly homogenous (low heterogeneity) may be less adaptable; this may be significant when considering the array of different stress factors which yeast are subjected to over the course of yeast handling and serial repitching (Chapter 1.4.3).

Analysis of the hillslope gradients from the sigmoidal curves shown in Figures 5.3, 5.5 and 5.7 was conducted using Graphpad Prism software (Chapter 2.4.3 and 2.6). This analysis allowed values to be determined for each strain based on the gradient of the curves obtained; data was also compared statistically using a two-way ANOVA (Chapter 2.4.3 and 2.6). Consequently, the degree of heterogeneity for each strain in response to each stress factor was calculated (Figure 5.9).

When comparing the response of yeast to H_2O_2 induced oxidative stress it can be seen that there was no significant difference in heterogeneity between strains. This was surprising given that individual limits of tolerance and inflection points differed, however it may reflect the nature of the stress which caused all of the strains to lose viability rapidly once the maximum asymptotes had been reached. This is significant for brewing since all yeast cultures are exposed to reactive oxygen species (ROS), which are either generated during aerobic metabolism (yeast propagation), or via the activity of cytochrome P450 enzymes during fermentation (Bogaert *et al.,* 2011). Furthermore, it has been shown that yeast are increasingly exposed to free radicals as fermentations progress, and that high gravity fermentations can lead to elevated exposure (Mott, 2017). Consequently, it is likely that defence mechanisms are broadly similar between industrial yeast strains since all strains are able to respond to ROS either by detoxification, by reducing the rate at which they are produced, or by repairing damage caused. Furthermore, although the response to ROS can be variable based on the type of free radical, yeast can also react through the general stress response pathway, associated with a diverse range of stress conditions (Morano *et al.,* 2012). The fact that oxidative stress tolerance is so closely linked to other stress factors suggests that this may not be a random (stochastic) process, but that upregulation of specific genes is an innate characteristic (Sumner *et al.,* 2003). Although there was no significant difference between strains, W34/70 did appear to be marginally more heterogenous than CBS 1174. However, what the phenotypic heterogeneity analysis might ultimately indicate is that brewing yeast are able to respond

effectively to ROS, however, once the defence systems have been breached damage is inflicted suddenly and severely such that cells can no longer survive. Under sorbitol induced osmotic stress, the ale strain NCYC 1332 showed significantly less phenotypic heterogeneity ($P<0.0001$) compared to the other four strains investigated. However, analysis of the hill slope gradients of strains W34/70, M2, CBS 1260 and CBS 1174 did not show any significant differences within this group. The data obtained for NCYC 1332 is difficult to explain, since *Saccharomyces* yeasts by their very nature should encounter a wide range of osmotically challenging environments. However, it is known that ale strains are a particularly diverse group of organisms, reflective of their origins and use in different beer styles. Consequently, this difference in character perhaps reflects the evolutionary history of this strain. Alternatively, it is possible that since this yeast strain showed relatively high overall tolerance to osmotic stress, the potential for this yeast had been reached with all cells showing similar functionality.

In contrast, the relatively higher heterogeneity of the remaining yeasts to osmotic stress may be representative of the innate adaptability of these organisms, both from the perspective of the brewing and the natural environment. A systems biology approach has previously been used to achieve quantitative phenotypic descriptions of biological systems. Essential genes encode for proteins which are essential for growth which include the metabolic enzymes which catalyse biosynthesis of biomass (Dikicioglu *et al.,* 2013). An example of this is when mutant laboratory strains of yeast are produced in order to make them auxotrophic, for instance the uracil synthesis pathway

gene can be deactivated (knocked out) and consequently uracil must be supplemented into the media in order for that mutant to grow (this aids strain selection in genetic engineering studies). A study of the effects of yeast auxotrophy on growth and aging was conducted by Mülleder *et al.* (2012) where knocked out genes were reintroduced on mini-chromosomes and comparisons with the original auxotrophic strain found that essential genes have control over highly interconnected metabolic networks and their regulation in that network are context dependant and can have unpredictable outcomes. This occurrence of the effect of one gene being dependant on a genetic background is known as epitasis. Musso *et al.* (2008) focussed on epistasis in 399 paralogous pair of metabolic enzymes among whole-genome duplicates in yeast. They found high functional overlapping and that no functional redundancy was lost (which is unfavourable to do in evolutionary terms). In addition to this, the ratio of epistatic pairing increased under stress such as osmotic pressure meaning that under this selective pressure the cell increases the overlapping in its metabolic network with more essential genes. The retention of paralogous pairs in the event of functional redundancy is an example of a bet-hedging strategy employed by the yeast in the anticipation of future use. This could explain the high tolerance of the strains in this study when under osmotic stress and why nearly all the strains react in a very similar way. Changes in tolerance will be down to the way each strain regulates this network and how many genes they have to overlap. Brewing yeast experience changes in water activity and solute concentration throughout the brewing process. Under osmotic stress the initial cellular response by yeast cells is to

shrink due to water loss. The reduction in cell volume increases the concentration of the substances present in the cytoplasm which can lead to cytoplasmic crowding (Babazadeh *et al.,* 2013), as well as membrane (Hohmann, 2002) and protein damage (Gasch *et al.,* 2000). Adaptation avoids viability loss, typically by recovering cell turgor via water ingress and returning the cell cytoplasm to an environment suitable for optimal biochemical reactions (Hohmann, 2002). Related to this, it is known that the "environmental stress response", ESR, involves the induction of around 300 genes (Gasch *et al.,* 2000), and a feature of this is to differentially produce isoenzymes (enzymes which perform identical functions but differ structurally). Causton *et al.* (2001) found that 74 of the ESR genes encoded for 37 pairs of highly homologous proteins that were differentially expressed under stress. 12 of these pairs were identified as participating in sugar or energy metabolism, and these isogenes all contained a 'strongly' verses 'poorly' expressed counterpart. Interestingly after the application of osmotic stress, poorly expressed counterparts are upregulated (Rep *et al.,* 2000). These differential expressions could begin to explain differences in heterogeneity between yeast strains.

Similar to osmotic stress, analysis of the response of cells to ethanol revealed significant differences between strains. Strains NCYC 1332 and CBS 1174 exhibited significantly less phenotypic heterogeneity ($P<0.0001$) compared to the other three strains. Analysis of the hill slope gradients of strains W34/70, M2 and CBS 1260 did not show any significant differences between one another within this group. Analysis of NCYC 1332 and CBS 1174 indicated that the latter was significantly less heterogenous in response to ethanol stress ($P\leq0.05$). xx

It is possible that the characteristics revealed here for the ale strain NCYC 1332 are related to its capacity to tolerate osmotic stress, however the data obtained for the lager strain CBS 1174 is harder to rationalise. It is possible that it is reflective of the high ethanol tolerance of this strain. Although the majority of cells were killed within a small range of ethanol, the fact that they were able to tolerate greater concentrations initially indicates a potential weighting away from bet-hedging. This indicates that this strain can perhaps be identified as being a specialist in the area of ethanol tolerance.

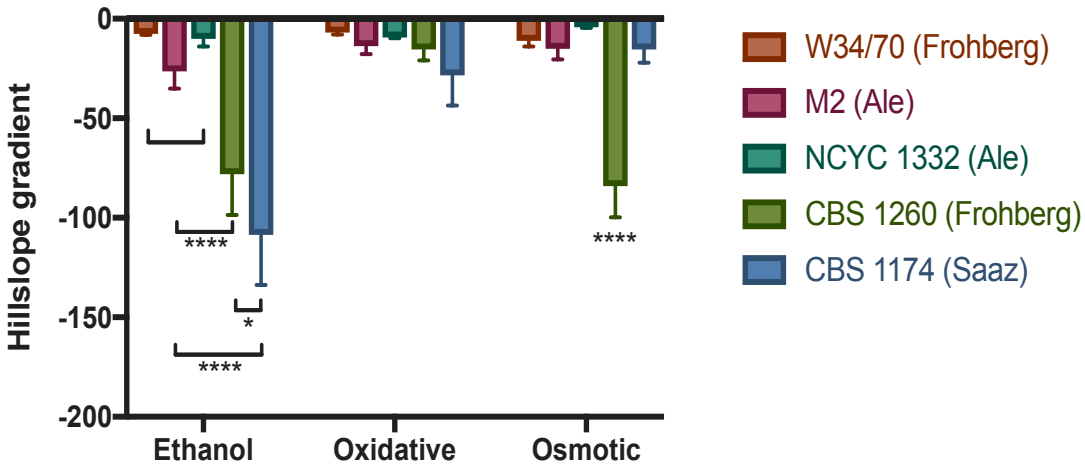


Figure 5.9 Hillslope gradients based on data obtained from dose-response analysis. Smaller values are the result of gradual slopes and therefore indicate a high degree of phenotypic heterogeneity; larger values indicate steep curves demonstrating low phenotypic heterogeneity. Error bars representative of standard deviation. (* = P≤0.05; significant, **** = P<0.0001; extremely significant due to a much higher calculated threshold of significance level).

5.2.2 Yeast strain analysis via dose-response assay after fermentation

The data presented above was obtained through analysis of freshly-grown cultures of individual yeast strains under laboratory conditions. While this approach provides useful information regarding the innate heterogeneity of yeast strains in response to isolated stress factors, it does not consider the impact of a period of intensive stress on population variation and may not be reflective of industrial yeast cultures taken from the process. Specifically, the

impact of fermentation conditions on phenotypic heterogeneity remains unknown. This is important since heterogeneity is by definition an estimation of 'plasticity' and this may be impacted further by environmental conditions encountered by individual cells prior to stress test analysis. Consequently, the yeast strain W34/70 was selected for further analysis, since it typically exhibited a highly heterogeneous phenotype in response to each stress analysed. In addition, this strain has great commercial significance and is used worldwide to produce a range of beers at low and high alcohol, from a variety of substrates that differ in gravity and nutritional composition. Consequently, strain W34/70 was used to conduct small scale fermentations in 13^{o}P wort at 25^{o}C for 3 days and fermentation progression was measured by weight loss over time as described in Chapter 2.6.5.

The fermentation profiles for W34/70 cells are depicted in Figure 5.10. It should be noted that viability decreased from 98.6% pre-fermentation to 90.2% post-fermentation, indicating that cells had been subject to stress resulting in some cell death in the wort fermentation. While this was unfortunate, this does somewhat reflect the situation which may be encountered industrially.

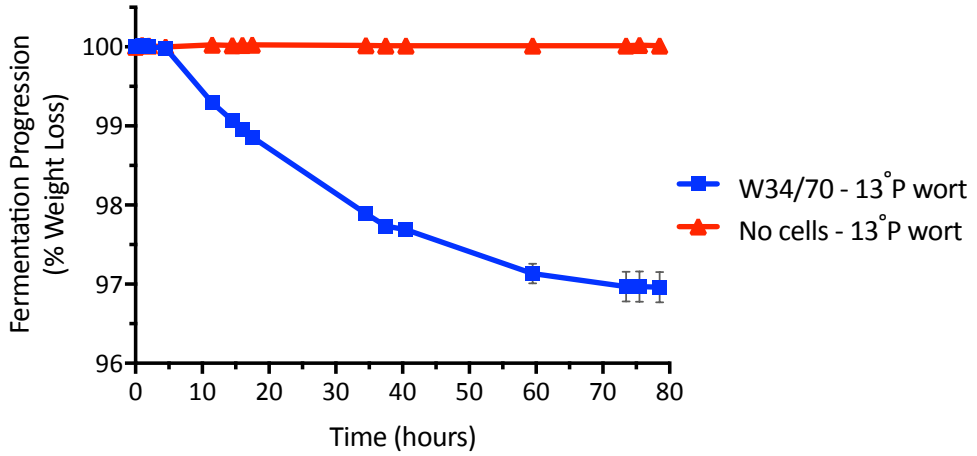


Figure 5.10 Average percentage weight loss during fermentations using strain W34/70 in 13°P wort plotted against time. Data points represent the mean of triplicates with error bars showing standard deviation.

Following fermentation, W34/70 cells were obtained from the beer and subjected to ethanol stress using heterogeneity plates as described previously (Chapter 2.4.3 and 5.2.1.1). The data generated was used to construct stress dose-response curves and compared to the 'control' data presented above; W34/70 cells taken straight from stock samples.

The response of strain W34/70 to ethanol before and after fermentation can be seen in Figures 5.11, 5.12 and 5.13. Based on the viable CFU counts a sigmoidal curve was fitted to model the effect of ethanol concentration (x) against viability (y) as described in Chapter 2.4.3. By overlapping the descending sigmoidal curves produced by the dose-response analysis for W34/70 before and after mini-fermentation in wort media, it was possible to observe that the maximum asymptotes altered slightly, as did the slope

gradient. The IC50 values for W34/70 towards ethanol altered significantly (P value 0.0036) from 12% (v/v) in the stock culture to 5.5% (v/v) in the post-fermentation sample. For post-fermentation samples, the maximum asymptote of the curve was higher on the y-axis, indicating that there was a positive correlation between viability and low doses of ethanol, however the curve shifted in a negative direction along the x-axis meaning the IC50 value for these cells decreased overall.

The hillslope gradient of each curve was calculated to indicate phenotypic heterogeneity as described previously (Chapter 2.5.4). In addition, data from pre- and post-fermentation yeast samples were compared using a paired t-test to observe the effects on phenotypic heterogeneity. It was evident that after a single fermentation there was a significant change in the phenotypic heterogeneity of W34/70. The gradient shifted from -7.6 to -4.6, deemed to be a significant difference (P value 0.0054). This data indicates an increase in phenotypic heterogeneity at the expense of decreased overall population viability at a relatively low level of ethanol. It is likely that to a certain extent this reflects the physiological condition of yeast that has been through fermentation. Since it can be assumed that heterogeneity already existed within the population, the extent of ‘damage’ may already have been highly variable before the dose-response analysis was conducted. This is supported by the lower tolerance to ethanol observed in post-fermentation yeast.

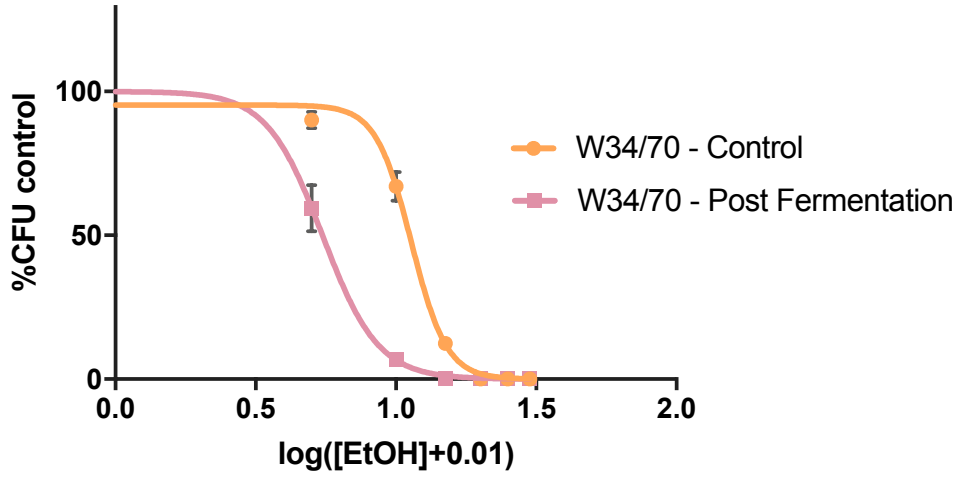


Figure 5.11 Heterogeneity analysis of brewing strain W34/70 in response to ethanol stress using cells obtained before and after fermentation. Data indicates triplicate independent experiments at each point. Error bars representative of standard deviation.

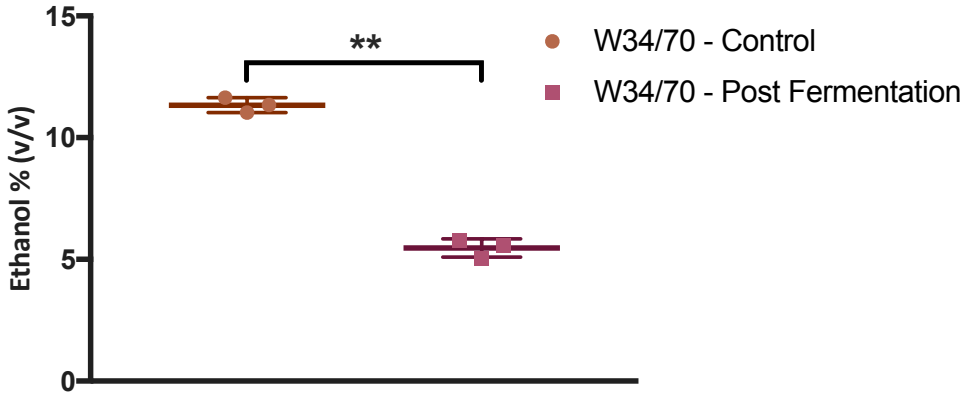


Figure 5.12 IC50 values based on data obtained from dose-response analysis. Fermentation can be seen to have caused the IC50 value of W34/70 towards ethanol to be reduced from 12% to 5.5% (v/v) Mean of the three independent data points represented with a line. Error bars representative of standard deviation. (= P value 0.0036).**

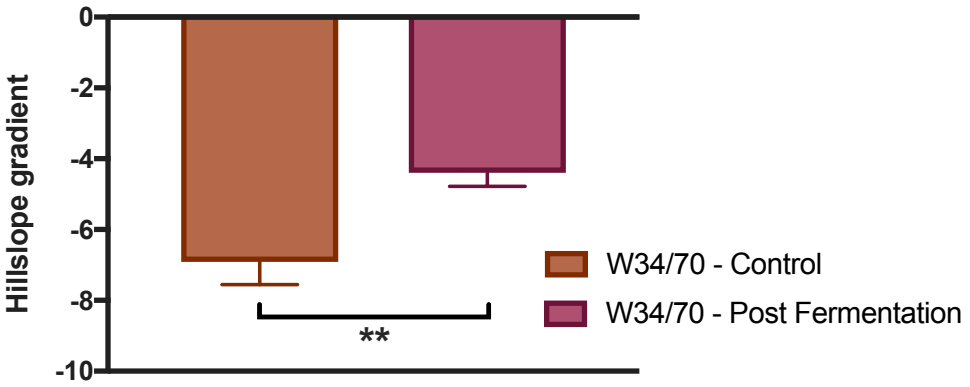


Figure 5.13 Hillslope gradients based on data obtained from dose-response analysis. Smaller values are the result of gradual slopes and therefore indicate a high degree of phenotypic heterogeneity; larger values indicate steep curves demonstrating low phenotypic heterogeneity. Error bars representative of standard deviation. (= P value 0.0054).**

5.3 Conclusion

In this chapter I aimed to investigate the extent of phenotypic heterogeneity in five brewing strains by exposure to increasing concentrations of stress (ethanol, oxidative and osmotic) in order to obtain dose-response curves. The range of stressors (concentrations of ethanol, H_2O_2 and sorbitol) applied were determined from previous data obtained from spot plate analysis for the same stress factors (Chapter 3) and the resulting data was interpreted by calculating IC50 values and hill-slope gradients. Although the IC50 values allowed valuable descriptions of the overall tolerance of a strain towards a particular stressor, these were not used to reflect population heterogeneity, since this function changes independently to the mean stressor resistance (IC50) (Holland *et al.,* 2014). However, based on the hillslope gradient data it was evident that there was a complex relationship between yeast strain, stress tolerance and heterogeneity. While each strain showed a different degree of phenotypic heterogeneity, there was no correlation between stress factors. For example, displaying a high degree of heterogeneity to one stress did not lead to a similar phenotype in response to other stresses. Furthermore, there were no similarities that can be related to species (*S. cerevisiae*/*S. pastorianus*). Under ethanol stress there did appear to be a link between the type of lager strain and stress resistance in that the Frohberg type yeast strains exhibited significantly ($P<000.1$) more heterogeneity compared to that of the Saaz type strain. However, this is too small a set strains being compared to conclusively adopt this conclusion.

It was interesting to observe that the variation in response of yeast strains to environmental challenges was dependent on the individual stress applied. For example, the lager CBS 1174 exhibited a relatively high degree of heterogeneity under oxidative and osmotic stress, but the opposite was true for ethanol stress. Similarly, the ale strain NCYC 1332 also exhibited a strain specific response, with high heterogeneity under oxidative stress and low heterogeneity under ethanol and osmotic stress. This was surprising considering the role of the General Stress Response (GSR) in stress resistance (Berry and Gasch, 2008; Bódi *et al.,* 2017; Gibson *et al.,* 2007; Holland *et al.,* 2014; James *et al.,* 2008; Levy *et al.,* 2012; Liu *et al.,* 2015), and the overlapping nature of many response mechanisms. For example, it is known that trehalose, is produced in response to a variety of different stress factors, and serves to protect a number of important cell organelles in a variety of ways (Lillie and Pringle, 1980; Mansure *et al.,* 1997; Singer and Lindquist, 1998; Wiemken, 1990). Nevertheless, individual strains must be able to elicit a tailored response to an environmental stress by upregulating specific genes and/or adapting metabolic activity in order to overcome a stress (Berry and Gasch, 2008; Bódi *et al.,* 2017; Gibson *et al.,* 2007; Holland *et al.,* 2014; James *et al.,* 2008; Levy *et al.,* 2012; Liu *et al.,* 2015), and it is known that brewing yeast cells respond to environmental stress by altering the expression of a number of genes (Gasch, 2003; Ruis and Schüller, 1995; Stewart, 2009; Thevelein, 1994; Toone and Jones, 1998). The variation in phenotypic heterogeneity observed indicates that the stress response may be more complex than at first thought. It is known that yeast can respond to stress factors in a number of different ways. Although

there are individual response mechanisms which act to counter or repair damage to specific stress factors, prolonged exposure to stress is likely to trigger the general stress response (GSR) in yeast. This is activated under a broad range of environmental stresses and serves to generate a range of compounds which protect against a range of stress factors (Chapter 1.5.2). Consequently, the GSR is a quick, non-specific response which can be useful to protect cells whilst they induce more specific responses to individual stresses (Gibson *et al.,* 2007; Ruis and Schüller, 1995). The genes involved in the GSR contain a stress response element (STRE) which requires activation in order for their upregulation (Chatterjee *et al.,* 2000; Costa and Moradas-Ferreira, 2001; Martínez-Pastor *et al.,* 1996). To perform well in an industrial fermentation, brewing yeast must be able to respond swiftly and suitably by adapting to the unique features of each stage of the brewing process. The hillslope gradients obtained indicate that the stress response is strain specific, although this is already widely accepted (Stanley *et al.,* 2010; White *et al.,* 2008). However the range of variation within each population is surprising and sheds new light on potential causes for viability loss during fermentation and yeast handling, as well as the versatility of certain strains for brewing.

Analysis of the phenotypic heterogeneity of the five brewing yeasts suggests that each strain may have a customised 'response program' for each environment, perhaps utilising specific gene products needed to help combat the effect of individual stresses (Gasch *et al.,* 2000). How the GSR contributes to the cellular resistance exhibited by yeast under various brewing related stresses is an important question in understanding its role in the brewing yeast

life cycle and its relevance in bet-hedging strategies (Berry and Gasch, 2008; Bódi *et al.,* 2017; Gasch *et al.,* 2000; Gibson *et al.,* 2007; Holland *et al.,* 2014; James *et al.,* 2008; Levy *et al.,* 2012; Liu *et al.,* 2015). In the future it would be useful to investigate this further, perhaps by targeting specific stress proteins using green fluorescent protein (GFP), or by using single cell RNAseq.

It was interesting to note that although heterogeneity could be accurately determined under laboratory conditions, it was not a consistent phenomenon when the pre-analysis environment changed. Analysis of yeast pre- and post-fermentation revealed that strain W34/70 was subject to an increase in phenotypic heterogeneity in response to ethanol. While this may simply be an indication that cells were in a poorer condition prior to stress dose-response analysis (i.e. more cells within the population exhibited 'weaker' characteristics), the change in phenotypic heterogeneity may also support the theory of a bet-hedging strategy. The broad array of phenotypes observed in this instance could potentially be a result of a trade-off with alternative survival strategies, although further analysis would be required to substantiate this hypothesis. From the perspective of brewing fermentations potential causes for this are also difficult to put forward. Under optimum conditions it may be expected that uneven segregation during budding may give rise to variation as part of a bet-hedging strategy (Beaumont *et al.,* 2009; Levy *et al.,* 2012). However, it is known that under fermentation conditions the extent of budding is restricted to 2-3 divisions. Although this may give rise to some heterogeneous cells, it seems likely that a greater number of divisions would be required to see a significant variation in phenotype.

In this Chapter I demonstrate that brewing yeast strains differ with respect to their phenotypic heterogeneity. This may be significant for current production streams, especially those which employ high intensity techniques such as high gravity brewing. Although the absolute tolerance of a strain to a specific stress factor will inevitably play a role in determining its suitability, it is possible that the variation within a population may also impact on performance. A highly homogenous population with good tolerance to multiple stress factors is theoretically likely to offer an advantage. However, in reality, there is likely to be a trade-off, complicated by the fact that yeast strains exhibit different heterogeneity curves in response to different stress factors. Consequently, a strong argument could be made for selecting a strain with a broader response system, which may be a reason why strain W34/70 is so widely used within the industry. Such strains are likely to be more adaptable to environmental fluctuations (Hewitt *et al.,* 2016) and perhaps more suitable for industrial fermentations. Despite this, and perhaps to confuse the issue further, it is likely that some characteristics of brewing yeast should be less variable than others. For example, aspects of yeast performance that have repercussions for final product characteristics should offer as little variation as possible, in order to ensure consistency. Based on the data presented here, it is likely that variation exists between cells in terms of their preference for different metabolic pathways. This could potentially lead to the production of undesirable metabolites (Papagianni, 2004; Xiao *et al.,* 2016) or inappropriate ratios of flavour compounds. Irrespective, the data provides new insight into the response of individual cells to stress and provides some rationale for the

variation which can be observed in brewing fermentations. A holistic examination of stress responses such as those provided here will give a much clearer picture of the performance potential of a strain under certain stress environments.

CHAPTER 6:

THE IMPACT OF SUB-LETHAL STRESS-CONDITIONING ON PHENOTYPIC HETEROGENEITY

6.1 Introduction

Previous analysis of brewing yeasts indicated that the degree of phenotypic heterogeneity in response to environmental challenges was strain specific (Chapter 5). Some yeast strains displayed a relatively high degree of heterogeneity in response to stress, while others were more homogeneous in nature. This is reflective of most organisms; there is a continuum from highly specialized species through to those that are broadly generalist (New *et al.,* 2014). The different ends of this spectrum can be considered to reflect a populations investment in 'bet-hedging' (Chapter 1.7). Bet hedging refers to the extent to which a population is willing to trade-off immediate 'fitness' with the capacity to adapt to stressful conditions. Hence specialists have a low degree of investment in bet hedging; they perform well in stable and defined conditions but are less competitive when conditions change. Conversely, generalists are not as competitive in a standard environment but are more adaptable and able to maintain performance under changeable conditions (Chapter 1.7).

Of the yeast strains examined, W34/70 exhibited a relatively high degree of heterogeneity to ethanol stress (Chapter 5.2.1.1). However, it was interesting to note that the population became even more heterogeneous once it had been used in a fermentation (Chapter 5.2.2). This supports previous suggestions that a strains investment in a specific strategy may be malleable and subject to evolution over time (Blake *et al.* 2006, Carlquist *et al.* 2012, Li *et al.* 2010). This phenomenon has implications for industrial fermentations; for example, if a strain cannot adapt across a process then this may raise questions

with regard to its suitability. This is particularly true for scenarios where the limits of yeast stress tolerance are tested, for example during the use of high gravity worts to produce high alcohol products. Conversely, if a strain is able to actively 'acquire' heterogeneity then this may lead to the development of subpopulations, which are likely to lead to a further shift in phenotypic heterogeneity. From a brewing perspective, this may provide a rationale for phenotypic drift and performance changes over time. However, it should also be noted that major genetic mutations typically occur after approximately 50 divisions in yeast strains, with viable variants able to predominate if certain environmental conditions are met (Paquin and Adams, 1983). Hence phenotypic heterogeneity should not be considered in isolation; changes to the genome are also likely to drive differences in performance.

While it is recognised that the presence of a small number of variants (genetic or phenotypic) may pass unnoticed if beer quality and fermentation profile remains consistent (Powell *et al.,* 2004), extreme environments typified by VHG brewing or extensive serial repitching may result in population changes that yield a product which is out of specification (Powell *et al.,* 2003; Quain *et al.,* 2001). In order to determine the potential for changes in heterogeneity to impact on fermentation-related characteristics, yeast strain W34/70 was subjected to an extended period of sub-lethal ethanol stress. It should be noted that in this context, sub-lethal is defined as being insufficient to completely destroy the yeast population, but that tests individual cells to their limits. Strain W34/70 was selected for analysis since it was identified as displaying a high degree of heterogeneity to ethanol stress (Chapter 5.2.1.1) and would

therefore have the potential to reveal changes in strategy more readily than strains more homogeneous in nature. The aim of the current study was therefore to determine if long term exposure to sub-lethal ethanol stress would lead to the development of phenotypic change. Furthermore, the variation encountered was characterised to elucidate if it could be attributed to genomic mutations, and to determine the potential for intrinsic changes to bet-hedging strategy in the lager yeast strain W34/70.

6.2 Results

6.2.1 The impact of extended ethanol stress on brewing yeast strain W34/70

The impact of sub-lethal ethanol stress on the lager yeast strain W34/70 was assessed using a chemostat fermentation system as described in Chapter 2.5.6. Populations of W34/70 cells were maintained in stirred 15L (10L working volume) fermentation vessels designed to support cellular division over 18 days. Continuous growth of the population was supported by the addition of fermentable sugars in the form of oxygenated wort over time (0.1L/hr) to maintain a gravity of approximately 2 – 3 °P. The wort reservoir was oxygenated throughout to ensure that fatty acids and sterols could be synthesised and did not become a limiting factor in the maintenance of cellular membrane structures (Lorenz and Parks, 1991). The addition of 5% ethanol (v/v) at the beginning of the fermentation was applied to induce a low level of ethanol stress (Chapter 2.6.6) and this concentration was also maintained throughout fermentation. While being supplemented with fresh wort, an identical quantity

of beer was removed from the chemostat at an equal rate of 0.1L/hr. These samples were used to determine fermentation progression and ethanol concentration, as well as yeast cell count, viability, and the extent of heterogeneity. Consequently, the key drivers of heterogeneity in this investigation (genetic or phenotypic) were investigated via interdelta PCR (Chapter 2.4.2) and stress dose response analysis (Chapter 2.5.4).

6.2.1.1 The effect of sub-lethal stress conditioning on yeast replication and physiology

Analysis of the media characteristics during the chemostat fermentation indicated that a steady decrease in gravity from 13 °P to 3 °P occurred during the first 5-6 days, reflected by an inversely proportional increase in alcohol concentration as would be expected. After this point feeding was adjusted to maintain a constant gravity of 2 °P (Figure 6.1), while ethanol was allowed to free rise to approximately 8%. It should be acknowledged that the environment created here was significantly different to those encountered during a batch-type fermentation and represents a more extreme environment than would be encountered during standard lager production. However, the goal was not to mimic industrial fermentations, but to create an environment which would allow cells to divide continuously under stressful conditions. Irrespective, conditions were maintained until day 6; at this point the concentration of ethanol reached a peak of 8.3%, after which it declined steadily to approximately 7% (v/v) at day 10 and remained relatively constant thereafter. The decline in ethanol concentration may have been a consequence of diauxic

shift, since yeast are able to metabolise ethanol as a carbon source for growth (Fiechter *et al.,* 1981). In addition, it is also possible that the production of ethanol became restricted as sugar concentrations reduced which may have caused an inhibition of the Crabtree effect triggering respiration in the yeast cells, especially since oxygen was not limiting at this time. Support for this also comes from evidence that yeast can actively utilise ethanol as a carbon source under certain conditions, as part of a 'make-accumulate-consume' strategy (Piškur *et al.,* 2006). Although ethanol stabilised at approximately 7% after 10 days, spikes in in production were observed at days 11, 14 and 18. It is possible that dosing at these times caused the concentration of fermentable sugar to reach a critical level triggering a regulatory cascade to initiate catabolite repression causing the yeast to shift back to a fermentative state until the fermentable sugars became a limiting factor again (Gancedo, 1998).

The characteristics of the yeast population and fermentation media (beer) over the course of the chemostat fermentation can also be seen in Figure 6.1. The demographics of strain W34/70 (cell concentration and viability) are indicated in Figure 6.1b and the corresponding beer characteristics (gravity and ethanol concentrations) are displayed in Figure 6.1a. Analysis of the total cell count during fermentation indicated that, after pitching with $1.3x10^7$ cells/ml, a period of rapid growth was established leading to the population stabilising at a concentration of $4x10^7$ cells/ml after approximately 6 days. After 10 days this gradually declined to around $3x10^7$ cells/ml with a final concentration of $2.8x10^7$ cells/ml. The calculation for the number of divisions was based on fact that the concentration of fermentable sugars plateaus and the rate at which

fresh wort was pumped into the chemostat becomes the limiting factor and can be used to calculate the cell generation factor (Chapter 2.5.6). To calculate the total generations the cell generation factor of 3.42 divisions per day was multiplied by the number of days in the chemostat. 3.42 x 18 days = 61.56 cell divisions. Consequently, it can be estimated that a total of 62 divisions (population doublings) occurred during the course of the 18 day fermentation. Although cell viability at pitching was approximately 99%, this decreased to 80% after approximately 3 days (Figure 6.1b). Interestingly this correlated with the period of rapid growth observed, indicating that despite population growth, there was variation in fitness between individuals. The extent to which viability decreased was unexpected, especially since it is known that that this strain is tolerant to ethanol (Chapter 3.2.2.3) and is typically robust during fermentations where high amounts of alcohol are produced. However, in this instance it is possible that subjecting cells to ethanol without any period for adaptation (Piper, 1993) resulted in a reduction in live cells. The viability of the culture remained at approximately 80% for 13 days, after which it deteriorated further to approximately 70% by day 18 (Figure 6.1b). Therefore, it can be seen that chemostat fermentation under the conditions applied was not inducive to healthy yeast; the decrease in viability was an indicator that the population was stressed. It is well known that yeast subjected to harsh fermentation environments may deteriorate in terms of their physiological state or lose their ability to replicate (Jenkins *et al.,* 2003). However, although the level of stress applied here was sufficient to impact the culture, it was not so severe that the entire population was completely destroyed.

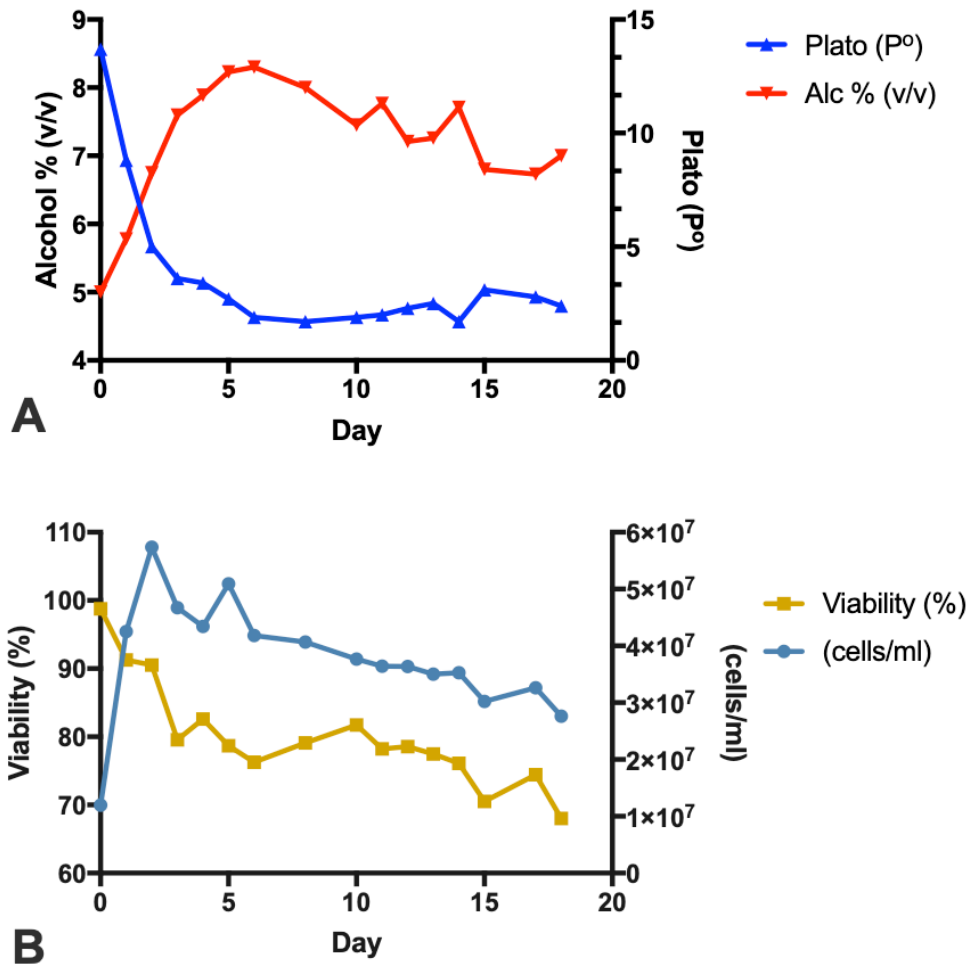


Figure 6.1 Chemostat fermentation characteristics of yeast W34/70 inoculated into ethanol-supplemented media over 18 days. A: alcohol concentration % (v/v) and Plato (Pº). B: yeast cell count (cells/ml) and yeast cell viability (%).

6.2.1.2 The impact of sub-lethal stress conditioning on yeast DNA integrity

In order to determine if the sub-lethal stress conditioning applied had impacted on the genetic make-up of lager strain W34/70, samples were taken pre- and post-fermentation and analysed using DNA fingerprinting. As described in Chapter 2.4.2.; Chapter 3.2.1.3, interdelta regions, known to be highly variable and indicative of genetic change (Dunn and Sherlock, 2008; Garfinkel, 2005; Rachidi *et al.,* 1999) were amplified to produce fingerprints of each culture. It was anticipated that investigating the number and size of amplicons obtained from PCR amplification of interdelta regions would provide an indication of the impact of sub-lethal stress on the genetic constitution of each culture.

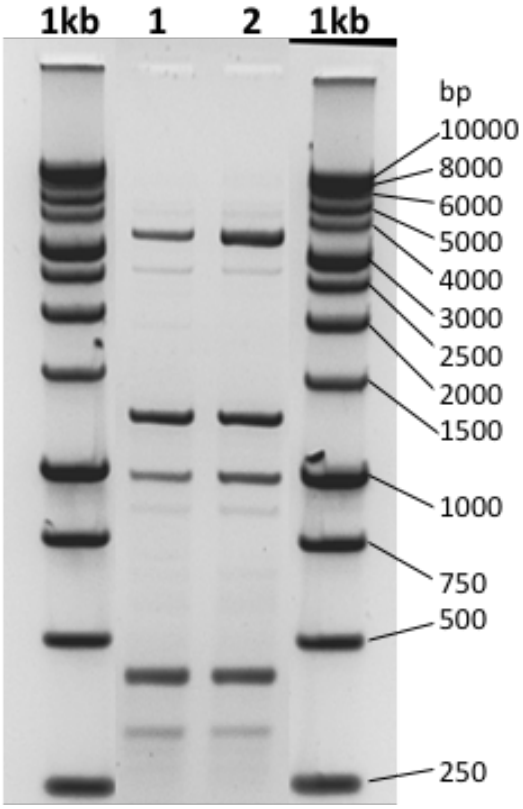


Figure 6.2 Interdelta PCR fingerprint analysis of W34/70 samples at Day 0 (Control) and Day 18 of long term sub-lethal ethanol stress. Lane 1: W34/70 Day 0. Lane 2: W34/70 Day 18. A 1kb ladder is shown and was used to determine fragment sizes in each instance.

The DNA fingerprint obtained through analysis of Day 0 (control) samples (Figure 6.2) was typical of the strain (Chapter 3.2.1.3); amplicons were observed in the expected region of 350-6000bp. For the lager strain W34/70, it was anticipated that amplicons would be observed at ~5000, ~3000, ~2500, ~2000, ~1300, ~1100, ~1000, ~440 and ~320 bp. It can be seen from Figure 6.2 that this was the case. However, in the analysis here (Figure 6.2) the bands for ~5000 and ~2000 bp were not as prominent as previously seen (Chapter 3.2.1.3), this can likely be attributed to the amount of template DNA available

pre-amplification, rather than being an indication of change to the genomic DNA. I can be confident that this is the case firstly because it is accepted that band intensity should not be considered when analysing interdelta PCR fingerprints (Innis *et al.,* 2012), and secondly because both the control sample and the experimental sample (Day 18) exhibited identical profiles. Consequently, I can conclude that the lack of variation in the banding patterns between each sample indicated that there had been no change to the frequency and location of interdelta sequences. While this is not absolute confirmation that genetic change had not occurred *per se*, the fact that interdelta regions are known to be impacted by stress factors is a good indication of genomic consistency. It is recognised that smaller point mutations may have occurred, and further analysis of key genes would be required to determine if this was the case and its significance in terms of functionality. Irrespective, based on the data obtained here, I can assume that no gross changes to the yeast genome had occurred in response to extended sub-lethal stress.

6.2.1.3 The effect of sub-lethal stress conditioning on yeast heterogeneity

Once yeast cells had been subjected to low-level ethanol stress for 18 Days in the chemostat, samples of the lager yeast strain W34/70 were taken (Day 18) and compared to control (Day 0) cultures. This was performed to compare the phenotypic response before and after long term exposure to sub-lethal concentrations of ethanol. Phenotypic heterogeneity was measured as previously described, via dose response curves using ethanol as a stressor (Section 2.5.4 and 5.2.1.1). Ethanol was selected since it was initially proven to be a useful stress factor to in determining heterogeneity (Chapter 5.2.1.4), while also being clearly demonstrable to the brewing sector.

The response of W34/70 populations (both from Days 0 and 18) to increasing ethanol concentrations can be seen in Figure 6.2. Based on the viable CFU's observed, a sigmoidal curve was fitted to model the effect of ethanol concentration (x) against viability (y) as described in Chapter 2.4.3. By overlapping the two descending sigmoidal curves produced by the dose-response analysis it was evident that there had been a shift in phenotypic heterogeneity over the course of the 18 Days. Under ethanol stress the maximum asymptotes of the curves can be seen to be variable; samples from Day 0 exhibited the smoothest curvature in contrast to samples from the end point of fermentation, which showed more angular curvature due to increased steepness of the slope. The IC50 values for W34/70 towards ethanol altered significantly (P value 0.0003) from 12% (v/v) in the stock culture to 14.8% (v/v) in the 18 day sample (Figure 6.3). For the 18 day samples, the maximum asymptote of the curve was lower on the y-axis, indicating that there was a

negative correlation between viability and low doses of ethanol, however the curve shifted in a positive direction along the x-axis, indicating that the IC50 value for these cells had increased ethanol tolerance overall. Consequently, it can be seen that after prolonged exposure to ethanol, W34/70 became more tolerant to higher concentrations of ethanol stress. However, the slope of the curve obtained during analysis of this culture was significantly steeper than that of the control sample as indicated by analysis of the hillslope gradient of each dose response curve (Figure 6.4).

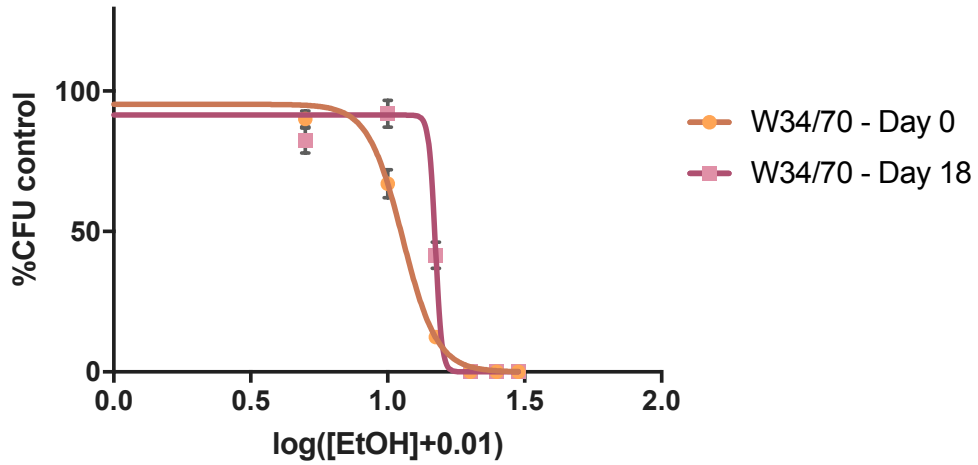


Figure 6.2 Heterogeneity analysis of strain W34/70 in response to ethanol stress, using samples obtained pre- and post-fermentation in a sub-lethal stress environment. Ethanol concentrations include 0, 5, 10, 15, 20, 25 and 30% (v/v) Data indicates triplicate independent experiments at each time point.

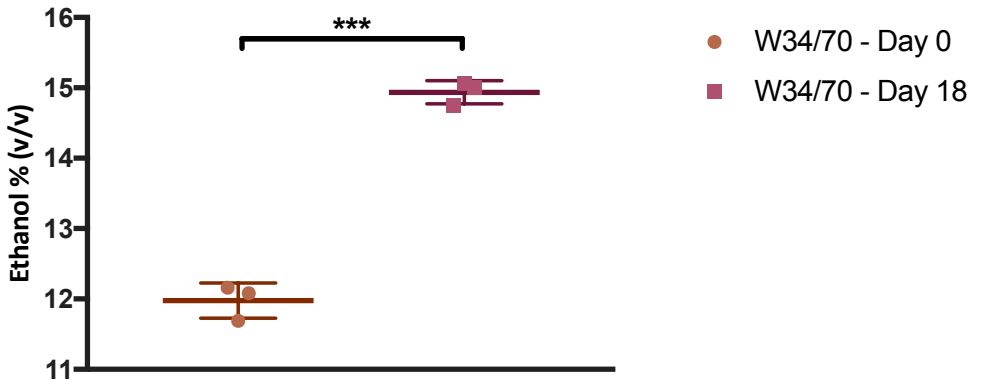


Figure 6.3 IC50 values extrapolated from data obtained from dose-response analysis. After 18 days under prolonged ethanol stress the IC50 value of W34/70 increased from 12% to 14.8% (v/v) Mean of the three independent data points represented with a line. Error bars representative of standard deviation. (* = P value 0.0003).**

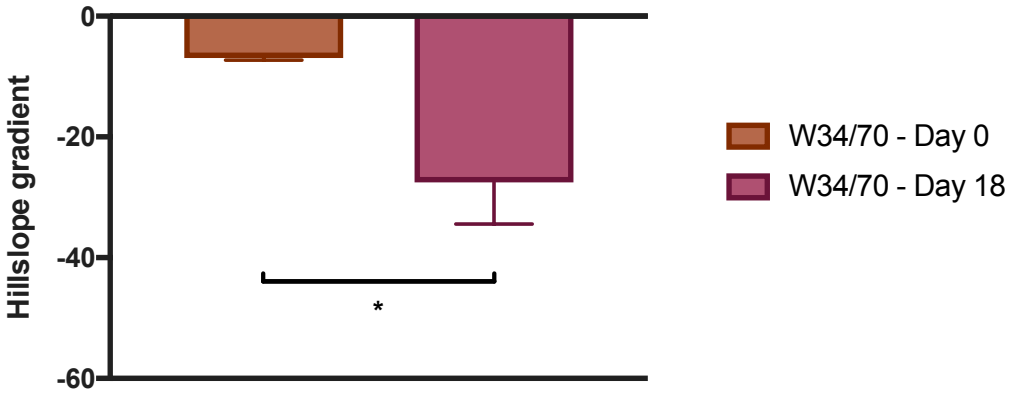


Figure 6.4 Hillslope gradients calculated based on dose-response analysis of W34/70 under ethanol stress. Smaller values indicate gentle curves and therefore show high phenotypic heterogeneity; larger values represent steep curves and therefore indicate a low degree of phenotypic heterogeneity. Error bars representative of standard deviation. (* = P value 0.054).

As described previously (Chapter 2.5.4; Chapter 5.2.1.1), by calculating the hillslope gradient from the data presented in Figure 6.2, the relative degree of heterogeneity could be calculated pre- and post-fermentation. Figure 6.4 represents the hillslope gradients taken directly from the sigmoidal curves obtained for lager yeast strain W34/70 after Day 0 (control) and Day 18. Statistical analysis (one-way ANNOVA; Chapter 2.5.4 and 2.7) of the curves obtained indicated a statistically significant shift in phenotypic heterogeneity, with the post-fermentation sample of strain W34/70 becoming less heterogenous in its response to ethanol stress. This can be seen by a change in gradient from -6.91 to -27.5; deemed to be significant (P value 0.0054). It is interesting to note that the data observed here conflicts with that obtained previously from analysis of yeast taken following a standard fermentation

(Chapter 5.2.2). Previously, post-fermentation samples were observed to show increased heterogeneity, but with a reduction in overall 'fitness' (Figure 5.7). The reduced heterogeneity observed in the current study is likely to be an artefact of the sustained stress placed on the population over time. It is possible that this may have arisen solely due to the death of 'weaker' individuals within the population, leaving a more ethanol-tolerant subpopulation.

6.2.1.4 Analysis of the capacity of a yeast strain to 'pass forward' acquired heterogeneity

As described above (Section 6.2.1.3), cultivating lager yeast strain W34/70 under a high ethanol environment for an extended period of time impacted on the heterogeneity of the yeast culture. Cells became more homogeneous in their response to ethanol, with the majority showing tolerance which was at the upper limit for the strain. It should be noted that an estimation of 62 generations represents a relatively large number of divisions for a brewing yeast culture, especially that of a lager strain. The majority of large international brewing companies producing lager type products tend to restrict the number of serial repitchings to 3-8, with many smaller 'craft' brewers limiting use to between 15-20 generations for ales (Powell and Diacetis, 2007; Smart and Whisker, 1996). The former is the equivalent of 9-24 yeast divisions and the latter 45-60, however, this is put into perspective by analysis of laboratory yeast strains, which have been shown to undergo viable mutational changes at a rate of approximately 50 divisions even under favourable conditions (Paquin and Adams, 1983). Given that lager yeast are hybrid

organisms, known to be genetically tractable (Libkind *et al.,* 2011), and that brewing fermentations are innately stressful (Bamforth, 2008; Briggs *et al.,* 2004), it might have been expected prolonged stress would lead to mutations. However, this was not observed in the current work and the change in stress tolerance related heterogeneity was attributed primarily to phenotype rather than gross chromosomal or DNA movements. However, it is acknowledged that further analysis of specific stress genes would provide much greater insight into the root causes of heterogeneity in this instance.

It should be noted that in natural 'wild' yeast populations, cells may be present *in situ* alongside stressors for significant periods of time (often years) and are therefore potentially subject to even greater evolutionary pressures. It might be expected that this would make adaptations more stable if phenotypic heterogeneity is a transient quality in population dynamics (Holland *et al.,* 2014). To test whether the reduced phenotypic heterogeneity exhibited by strain W34/70 after 18 Days ethanol stress was transiently expressed, or a permanent feature that could be 'passed forward', a subculture of this sample was grown on YPD agar, propagated in YPD media and exposed to ethanol stress dose response analysis as described previously (Section 2.5.4, 5.2.2 and 6.2.1.2). The results were compared to data obtained from pre- and post-fermentation (Day 0 control and 18 Days respectively), while the results from Chapter 5.2.2 (heterogeneity after a standard brewing fermentation) were also included as an additional reference.

It can be seen from Figure 6.5 that the response of each of the four different populations of W34/70 to increasing ethanol concentrations was varied; each

population was characterised by its immediate history. By overlapping the four descending sigmoidal curves produced by the dose response analysis it was evident that a population shift away from phenotypic heterogeneity was seen after 18 Days of exposure to ethanol stress (as already discussed above; Figure 6.2). Under ethanol stress the maximum asymptotes of the curves show differences between the four samples; the 'W34/70 Day 18 subculture' exhibited the lowest asymptote of all the curves positioning it lower on the y-axis, indicating that there was a negative correlation between viability and low doses of ethanol, however the curve shifted in a negative direction along the x-axis meaning the IC50 value for these cells had decreased ethanol tolerance overall compared to the 'Day 18' sample and was exhibiting tolerance more similar to that of the 'Day 0' control. As discussed previously, the position of the sigmoidal curve along the x-axis indicates changes in ethanol IC50 values. In Figure 6.6 it is possible to see that a single fermentation reduced the IC50 value of the W34/70 control by 6.5% (v/v), prolonged (18 days) exposure to low level doses of ethanol increased the IC50 value by 2.8% (v/v), but by sub-culturing the more tolerant W34/70 – Day 18 culture in YPD media, and removing the ethanol stress as a primary selective agent, the IC50 value was reduced by 2.3% to 12.5% (v/v); almost exactly to what it had previously been at Day 0. This suggests that the 'normal' level of population tolerance for W34/70 had been resumed and acquired tolerance had not been 'passed forwards'. Despite the concerns raised above that changing heterogeneity may have been based on a genetic element, such as individual stress gene structure and regulation, this does suggest that if any changes to the genome had

occurred, these became lost or outcompeted once 'normal' conditions had been reinstated. For a more accurate measure of the extent of phenotypic heterogeneity between these samples the hillslope gradient of the four dose response curves was compared as shown in Figure 6.7.

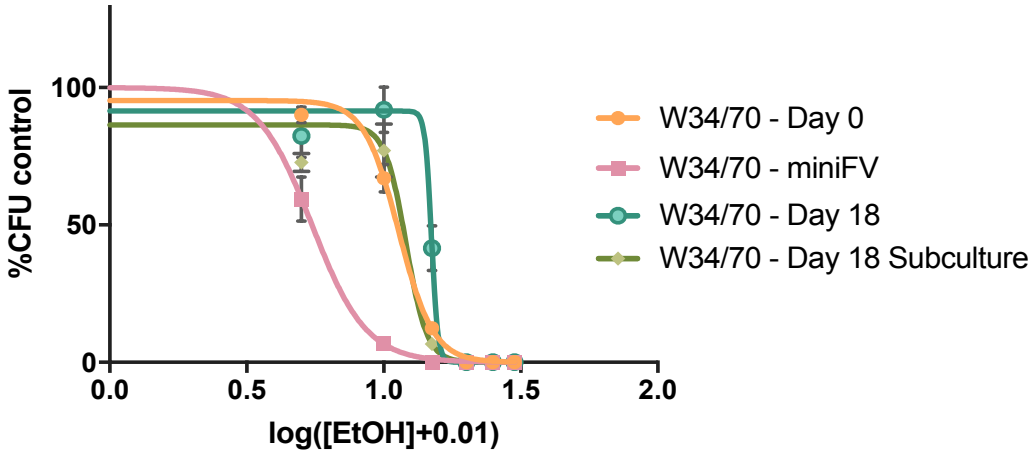


Figure 6.5 Heterogeneity analysis of strain W34/70 in response to ethanol stress, using samples obtained pre- and post-fermentation in a sub-lethal stress environment, a subcultured population of the post-fermentation sample and a sample obtained from a single miniFV was included for reference (Chapter 5.2.2). Data indicates triplicate independent experiments at each time point.

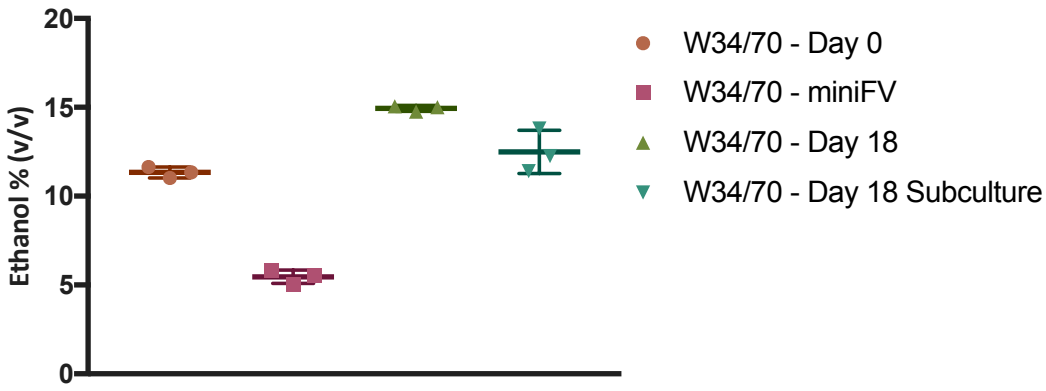


Figure 6.6 IC50 values extrapolated from heterogeneity profiles of samples obtained pre- and post-fermentation in a sub-lethal stress environment, subcultured population of the post-fermentation sample and the sample obtained from a single miniFV was included for reference (Chapter 5.2.2) in response to ethanol stress. Mean of the three independent data points represented with a line. Error bars representative of standard deviation.

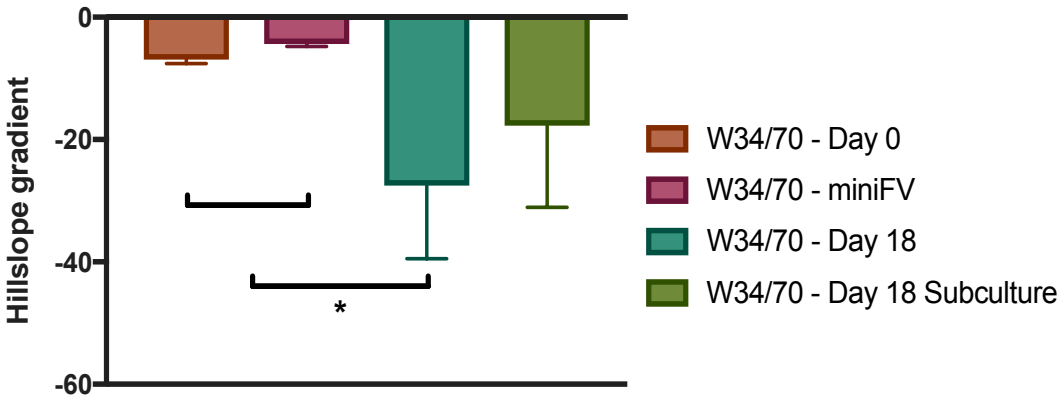


Figure 6.7 Hillslope gradients calculated based on dose-response analysis of W34/70 populations under ethanol stress. Smaller values indicate gentle curves and therefore show high phenotypic heterogeneity; larger values represent steep curves and therefore indicate a low degree of phenotypic heterogeneity. Error bars representative of standard deviation. (* = $P \leq 0.05$).

Analysis of the hillslope gradients for each sample group confirmed the transient nature of the phenotypic heterogeneity observed in Day 18 samples (Figure 6.5). However, the extent to which the subculturing of the Day 18 sample caused a return to the 'baseline' value (Day 0) from the heterogeneity slope analysis was not as pronounced in the IC50 values, suggesting that the subcultured W34/70 population was still in a period of full recovery from the previous ethanol stress. This may be explained by cell cycle and cell tolerance to stress as unequal segregation of certain molecular compounds between the mother and daughter cells prolong the effect of stress induced tolerance once the stress has been removed (i.e. it is not a binary switch that appears to be fundamental in bacteria) (Avery, 2006; Henderson and Gottschling, 2008; Levy *et al.,* 2012; Powell *et al.,* 2003). In Chapter 5, it was argued that an increase in

phenotypic heterogeneity in response to fermentation may have been due to a consequential investment in bet hedging. Further support for this hypothesis is provided by comparing data obtained from W34/70 Day 18 and the W34/70 Day 18 Subculture. The Day 18 sample represents a population extracted immediately from an environment of prolonged stress. It is suggested that the effect of this was to essentially eradicate 'weaker cells' within the population resulting in it comprising highly resistant cells. This can be seen since the density distribution shifted towards a higher mean with a smaller standard deviation (Figure 6.5). If this state was induced by a permanent switch in phenotype then upon removing the primary selective agent, in this case ethanol, then the previous cell ratio would still exist. However that was not observed; subculturing the Day 18 sample in nutrient rich media resulted in a shift back towards a more phenotypically heterogeneous population. Therefore, the phenotypic heterogeneity of W34/70 can be described as being 'tuned' to ethanol stress due to the way it quickly (within ~1 generation) switched between phenotypes at the same rate as frequency of the environmental change (Acar *et al.,* 2008; Holland *et al.,* 2014).

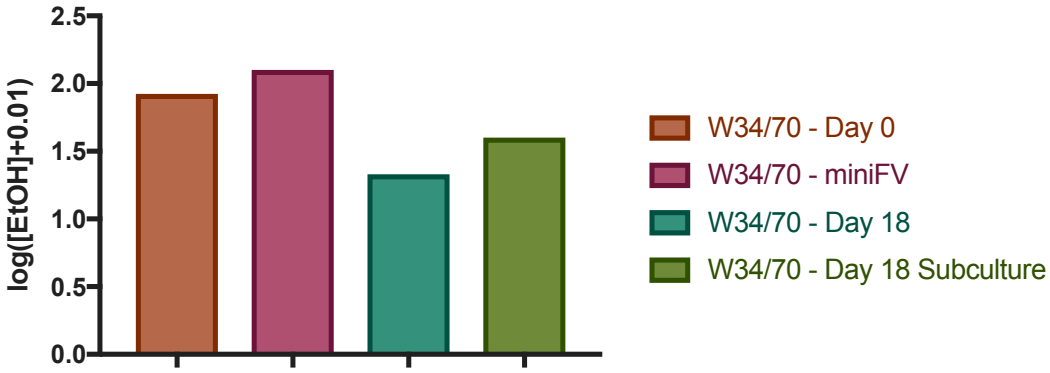


Figure 6.8 Interpolation of sigmoidal model intercept with x-axis. Model equation: Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

In order to fully investigate the upper limits of tolerance for each population, data was analysed to determine the intercept with the x-axis (ethanol concentration). However, due to the use of a logistic function to produce the model sigmoidal response curves for the measurement of phenotypic heterogeneity, the point at which the curve intercepts the x-axis does not actually exist. Therefore, when solving the sigmoidal four parameter logistic model equation, a value as close to zero as possible (and which gives a real number) was calculated and used. A comparison of the intercept gives an indication of overall ethanol tolerance exhibited by a population (Figure 6.8), where the higher the intercept, the more tolerant the sample was to ethanol toxicity. The results of the 'model x-axis intercept point' mirror the relationship between each sample and the extent of phenotypic heterogeneity (Figure 6.6; Figure 6.7), with the sample obtained from miniFV (W34/70 miniFV) exhibiting the highest tolerance to ethanol, and W34/70 Day 18 exhibiting the lowest tolerance to ethanol. This data was interesting since W34/70 Day 18 samples

initially appeared to show greater tolerance to ethanol, based on the inflection point prior to population death (Figure 6.4). Similarly, the theoretically 'poorer' population derived from miniFV showed individuals with increased tolerance. A rationale for this is hard to suggest, but this may simply be a reflection of the extent of variation associated with each population. Despite this, it should be noted that the maximum values for each strain (i.e. the limits of tolerance) were broadly similar. This indicates that although each population had undergone shifts in the distribution of cells, the maximum capacity of yeast to resistant stress had not become altered.

6.3 Conclusions

A fundamental question about phenotypic heterogeneity in brewing yeast brought forward from the previous chapters was related to the extent to which this phenomenon relates to industrial fermentations. Previous studies have shown the importance of phenotypic heterogeneity in nature (Ackermann, 2015; Holland *et al.,* 2014) where microbial communities *in situ* can experience fluctuations in the levels of environmental stress. Due to the non-motile nature of yeast, being able to elicit a physiological response (such as upregulating genes) to adapt or tolerate these changing environments, is essential (Gibson *et al.,* 2007). As described previously, it is accepted that there are a multitude of fluctuating environmental stresses imposed by brewery fermentations, and the added pressure imposed by serially repitching brewing yeast can cause the yeast population to accumulate variants which may be either genetic or

phenotypic in nature. If these are selected during yeast recycling this can result in a product which does not meet specification for the brand (Gibson *et al.,* 2007; Powell and Diacetis, 2007; Quain *et al.,* 2001). In this Chapter a small scale fermentation was conducted to provide a challenging environment to a yeast culture, with the goal of encouraging population changes. The lager yeast strain W34/70 was identified previously as displaying a high degree of heterogeneity to ethanol stress (Section 5.2.1.4) and was therefore selected as a potential candidate to reveal changes in bet-hedging strategy more readily than strains that were more homogeneous in nature. The addition of a sub-lethal ethanol stress was used as a primary selective agent for phenotypic heterogeneity analysis and to accelerate the potential for change.

During the course of an 18 day fermentation, an initial drop in viability was observed, indicating that the population was negatively affected by the continuous exposure to sub-lethal ethanol concentrations. However a significant proportion of the population was able to survive and persist through the remainder of the experiment. Consequently, samples taken at Day 0 (control) and Day 18 were taken to determine if long term stress conditioning would cause a shift in phenotypic heterogeneity. After ethanol stress dose response analysis, it was evident that prolonged exposure to ethanol had caused strain W34/70 to become more homogeneous in nature. Day 18 samples comprised mainly highly resistant cells, shifting the density distribution towards a higher mean and smaller standard deviation, supported by a steeper hillslope gradient. A secondary hypothesis was also tested to identify if the extent of investment in bet-hedging would cause a permanent change within

the population, or if it had the potential to revert to 'type'. Specifically, if the ethanol tolerant 'state' observed in Day 18 populations was induced by a permanent switch in phenotype then, upon removing the primary selective agent (in this case ethanol), the previous cell ratio would still exist. However that was not the result observed in this investigation; subculturing the Day 18 sample in nutrient rich media without the pressures of ethanol stress resulted in a shift back towards the typical stress dose-response analysis curve for the strain. It should be noted that the hillslope gradient for this strain appeared to be in-between that observed for stressed (Day 18) and non-stressed cultures (Day 0). However, analysis of the upper limits of tolerance (intercept with the x-axis) revealed that there was no substantial change within the population. Therefore the phenotypic heterogeneity of W34/70 can be described as being 'tuned' to ethanol stress since it was observed to quickly switch based on the precise environmental make-up. This supports previous analysis indicating that phenotypic heterogeneity is a selected trait in natural yeast populations subject to environmental stress (Acar *et al.,* 2008; Holland *et al.,* 2014). The implications of these data for brewing fermentations remain unclear. However, it is suggested that the variation observed in phenotypes sheds new light on the potential root causes of viability loss, and why some strains are more suitable for certain fermentations, while others do not respond well. Related to this, a detailed study of phenotypic heterogeneity may prove to be a useful selection criterion for novel yeast strains, or indeed, for selecting parental strains in breeding projects. Irrespective, arguably the most important observation is that strain W34/70 appears to be innately heterogenous in

response to fermentation-related stress factors. It could be argued that the intrinsic adaptability of strain W34/70 is perhaps a direct reason why this strain is so widely used in lager fermentations worldwide.

CHAPTER 7:

CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

In the brewing industry it is common practice for both ale and lager yeast to be cropped post fermentation and serially repitched into subsequent fermentations. The number of times this is occurs is restricted based on yeast strain and wort composition. A brewer may become aware of the effects of continual serial repitching due to the changes in fermentation performance, end product deviation and yeast flocculation potential. Previous work has shown that the repeated stress of being repitched into the stressful environment of an industrial fermentation along with the stress of being contained within a sediment at the end of the fermentation leads to cell deterioration including the production of genetic variants (Lawrence *et al.,* 2013; Powell *et al.,* 2004; Powell and Diacetis, 2007; Smart and Whisker, 1996). As the practice of serial repitching is cost effective to the brewing industry due to mitigating the costs of freshly propagating enough brewing yeast for each brew. Also, the first batch of freshly propagated yeast has a lower fermentation efficiency than from the second onwards (before it deteriorates).

This difference between the fermentation efficiency of the freshly propagated yeast and the first population of repitched yeast offers the first glimpse of phenotypic heterogeneity in the brewing industry. Both of these yeast populations are isogenic yet perform differently under the same environmental conditions (Powell *et al.,* 2003; Smart, 2001). If a phenotypic shift can occur from the population in the first fermentation to the second then it is also highly likely that phenotypic variation also occurs after many repitches before any genetic variation develops. This may become an even more important

consideration with the onset of brewing intensification becoming more prevalent in the industry. High gravity and very high gravity brewing involves the use of highly concentrated wort to produce high ethanol beer which will require diluting at the end of the process. This process applies more extreme environmental pressures on the brewing yeast during fermentation.

In this study I chose five industrially important brewing yeast strains based on product variety (ale and lager yeasts) and strain type (Frohberg and Saaz type lager yeast) in order to determine the extent of phenotypic heterogeneity of these strains when exposed to stresses relevant to industrial brewing practices (ethanol, osmotic and oxidative stress). Firstly, each strain was characterised both genetically and phenotypically (Chapter 3) and subsequently a series of laboratory scale tests looked at the propensity each strain had to produce morphological variants and if this was linked to stress resistance (Chapter 4). This led to an investigation into phenotypic heterogeneity via the use of dose response curves (Chapter 5) and the most heterogeneous strain being selected for long term, low level stress exposure to ascertain if this causes changes in the level of heterogeneity and if it can recover once the selective pressure was removed (Chapter 6).

In order to determine the effect brewing related stresses has on industrial brewing yeasts, the genetic and phenotypic characterisation of each strain first needs to benchmarked. In this study two ale strains (M2 and NCYC 1332) and three lager strains (W34/70, CBS 1260 and CBS 1174) were characterised based on growth temperatures, kinetic growth curve analysis, growth on solid agar containing rising concentrations of stressors (ethanol, H_2O_2 induced oxidative

stress, and sorbitol induced osmotic stress), ITS PCR-RFLP, and interdelta PCR. The combination of these analyses meant that the yeasts could be split according to classification (ale/lager), type (Frohberg/Saaz) and because interdelta PCR gave a clear individual fingerprint, they could be confirmed no matter what their genealogy. The spot plates used to asses stress tolerance on solid agar medium ascertained the upper limits of each stress as the strains went into decline and growth capacity became inhibited. While it is acknowledged that there are other methods which can be applied to assess tolerance to stress (such as metabolic activity), for the purposes of this work, and from the brewing perspective, the capacity to divide provides sufficient information. From this investigation it was possible to see that all strains were susceptible to elevated concentrations of stresses, however the way in which they reacted was both 'type' and strain dependant. The ale strains were generally less tolerant of oxidative stress than the lager strains (Chapter 3.2.2.1). All of the strains were able to tolerate high concentrations of osmotic stress, with growth observed for all strains at concentrations of up to 40% sorbitol (w/v). Analysis of the response of yeast to alcohol stress indicated that the lager strains that were generally less tolerant of ethanol stress than the ale strains. These preliminary examinations provided an overview of the phenotypic characteristics of each brewing strain selected and the benchmarked attributes (such as maximum stress tolerance) was used to support further investigations into colony variance (Chapter 4) and phenotypic heterogeneity (Chapters 5-6) under these brewery related stresses. An important observation arising from the preliminary characterisation work was

that when stresses were applied, sub-populations within each strain became evident.

Levy *et al.* (2012) stated that due to some sub-populations of isogenic cells retaining a bet-hedging strategy which protects cells against unpredictable environments, it is possible for cell – cell heterogeneity to occur even in benign environments. Morphological variants can be detected via the use of WLN agar and allowing the growth of giant colonies (Chapter 4). The frequency of morphological variants appears to be strain dependant based on this subset of brewing strains tested. The lager stains produce more morphological variants when compared to the ale strains. This may be due to the hybrid, aneuploid nature of lager strains making them more susceptible to phenotypic variation. The history of the particular brewing strain may also be of importance. Ale strains have had thousands of years to stabilise their genome whilst lager strains presenting more genomic variation could simply be down to them being in an early part of their existence (Section 1.3.3, Byrnes *et al.,* 2006; Dunn and Sherlock, 2008; Scannell *et al.,* 2006). While lager strains 'work themselves out' it makes sense that they would be more well equipped to exploit new environmental niches. Interdelta PCR confirmed the morphological heterogeneity to be based on phenotype rather than a genomic mutation. Differential gene expression such as that of *FLO11* which produces proteins associated with cell adhesion and hydrophobicity (Bayly *et al.,* 2005; Halme *et al.,* 2004; Reynolds and Fink, 2001; Voordeckers *et al.,* 2012) are likely to be involved with colony variation within a strain. Although in this case the variation on colony morphology was not found to have a clear link between

strain and stress resistance it is possible that there is a link with nutrient limitation. The ability for a colony to adhere to a surface in times of nutrient limitation would allow for that a sub-set of phenotypically diverse cells are able to maintain viability until they next come into contact with a nutrient rich environment.

There are numerous studies which investigate the effects of the environmental pressures involved in industrial scale brewing (Barker and Smart, 1996; Gibson *et al.,* 2007; González *et al.,* 2015; Paquin and Adams, 1983; Powell *et al.,* 2004; Powell and Diacetis, 2007; Smart and Whisker, 1996) but the experimental procedures look at whole populations of brewing yeast making the result a mean of all the cellular responses. By taking into account individual cells it is possible to measure the heterogeneity of a strain under a particular stress (Chapter 5). Based on the hillslope gradient data provided by dose-response curves it was evident that there was a complex relationship between yeast strain, stress tolerance and heterogeneity. While each strain showed a different degree of phenotypic heterogeneity, there was no correlation between stress factors. For example, displaying a high degree of heterogeneity to one stress did not lead to a similar phenotype in response to other stresses. Furthermore, there were no similarities that can be related to species (*S. cerevisiae*/*S. pastorianus*). Under ethanol stress there did appear to be a link between the type of lager strain and stress resistance in that the Frohberg type yeast strains exhibited significantly ($P<000.1$) more heterogeneity compared to that of the Saaz type strain. However, this is too small a set strains being compared to conclusively adopt this conclusion. Analysis of the phenotypic heterogeneity of

the five brewing yeasts suggests that each strain may have a customised 'response program' for each environment, perhaps utilising specific gene products needed to help combat the effect of individual stresses (Gasch *et al.,* 2000). This may be significant for current production streams, especially those which employ high intensity techniques such as high gravity brewing. Although the absolute tolerance of a strain to a specific stress factor will inevitably play a role in determining its suitability, it is possible that the variation within a population may also impact on performance. A highly homogenous population with good tolerance to multiple stress factors is theoretically likely to offer an advantage. However, in reality, there is likely to be a trade-off, complicated by the fact that yeast strains exhibit different heterogeneity curves in response to different stress factors. Consequently, a strong argument could be made for selecting a strain with a broader response system as such strains are likely to be more adaptable to environmental fluctuations (Hewitt *et al.,* 2016) and perhaps more suitable for industrial fermentations. In contrary to this is the opinion that some characteristics of brewing yeast should be less variable than others. For example, aspects of yeast performance that have repercussions for final product characteristics should offer as little variation as possible, in order to ensure consistency.

The final question relating to the extent phenotypic heterogeneity impacts on brewing related processes is; does the act of serial repitching act as a selective pressure on sub-populations in heterogeneous yeast populations? To investigate this a small scale, long term fermentation was conducted to provide a challenging environment to a yeast culture, with the goal of encouraging

population changes (Chapter 6). The lager yeast strain W34/70 was identified as displaying a high degree of heterogeneity to ethanol stress (Section 5.2.1.4) and was therefore selected as a potential candidate to reveal changes in bet-hedging strategy more readily than strains that were more homogeneous in nature. The addition of a sub-lethal ethanol stress was used as a primary selective agent for phenotypic heterogeneity analysis and to accelerate the potential for change. Yeast was extracted from the chemostat after 18 days of fermentation and ethanol stress dose response analysis used to deduce phenotypic heterogeneity revealed that prolonged exposure to ethanol had caused strain W34/70 to become more homogeneous in nature with an IC50 value higher than that of the control (more resistant to ethanol). To identify if the extent of the investment in bet-hedging would cause a permanent change within the population, or if it had the potential to revert to 'type', the Day 18 sample was subcultured in nutrient rich media without the pressures of ethanol stress. The phenotypic heterogeneity analysis revealed a shift back towards the typical stress dose-response analysis curve for the strain. It should be noted that the hillslope gradient for this strain appeared to be in-between that observed for stressed (Day 18) and non-stressed cultures (Day 0). However, analysis of the upper limits of tolerance (intercept with the x-axis) revealed that there was no substantial change within the population suggesting it was still in a transitional phase. Therefore, the phenotypic heterogeneity of W34/70 can be described as being 'tuned' to ethanol stress since it was observed to quickly switch based on the precise environmental make-up. This supports previous studies indicating that phenotypic heterogeneity is a selected trait in natural

yeast populations subject to environmental stress (Acar *et al.,* 2008; Holland *et al.,* 2014). Strain W34/70 appears to be innately heterogenous in response to fermentation-related stress factors. It could be argued that the intrinsic adaptability of strain W34/70 is perhaps a direct reason why this strain is so widely used in lager fermentations worldwide.

In summary, this work provides evidence based on the strains studied, that there are complex links between stress tolerance and phenotypic heterogeneity. There is a need for more research into this area so as to understand the molecular basis for phenotypic heterogeneity which would result in possibilities to mediate its control. The role of heterogeneity in stress resistance appears to be regulated by more deterministic mechanisms (Avery, 2006). In the case of the epigenetic regulation of gene expression in isogenic cells it is still unknown as to why some cells are activated and some remain silenced even though they have come into contact with the same environment (Avery, 2006; Halme *et al.,* 2004). Molecular sources of gene expression must also come from cell cycle heterogeneity within the population. In the natural environment it has been found that evolution and selection drive increased phenotypic heterogeneity in adverse conditions (Holland *et al.,* 2014). Human intervention may have reduced the instances of genomic variation in brewing yeast due to selection of cropped yeast from successful fermentations and tight process regulation but the relatively stressful environments which brewing yeast is cycled through in the act of fermentation and serial repitching may increase cell – cell heterogeneity sub-populations develop by bet-hedging strategies put into place by cells which distribute risk coping mechanisms from

mother to daughter cell via asymmetric division (Beaumont *et al.,* 2009; Holland *et al.,* 2014; Ito *et al.,* 2009; Kussell and Leibler, 2005; Levy *et al.,* 2012). As phenotypic heterogeneity enhances the competitiveness of a population, brewers could use the continuous, low stress technique (Chapter 6) to select for populations of highly resistant cells which could be used in more intensive brewing practices such as high gravity or very high gravity brewing. The same technique could also be applied in the search for high performing cells or cells which produce a particular metabolite of interest.

The use of agar plates represented some drawbacks in this study. In order to obtain statistically viable data each data point was sampled in triplicate and the whole set experiments was then sampled in triplicate on separate occasions. This represented a large workload and was time consuming with the maximum number of CFU's which could be reliably counted being in the range of 100-300. The dose response analysis on agar media offers a great first insight into phenotypic heterogeneity in brewing yeast strain but for the consequences to be precisely monitored techniques such as the use of fluorescent gene targets and flow cytometry would allow for the monitoring of thousands of cells at once in a more precise manner. This would help further the understanding of how yeast interact with their environment and each other. In the case of the morphological variant analysis, it would have been interesting to test the individual variants as well as the 'parent' strain under brewery related stresses to ascertain if there were any other phenotypic traits which could be linked to the variance in morphology.

The use of the chemostat to induce low level ethanol stress for an extended period of time (Chapter 6) revealed insights into the relationship between the stresses of fermentation and phenotypic heterogeneity which would not have been present in smaller scale lab-bench fermentations. To further explore this relationship, I suggest that yeast samples taken from a brewery would be the final step in exploring the nature of heterogeneity in brewing yeast. In this case it would be interesting to compare smaller scale breweries to larger and also see if yeast storage procedures/timescales are another root for increased heterogeneity in brewing yeast.

7.2 Future work

The next steps I would like to take to expand on the findings in this research is to look at the relationship between cell divisional age and population heterogeneity. Specifically, I would isolate new daughter cells via flow cytometry and cell sorting technology to determine the intrinsic variation in stress resistance when compared to mixed age yeast populations. This will reveal whether or not daughter cells inherit the same properties as parental cells. Previous research has found that an asymmetric division of metabolites can take place between the mother and daughter cell (Henderson and Gottschling, 2008; Levy *et al.,* 2012) and that daughter cells can differ in their ability to assimilate wort sugars and reduce diacetyl when compared to normal (mixed age) populations (Powell *et al.,* 2003). Given that brewing yeast populations by nature will always comprise of around 50% daughter cells, this information would be highly relevant in exploring potential reasons and cellular

targets involved in viability loss relating to stresses such as fermentation intensification (high/very high gravity brewing), and over the course of serial repitching. An improved understanding of the causes of cell death in different cell types during fermentation may lead to process enhancements which would remove or mitigate stress factors, either by preventing cell damage in the first place or by stimulating cellular repair mechanisms.

There are several other opportunities based on published literature to expand on the data described in the results chapters:

1) Increased ethanol concentrations will impact on brewing yeast health if high/very high gravity brewing procedures are employed. Ethanol is an active membrane solvent and its mutagenic effects have been attributed to mitochondrial membrane alterations leading to mtDNA loss (Castrejón *et al.,* 2002). Mitochondrial function is important for, amongst other things, the final step in pyruvate oxidation from the metabolism of glucose yielding intermediates of the TCA cycle which go on to produce new cell material and produce energy in the form of ATP (Ratledge, 1991). Previous research has shown that the mitochondrial genome is responsible for viability under increased ethanol concentrations (Castrejón *et al.,* 2002; Jiménez and Benítez, 1988) and that mitochondrial activity is likely to be an important eukaryotic variable that drives heterogeneity (Sumner and Avery, 2002; Zeyl and DeVisser, 2001). Therefore, studying the mitochondria in different brewing yeast populations whilst under ethanol stress may reveal some yet undiscovered metabolic drivers behind loss of viability in some cells

which could lead to process changes which would improve yeast viability during intensified brewing practices. A method which could potentially measure heterogeneity within a population would be to assess the mitochondrial membrane potential (a measure of membrane integrity) via the use of rhodamine 123 (Rh123) and flow cytometry (Ludovico *et al.,* 2001).

2) Oxidative damage to cellular constituents occurs during aerobic respiration from the formation of free radicals (Jamieson, 1998; Powell *et al.,* 2000). Cells exhibit antioxidant defences in order to mitigate this damage with superoxide dismutase (SOD) enzymes such as MnSOD being found to be essential for healthy aerobic life (Gutteridge and Halliwell, 2000) with upregulation also being associated with increased ethanol tolerance (Zyrina *et al.,* 2017). It would be interesting to investigate the role of MnSOD in brewing yeast populations and ascertain if and why in levels of steady-state oxidative damage to DNA and lipids is there variation between individuals? (Halliwell, 2009). Any subpopulations of cells which are able to upregulate MnSOD could be selected for to produce a more homogeneous population of cells which are more tolerant to oxidative and ethanol stress.
3) The first metabolite of ethanol production, acetaldehyde, can induce severe DNA damage (Ristow *et al.,* 1995) and negatively impacts on flavour stability in high concentrations (Wang *et al.,* 2013). In addition to being an off-flavour compound, acetaldehyde affects beer staling and therefore reduces shelf-life (Saison *et al.,* 2009; Vanderhaegen *et al.,*

2006). Previously a strain with low acetaldehyde production was developed by UV irradiation induced mutagenesis (Wang *et al.,* 2013) however, heterogeneity within current brewing strains may provide subpopulations of low acetaldehyde production. A heterogeneity screen could be used to select for these cells with the potential of improving flavour stability in beer.

4) Variation in the flocculation potential of brewing yeast has been found to occur during serial repitching (Barker and Smart, 1996; Powell *et al.,* 2003). Flocculation of brewing yeast at the end of a fermentation is of huge importance in industrial beer production with unexpected changes resulting in increased processing and costs. The use of florescent stains which target flocculin encoding genes (Mulders *et al.,* 2010; Verstrepen *et al.,* 2003) and other genes associated with fermentation performance (such as FLO1, HXK1 and MAL4) (Powell and Diacetis, 2007) will offer insights as to what brewery related stresses cause alterations in gene activity.

Of course, heterogeneity will be present in other brewing process 'ingredients'. Individual grain analyses found the inherent biological variation in germinating barley seeds (Kleinwächter *et al.,* 2014) and substantial intraspecific variation in the vegetative morphology of hop plants cannot be attributed to the same levels of genomic variation (Pillay and Kenny, 1996). Further research into the fundamentals of phenotypic heterogeneity could therefore have additional benefits beyond improving brewing yeast alone. Indeed, the benefits are far reaching beyond the world of brewing, the amount of food spoilage could be

vastly reduced with improved understanding of persister cells and the correct use of preservatives (Stratford *et al.,* 2014), other biotechnological industries could apply this knowledge to improve production of industrially, medically and agriculturally useful products, pathogenicity studies may be able to ascertain links between why some cells become resistant to drugs (LaFleur *et al.,* 2006) and gene expression/phenotypic switching studies could lead to new treatments or materials which prevent biofilm formation which could also have industrial and medical benefits.

References

Acar, M., Mettetal, J.T. and Van Oudenaarden, A., 2008. Stochastic switching as a survival strategy in fluctuating environments. *Nature Genetics*, 40(4), p.471.

Ackermann, M., 2015. A functional perspective on phenotypic heterogeneity in microorganisms. *Nature Reviews Microbiology*, 13(8), p.497.

Adams, J., Puskas-Rozsa, S., Simlar, J. and Wilke, C.M., 1992. Adaptation and major chromosomal changes in populations of Saccharomyces cerevisiae. *Current Genetics*, 22(1), p.13-19.

Alba-Lois, L. and Segal-Kischinevzky, C., 2008. Beer & wine makers. *Nature*.

Alexandre, H., Plourde, L., Charpentier, C. and François, J., 1998. Lack of correlation between trehalose accumulation, cell viability and intracellular acidification as induced by various stresses in Saccharomyces cerevisiae. *Microbiology*, 144(4), p.1103-1111

Attfield, P.V., Choi, H.Y., Veal, D.A. and Bell, P.J., 2001. Heterogeneity of stress gene expression and stress resistance among individual cells of Saccharomyces cerevisiae. *Molecular Microbiology*, 40(4), p.1000-1008.

Avery, S.V., 2001. Metal toxicity in yeasts and the role of oxidative stress. *Advances in Applied Microbiology*, 49, p.111-142.

Avery, S.V., 2006. Microbial cell individuality and the underlying sources of heterogeneity. *Nature Reviews Microbiology*, 4(8), p.577.

Babazadeh, R., Adiels, C.B., Smedh, M., Petelenz-Kurdziel, E., Goksör, M. and Hohmann, S., 2013. Osmostress-induced cell volume loss delays yeast Hog1 signalling by limiting diffusion processes and by Hog1-specific effects. *PLoS One*, 8(11).

Baker, E., Wang, B., Bellora, N., Peris, D., Hulfachor, A.B., Koshalek, J.A., Adams, M., Libkind, D. and Hittinger, C.T., 2015. The genome sequence of Saccharomyces eubayanus and the domestication of lager-brewing yeasts. *Molecular Biology and Evolution*, 32(11), p.2818-2831.

Bamforth, C.W., 2008. Wort composition and beer quality. *Brewing Yeast Fermentation Performance*, p.77-84. Blackwell Science, Oxford.

Bandara, A., Fraser, S., Chambers, P.J. and Stanley, G.A., 2009. Trehalose promotes the survival of Saccharomyces cerevisiae during lethal ethanol stress but does not influence growth under sublethal ethanol stress. *FEMS yeast research*, 9(8), p.1208-1216.

Barker, M.G. and Smart, K.A., 1996. Morphological changes associated with the cellular aging of a brewing yeast strain. *Journal of the American Society of Brewing Chemists*, 54(2), p.121-126.

Bartholomew, J.W. and Mittwer, T., 1953. Demonstration of yeast bud scars with the electron microscope. *Journal of Bacteriology*, 65(3), p.272.

Bayly, J.C., Douglas, L.M., Pretorius, I.S., Bauer, F.F. and Dranginis, A.M., 2005. Characteristics of Flo11-dependent flocculation in Saccharomyces cerevisiae. *FEMS yeast research*, 5(12), p.1151-1156.

Beaumont, H.J., Gallie, J., Kost, C., Ferguson, G.C. and Rainey, P.B., 2009. Experimental evolution of bet hedging. *Nature*, 462(7269), p.90.

Berry, D.B. and Gasch, A.P., 2008. Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. *Molecular Biology of the Cell*, 19(11), p.4580-4587.

Berry, D.R. and Slaughter, J.C., 2003. Alcoholic beverage fermentations. *Fermented Beverage Production*. p.25-39. Springer, Boston, MA.

Bing, J., Han, P.J., Liu, W.Q., Wang, Q.M. and Bai, F.Y., 2014. Evidence for a Far East Asian origin of lager beer yeast. *Current Biology*, 24(10), p.380-381.

Bishop, A.L., Rab, F.A., Sumner, E.R. and Avery, S.V., 2007. Phenotypic heterogeneity can enhance rare-cell survival in 'stress-sensitive' yeast populations. *Molecular Microbiology*, 63(2), p.507-520.

Bisson, L.F., 1999. Stuck and sluggish fermentations. *American Journal of Enology and Viticulture*, 50(1), p.107-119.

Blake, W.J., Balázsi, G., Kohanski, M.A., Isaacs, F.J., Murphy, K.F., Kuang, Y., Cantor, C.R., Walt, D.R. and Collins, J.J., 2006. Phenotypic consequences of promoter-mediated transcriptional noise. *Molecular Cell*, 24(6), p.853-865.

Bódi, Z., Farkas, Z., Nevozhay, D., Kalapis, D., Lázár, V., Csörgő, B., Nyerges, Á., Szamecz, B., Fekete, G., Papp, B. and Araújo, H., 2017. Correction: Phenotypic heterogeneity promotes adaptive evolution. *PLoS Biology*, 15(6).

Bogaert, I.N.V, Groeneboer, S., Saerens, K. and Soetaert, W., 2011. The role of cytochrome P450 monooxygenases in microbial fatty acid metabolism. *The FEBS journal*, 278(2), p.206-221.

Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J. and Lindquist, S., 1989. hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Molecular and Cellular Biology*, *9*(9), p.3919-3930.

Borneman, A.R., Desany, B.A., Riches, D., Affourtit, J.P., Forgan, A.H., Pretorius, I.S., Egholm, M. and Chambers, P.J., 2011. Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of Saccharomyces cerevisiae. *PLoS Genetics*, *7*(2).

Boulton, C. and Quain, D., 2006. Brewing Yeast and Fermentation. *John Wiley & Sons.*

Boulton, C.A., 1991. Yeast management and the control of brewery fermentations. *Brew. Guardian*, 120(3), p.25-29.

Boulton, C.A., 2015. Advances in metabolic engineering of yeasts. *Brewing Microbiology*. p.47-64.

Box, W., Bendiak, D., Castonguay, L., Feliciano, S., Fischborn, T., Gibson, B., Kuenker, M., Lodolo, B., Miller, M., Nicholls, S. and Thiele, F., 2012. Differentiation of Ale and Lager Yeast Strains by Rapid X-alpha-Gal Analysis. *Journal of the American Society of Brewing Chemists*, 70(4), p.313-315.

Brady, G., 2000. Expression profiling of single mammalian cells–small is beautiful. *International Journal of Genomics*, 1(3), p.211-217.

Briggs, D.E., Brookes, P.A., Stevens, R. and Boulton, C.A., 2004. *Brewing: Science and Practice*. Elsevier.

Briggs, D.E., Hough, J.S., Stevens, R. and Young, T.W., 1981. The biochemistry of malting grain. *Malting and Brewing Science*, 1, p.57-107.

Briggs, D.E., Hough, J.S., Stevens, R. and Young, T.W., 1982. *Malting and Brewing Science: Hopped Wort and Beer* (Vol. 2). Springer Science & Business Media.

Byrnes, J.K., Morris, G.P. and Li, W.H., 2006. Reorganization of adjacent gene relationships in yeast genomes by whole-genome duplication and gene deletion. *Molecular Biology and Evolution*, 23(6), p.1136-1143.

Cahill, G., Murray, D.M., Walsh, P.K. and Donnelly, D., 2000. Effect of the concentration of propagation wort on yeast cell volume and fermentation performance. *Journal of the American Society of Brewing Chemists*, 58(1), p.14-20.

Cahill, G., Walsh, P.K. and Donnelly, D., 1999. Improved control of brewery yeast pitching using image analysis. *Journal of the American Society of Brewing Chemists*, 57(2), p.72-78.

Carlquist, M., Fernandes, R.L., Helmark, S., Heins, A.L., Lundin, L., Sørensen, S.J., Gernaey, K.V. and Lantz, A.E., 2012. Physiological heterogeneities in microbial populations and implications for physical stress tolerance. *Microbial Cell Factories*, 11(1), p.94.

Carrasco, P. and Querol, A., 2001. Analysis of the stress resistance of commercial wine yeast strains. *Archives of Microbiology*, 175(6), p.450-457.

Casey, P.G., 1996. Practical applications of pulsed field electrophoresis and yeast chromosome fingerprinting in brewing QA and R&D. *Tech. Q.-Master Brew. Assoc. Am.*, 33, p.1-10.

Castrejón, F., Codón, A.C., Cubero, B. and Benítez, T., 2002. Acetaldehyde and ethanol are responsible for mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) in flor yeasts. *Systematic and Applied Microbiology*, 25(3), p.462-467.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S. and Young, R.A., 2001. Remodeling of yeast genome expression in response to environmental changes. *Molecular Biology of the Cell*, 12(2), p.323-337.

Chatterjee, M.T., Khalawan, S.A. and Curran, B.P., 2000. Cellular lipid composition influences stress activation of the yeast general stress response element (STRE). *Microbiology*, 146(4), p.877-884.

Costa, V. and Moradas-Ferreira, P., 2001. Oxidative stress and signal transduction in Saccharomyces cerevisiae: insights into ageing, apoptosis and diseases. *Molecular Aspects of Medicine*, 22(4-5), p.217-246.

Costa, V., Reis, E., Quintanilha, A. and Moradasferreira, P., 1993. Acquisition of ethanol tolerance in Saccharomyces cerevisiae: the key role of the mitochondrial superoxide dismutase. *Archives of Biochemistry and Biophysics*, 300(2), p.608-614.

D'Amore, T., 1992. Cambridge prize lecture improving yeast fermentation performance. *Journal of the Institute of Brewing*, 98(5), p.375-382.

Dahout-Gonzalez, C., Nury, H., Trézéguet, V., Lauquin, G.M., Pebay-Peyroula, E.A. and Brandolin, G., 2006. Molecular, functional, and pathological aspects of the mitochondrial ADP/ATP carrier. *Physiology*, 21(4), p.242-249.

Dashko, S., Zhou, N., Compagno, C. and Piškur, J., 2014. Why, when, and how did yeast evolve alcoholic fermentation? *FEMS yeast research*, 14(6), p.826-832.

de Barros Lopes, M., Soden, A., Martens, A.L., Henschke, P.A. and Langridge, P., 1998. Differentiation and species identification of yeasts using PCR. *International Journal of Systematic and Evolutionary Microbiology*, 48(1), p.279-286.

De Deken, R.H., 1966. The Crabtree effect: a regulatory system in yeast. *Microbiology*, *44*(2), p.149-156.

de Vries, A.R.G., Pronk, J.T. and Daran, J.M.G., 2017. Industrial relevance of chromosomal copy number variation in Saccharomyces yeasts. *Appl. Environ. Microbiol.*, *83*(11), e03206-16.

Dikicioglu, D., Pir, P. and Oliver, S.G., 2013. Predicting complex phenotype–genotype interactions to enable yeast engineering: Saccharomyces cerevisiae as a model organism and a cell factory. *Biotechnology Journal*, 8(9), p.1017-1034.

Dunn, B. and Sherlock, G., 2008. Reconstruction of the genome origins and evolution of the hybrid lager yeast Saccharomyces pastorianus. *Genome Research*.

Egilmez, N.K., Chen, J.B. and Jazwinski, S.M., 1989. Specific alterations in transcript prevalence during the yeast life span. *Journal of Biological Chemistry*, 264(24), p.14312-14317.

Egilmez, N.K., Chen, J.B. and Jazwinski, S.M., 1990. Preparation and partial characterization of old yeast cells. *Journal of Gerontology*, 45(1), p.9-17.

Engel, S.R., Dietrich, F.S., Fisk, D.G., Binkley, G., Balakrishnan, R., Costanzo, M.C., Dwight, S.S., Hitz, B.C., Karra, K., Nash, R.S. and Weng, S., 2014. The reference

genome sequence of Saccharomyces cerevisiae: then and now. *G3: Genes, Genomes, Genetics*, 4(3), p.389-398.

Escribá, P.V., González-Ros, J.M., Goñi, F.M., Kinnunen, P.K., Vigh, L., Sánchez-Magraner, L., Fernández, A.M., Busquets, X., Horváth, I. and Barceló-Coblijn, G., 2008. Membranes: a meeting point for lipids, proteins and therapies. *Journal of Cellular and Molecular Medicine*, 12(3), p.829-875.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8 S rRNA gene and the two ribosomal internal transcribed spacers. *International Journal of Systematic and Evolutionary Microbiology*, 49(1), p.329-337.

Fahy, E., Cotter, D., Sud, M. and Subramaniam, S., 2011. Lipid classification, structures and tools. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1811*(11), p.637-647.

Fiechter, A., Fuhrmann, G.F. and Käppeli, O., 1981. Regulation of glucose metabolism in growing yeast cells. *Advances in Microbial Physiology*, 22, p.123-183. Academic Press.

Fischer, E. and Sauer, U., 2005. Large-scale in vivo flux analysis shows rigidity and suboptimal performance of Bacillus subtilis metabolism. *Nature Genetics*, 37(6), p.636.

Fleming, J.E., Walton, J.K., Dubitsky, R. and Bensch, K.G., 1988. Aging results in an unusual expression of Drosophila heat shock proteins. *Proceedings of the National Academy of Sciences*, 85(11), p.4099-4103.

Gabriel, W., Lynch, M. and Bürger, R., 1993. Muller's ratchet and mutational meltdowns. *Evolution*, 47(6), p.1744-1757.

Galitski, T., Saldanha, A.J., Styles, C.A., Lander, E.S. and Fink, G.R., 1999. Ploidy regulation of gene expression. *Science*, 285(5425), p.251-254.

Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., Merlevede, A., Roncoroni, M., Voordeckers, K., Miraglia, L. and Teiling, C., 2016. Domestication and divergence of Saccharomyces cerevisiae beer yeasts. *Cell*, 166(6), p.1397-1410.

Gallone, B., Mertens, S., Gordon, J.L., Maere, S., Verstrepen, K.J. and Steensels, J., 2018. Origins, evolution, domestication and diversity of Saccharomyces beer yeasts. *Current opinion in biotechnology*, *49*, p.148-155.

Garfinkel, D.J., 2005. Genome evolution mediated by Ty elements in Saccharomyces. *Cytogenetic and Genome Research*, 110(1-4), p.63-69.

Gasch, A.P. and Werner-Washburne, M., 2002. The genomics of yeast responses to environmental stress and starvation. *Functional & Integrative Genomics*, 2(4-5), p.181-192.

Gasch, A.P., 2003. The environmental stress response: a common yeast response to diverse environmental stresses. *Yeast Stress Responses*, p.11-70. Springer, Berlin, Heidelberg.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O., 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell*, 11(12), p.4241-4257.

Gibson, B. and Liti, G., 2015. Saccharomyces pastorianus: genomic insights inspiring innovation for industry. *Yeast*, 32(1), p.17-27.

Gibson, B., Geertman, J., Hittinger, C.T., Krogerus, K., Libkind, D., Louis, E.J., Magalhães, F. and Sampaio, J.P., 2017. New yeasts—new brews: modern approaches to brewing yeast design and development. *FEMS Yeast Research*, 17(4).

Gibson, B.R., Boulton, C.A., Box, W.G., Graham, N.S., Lawrence, S.J., Linforth, R.S. and Smart, K.A., 2008. Carbohydrate utilization and the lager yeast transcriptome during brewery fermentation. *Yeast*, 25(8), p.549-562.

Gibson, B.R., Lawrence, S.J., Leclaire, J.P., Powell, C.D. and Smart, K.A., 2007. Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiology Reviews*, 31(5), p.535-569.

Gibson, B.R., Prescott, K.A. and Smart, K.A., 2008. Petite mutation in aged and oxidatively stressed ale and lager brewing yeast. *Letters in Applied Microbiology*, 46(6), p.636-642.

Gibson, B.R., Storgårds, E., Krogerus, K. and Vidgren, V., 2013. Comparative physiology and fermentation performance of Saaz and Frohberg lager yeast strains and the parental species Saccharomyces eubayanus. *Yeast*, 30(7), p.255-266.

Gilliland, R.B., 1962. Yeast reproduction during fermentation. *Journal of the Institute of Brewing*, *68*(3), p.271-275.

Gilliland, R.B., 1971. Yeast classification. *Journal of the Institute of Brewing*, *77*(3), p.276-284

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M. and Louis, E.J., 1996. Life with 6000 genes. *Science*, 274(5287), p.546-567.

Gorter de Vries, A.R., Voskamp, M.A., van Aalst, A.C., Kristensen, L.H., Jansen, L., Van den Broek, M., Salazar, A.N., Brouwers, N., Abeel, T., Pronk, J.T. and Daran, J.M.G., 2019. Laboratory Evolution of a Saccharomyces cerevisiae× S. eubayanus Hybrid Under Simulated Lager-Brewing Conditions. *Frontiers in genetics*, *10*, p.242.

Gonçalves, M., Pontes, A., Almeida, P., Barbosa, R., Serra, M., Libkind, D., Hutzler, M., Gonçalves, P. and Sampaio, J.P., 2016. Distinct domestication trajectories in top-fermenting beer yeasts and wine yeasts. *Current Biology*, 26(20), p.2750-2761.

González, C., Ray, J.C.J., Manhart, M., Adams, R.M., Nevozhay, D., Morozov, A.V. and Balázsi, G., 2015. Stress-response balance drives the evolution of a network module and its host genome. *Molecular Systems Biology*, 11(8), p.827.

Goossens, K.V., Stassen, C., Stals, I., Donohue, D.S., Devreese, B., De Greve, H. and Willaert, R.G., 2011. The N-terminal domain of the Flo1 flocculation protein from Saccharomyces cerevisiae binds specifically to mannose carbohydrates. *Eukaryotic Cell*, 10(1), p.110-117.

Guillamón Navarro, J.M. and Barrio Esparducer, E., 2017. Genetic polymorphism in wine yeasts: mechanisms and methods for its detection. *Frontiers in Microbiology,* 8, p.806.

Gutteridge, J.M. and Halliwell, B., 2000. Free radicals and antioxidants in the year 2000: a historical look to the future. *Annals of the New York Academy of Sciences*, *899*(1), p.136-147.

Hall, J.F., 1971. Detection of wild yeasts in the brewery. *Journal of the Institute of Brewing*, 77(6), p.513-516.

Halliwell, B., 1999. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). *Free Radical Research*, 31(4), p.261-272.

Halliwell, B., 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiology*, 141(2), p.312-322.

Halme, A., Bumgarner, S., Styles, C. and Fink, G.R., 2004. Genetic and epigenetic regulation of the FLO gene family generates cell-surface variation in yeast. *Cell*, 116(3), p.405-415.

Hammond, J.R., 1993. Brewer's yeast. *The Yeasts*, p.7-67.

Hammond, J.R., 1995. Genetically-modified brewing yeasts for the 21st century. Progress to date. *Yeast*, 11(16), p.1613-1627.

Hammond, J.R., 1996. Yeast genetics. *Brewing Microbiology*. p.43-82. Springer, Boston, MA.

Hammond, J.R., 2000. Yeast growth and nutrition. *Brewing Yeast Fermentation Performance*, p.77-85.

Hatfield E, Barbero F, Bendiak D, *et al.,* 1988. Report of subcommittee on improved microscopic yeast cell counting. *Journal of the American Society of Brewing Chemists.* 46. p.123-125.

Hayflick, L., 1965. The limited in vitro lifetime of human diploid cell strains. *Experimental Cell Research*, 37(3), p.614-636.

Henderson, K.A. and Gottschling, D.E., 2008. A mother's sacrifice: what is she keeping for herself? *Current opinion in Cell Biology*, 20(6), p.723-728.

Hewitt, S.K., Foster, D.S., Dyer, P.S. and Avery, S.V., 2016. Phenotypic heterogeneity in fungi: importance and methodology. *Fungal Biology Reviews*, 30(4), p.176-184.

Hohmann, S., 2002. Osmotic stress signalling and osmoadaptation in yeasts. *Microbiology and Molecular Biology Reviews*, 66(2), p.300-372.

Holland, S.L., Reader, T., Dyer, P.S. and Avery, S.V., 2014. Phenotypic heterogeneity is a selected trait in natural yeast populations subject to environmental stress. *Environmental Microbiology*, 16(6), p.1729-1740.

Honigberg, S.M., 2011. Cell signals, cell contacts, and the organization of yeast communities. *Eukaryotic Cell*. 10, p.466-473.

Howlett, N.G. and Avery, S.V., 1999. Flow cytometric investigation of heterogeneous copper-sensitivity in asynchronously grown Saccharomyces cerevisiae. *FEMS Microbiology Letters*, 176(2), pp.379-386.

Hulse, G., Bihl, G., Morakile, G., Axcell, B. and Smart, K., 2000. Optimisation of storage and propagation for consistent lager fermentations. *Brewing Yeast Fermentation Performance,* p.161-169. Oxford, Blackwell Science.

Hulse, G.A., 2003. Yeast propagation. *Brewing Yeast Fermentation Performance*, p.249-256.

Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. eds., 2012. *PCR protocols: a guide to methods and applications*. Academic Press.

Iserentant, D., Geenens, W. & Verachtert, H., 1996. Titrated Acidification Power: A Simple and Sensitive Method to Measure Yeast Vitality and its Relation to

Other Vitality Measurements, *Journal of the American Society of Brewing Chemists*, 54:2, p.110-114

Ito, Y., Toyota, H., Kaneko, K. and Yomo, T., 2009. How selection affects phenotypic fluctuation. *Molecular Systems Biology*, 5(1), p.264.

James, T.C., Usher, J., Campbell, S. and Bond, U., 2008. Lager yeasts possess dynamic genomes that undergo rearrangements and gene amplification in response to stress. *Current Genetics*, 53(3), p.139-152.

Jamieson, D.J., 1998. Oxidative stress responses of the yeast Saccharomyces cerevisiae. *Yeast*, 14(16), p.1511-1527.

Jenkins, C.L., Kennedy, A.I., Hodgson, J.A., Thurston, P. and Smart, K.A., 2003. Impact of serial repitching on lager brewing yeast quality. *Journal of the American Society of Brewing Chemists*, 61(1), p.1-9.

Jenkins, C.L., Kennedy, A.I., Thurston, P., Hodgson, J.A. and Smart, K.A., 2008. Serial Repitching Fermentation Performance and Functional Biomarkers. *Brewing Yeast Fermentation Performance*, p.257.

Jimenez, J., Longo, E. and Benitez, T., 1988. Induction of petite yeast mutants by membrane-active agents. *Applied and Environmental Microbiology*, 54(12), p.3126-3132.

Kale, S.P. and Jazwinski, S.M., 1996. Differential response to UV stress and DNA damage during the yeast replicative life span. *Developmental Genetics*, 18(2), p.154-160.

Kandror, O., Bretschneider, N., Kreydin, E., Cavalieri, D. and Goldberg, A.L., 2004. Yeast adapt to near-freezing temperatures by STRE/Msn2, 4-dependent induction of trehalose synthesis and certain molecular chaperones. *Molecular Cell*, 13(6), p.771-781.

Kassen, R., 2002. The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology*, 15(2), p.173-190.

Kennedy, A. and Smart, K., 2000. Yeast handling in the brewery. *Brewing Yeast Fermentation Performance*, p.129-134.

Kerby, C. and Vriesekoop, F., 2017. An overview of the utilisation of brewery by-products as generated by British craft breweries. *Beverages*, 3(2), p.24.

Kleinwächter, M., Müller, C., Methner, F.J. and Selmar, D., 2014. Biochemical heterogeneity of malt is caused by both biological variation and differences in processing: I. Individual grain analyses of biochemical parameters in differently steeped barley (Hordeum vulgare L.) malts. *Food Chemistry*, 147, p.25-33.

Klug, L. and Daum, G., 2014. Yeast lipid metabolism at a glance. *FEMS yeast research*, 14(3), p.369-388.

Knudsen, F.B., 1985. Fermentation variables and their control. *Journal of the American Society of Brewing Chemists*, 43(2), p.91-95.

Krogerus, K. and Gibson, B.R., 2013. 125th anniversary review: diacetyl and its control during brewery fermentation. *Journal of the Institute of Brewing*, 119(3), p.86-97.

Kumar, A. and Snyder, M., 2001. Emerging technologies in yeast genomics. *Nature Reviews Genetics*, 2(4), p.302.

Kunz, B.A., Ramachandran, K. and Vonarx, E.J., 1998. DNA sequence analysis of spontaneous mutagenesis in Saccharomyces cerevisiae. *Genetics*, 148(4), p.1491-1505.

Kuřec, M., Baszczyński, M., Lehnert, R., Mota, A., Teixeira, J.A. and Brányik, T., 2009. Flow cytometry for age assessment of a yeast population and its application in beer fermentations. *Journal of the Institute of Brewing*, 115(3), p.253-258.

Kurtzman, C.P. and Robnett, C.J., 1991. Phylogenetic relationships among species of Saccharomyces, Schizosaccharomyces, Debaryomyces and Schwanniomyces determined from partial ribosomal RNA sequences. *Yeast*, 7(1), p.61-72.

Kussell, E. and Leibler, S., 2005. Phenotypic diversity, population growth, and information in fluctuating environments. *Science*, 309(5743), p.2075-2078.

LaFleur, M.D., Kumamoto, C.A. and Lewis, K., 2006. Candida albicans biofilms produce antifungal-tolerant persister cells. *Antimicrobial agents and chemotherapy*, 50(11), p.3839-3846.

Landolfo, S., Politi, H., Angelozzi, D. and Mannazzu, I., 2008. ROS accumulation and oxidative damage to cell structures in Saccharomyces cerevisiae wine strains during fermentation of high-sugar-containing medium. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1780(6), p.892-898.

Latterich, M. and Watson, M.D., 1993. Evidence for a dual osmoregulatory mechanism in the yeast Saccharomyces cerevisiae. *Biochemical and Biophysical Research Communications*, 191(3), p.1111-1117.

Lawrence, S.J., Nicholls, S., Box, W.G., Sbuelz, R., Bealin-Kelly, F., Axcell, B. and Smart, K.A., 2013. The Relationship between Yeast Cell Age, Fermenter Cone Environment, and Petite Mutant Formation in Lager Fermentations. *Journal of the American Society of Brewing Chemists*, 71(2), p.90-96.

Lees, N.D., Skaggs, B., Kirsch, D.R. and Bard, M., 1995. Cloning of the late genes in the ergosterol biosynthetic pathway of Saccharomyces cerevisiae—A review. *Lipids*, 30(3), p.221-226.

Legras, J.L. and Karst, F., 2003. Optimisation of interdelta analysis for Saccharomyces cerevisiae strain characterisation. *FEMS microbiology letters*, 221(2), p.249-255.

Lentini, A., Rogers, P., Higgins, V., Dawes, I., Chandler, M., Stanley, G., Chambers, P. and Smart, K., 2003. The impact of ethanol stress on yeast physiology. *Brewing Yeast Fermentation Performance*, p.25-38.

Levic, J., Djuragic, O. and Sredanovic, S., 2010. Use of new feed from brewery by-products for breeding layers. *Romanian Biotechnological Letters*, 15(5), p.5559-5565.

Levy, S.F., Ziv, N. and Siegal, M.L., 2012. Bet hedging in yeast by heterogeneous, age-correlated expression of a stress protectant. *PLoS Biology*, 10(5).

Lewis, J.A., Elkon, I.M., McGee, M.A., Higbee, A.J. and Gasch, A.P., 2010. Exploiting natural variation in Saccharomyces cerevisiae to identify genes for increased ethanol resistance. *Genetics*, 186(4), p.1197-1205.

Lewis, J.G., Northcott, C.J., Learmonth, R.P., Attfield, P.V. and Watson, K., 1993. The need for consistent nomenclature and assessment of growth phases in diauxic cultures of Saccharomyces cerevisiae. *Microbiology*, 139(4), p.835-839.

Li, L., Miles, S., Melville, Z., Prasad, A., Bradley, G. and Breeden, L.L., 2013. Key events during the transition from rapid growth to quiescence in budding yeast require posttranscriptional regulators. *Molecular Biology of the Cell*, 24(23), p.3697-3709.

Libkind, D., Hittinger, C.T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., Gonçalves, P. and Sampaio, J.P., 2011. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences*.

Lillie, S.H. and Pringle, J.R., 1980. Reserve carbohydrate metabolism in Saccharomyces cerevisiae: responses to nutrient limitation. *Journal of Bacteriology*, 143(3), p.1384-1394.

Lindquist, S. and Craig, E.A., 1988. The heat-shock proteins. *Annual review of Genetics*, 22(1), p.631-677.

Lipinski, K.A., Kaniak-Golik, A. and Golik, P., 2010. Maintenance and expression of the *S. cerevisiae* mitochondrial genome—from genetics to evolution and

systems biology. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1797(6-7), p.1086-1098.

Liu, J., Martin-Yken, H., Bigey, F., Dequin, S., François, J.M. and Capp, J.P., 2015. Natural yeast promoter variants reveal epistasis in the generation of transcriptional-mediated noise and its potential benefit in stressful conditions. *Genome Biology and Evolution*, 7(4), p.969-984.

Lodolo, E.J., Kock, J.L., Axcell, B.C. and Brooks, M., 2008. The yeast Saccharomyces cerevisiae–the main character in beer brewing. *FEMS Yeast Research*, 8(7), p.1018-1036.

Lorenz, R.T. and Parks, L.W., 1991. Involvement of heme components in sterol metabolism of Saccharomyces cerevisiae. *Lipids*, 26(8), p.598-603.

Loveridge, D., Ruddlesden, J.D., Noble, C.S. and Quain, D.E., 1999. Improvements in brewery fermentation performance by 'early' yeast cropping and reduced yeast storage time. *Proc. 7th Inst. Brew. Congr. Africa Sect*, p.95-99.

Ludovico, P., Sansonetty, F. and Côrte-Real, M., 2001. Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. *Microbiology*, 147(12), p.3335-3343.

MacLean, M., Harris, N. and Piper, P.W., 2001. Chronological lifespan of stationary phase yeast cells; a model for investigating the factors that might influence the ageing of postmitotic tissues in higher organisms. *Yeast*, 18(6), p.499-509.

Mager, W.H. and Varela, J.C., 1993. Osmostress response of the yeast Saccharomyces. *Molecular Microbiology*, 10(2), p.253-258.

Mansure, J.J., Souza, R.C. and Panek, A.D., 1997. Trehalose metabolism in Saccharomyces cerevisiae during alcoholic fermentation. *Biotechnology letters*, 19(12), p.1201-1203.

Martin, V., Quain, D.E., Smart, K.A., 2008. *Brewing Yeast Fermentation Performance*, Second Edition. p.61–73. John Wiley & Sons.

Martinez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H. and Estruch, F., 1996. The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *The EMBO journal*, 15(9), p.2227-2235.

Meier-Dörnberg, T., Hutzler, M., Michel, M., Methner, F.J. and Jacob, F., 2017. The importance of a comparative characterization of Saccharomyces Cerevisiae and Saccharomyces Pastorianus strains for brewing. *Fermentation*, 3(3), p.41.

Meier-Dörnberg, T., Hutzler, M., Michel, M., Methner, F.J. and Jacob, F., 2017. The importance of a comparative characterization of Saccharomyces Cerevisiae and Saccharomyces Pastorianus strains for brewing. *Fermentation*, 3(3), p.41.

Meilgaard, M.C., 1975. Flavor chemistry of beer. II. Flavor and threshold of 239 aroma volatiles. *Tech. Quart. Master. Brew. Assoc. Am.*, 12, p.151-168.

Miller, K.J., Box, W.G., Boulton, C.A. and Smart, K.A., 2012. Cell cycle synchrony of propagated and recycled lager yeast and its impact on lag phase in fermenter. *Journal of the American Society of Brewing Chemists*, 70(1), p.1-9.

Miller, K.J., Box, W.G., Jenkins, D.M., Boulton, C.A., Linforth, R. and Smart, K.A., 2013. Does generation number matter? The impact of repitching on wort utilization. *Journal of the American Society of Brewing Chemists*, 71(4), p.233-241.

Mináriková, L., Kuthan, M., Řičicová, M., Forstová, J. and Palková, Z., 2001. Differentiated gene expression in cells within yeast colonies. *Experimental Cell Research*, 271(2), p.296-304.

Miyazaki, S., Nevo, E. and Bohnert, H.J., 2004. Adaptive oxidative stress in yeast Saccharomyces cerevisiae: interslope genetic divergence in 'Evolution Canyon'. *Biological Journal of the Linnean Society*, 84(1), p.103-117.

Mizushima, N. and Klionsky, D.J., 2007. Protein turnover via autophagy: implications for metabolism. *Annual Review Nutrition*, 27, p.19-40.

Moench, D., Krueger, E. and Stahl, U., 1995. Effects of stress on brewery yeasts. *Monatsschrift fuer Brauwissenschaft (Germany)*.

Monerawela, C. and Bond, U., 2018. The hybrid genomes of Saccharomyces pastorianus: A current perspective. *Yeast*, *35*(1), p.39-50.

Morano, K.A., Grant, C.M. and Moye-Rowley, W.S., 2012. The response to heat shock and oxidative stress in Saccharomyces cerevisiae. *Genetics*, 190(4), p.1157-1195.

Mortimer, R.K. and Johnston, J.R., 1959. Life Span of Individual Yeast Cells. *Nature*, 183, p.1751-1752.

Mortimer, R.K. and Johnston, J.R., 1986. Genealogy of principal strains of the yeast genetic stock centre. *Genetics*, 113(1), p.35-43.

Mott, A.C., 2017. *The relationship between very high gravity fermentations and oxidative stress in the lager yeast Saccharomyces pastorianus* (Doctoral dissertation, University of Nottingham).

Mulders, S.E.V, Ghequire, M., Daenen, L., Verbelen, P.J., Verstrepen, K.J. and Delvaux, F.R., 2010. Flocculation gene variability in industrial brewer's yeast strains. *Applied Microbiology and Biotechnology*, 88(6), p.1321-1331.

Mülleder, M., Capuano, F., Pir, P., Christen, S., Sauer, U., Oliver, S.G. and Ralser, M., 2012. A prototrophic deletion mutant collection for yeast metabolomics and systems biology. *Nature Biotechnology*, 30(12), p.1176.

Muller, H.J., 1964. The relation of recombination to mutational advance. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 1(1), p.2-9.

Müller, I., 1971. Experiments on ageing in single cells of Saccharomyces cerevisiae. *Archiv für Mikrobiologie*, *77*(1), p.20-25.

Musso, G., Costanzo, M., Huangfu, M., Smith, A.M., Paw, J., San Luis, B.J., Boone, C., Giaever, G., Nislow, C., Emili, A. and Zhang, Z., 2008. The extensive and condition-dependent nature of epistasis among whole-genome duplicates in yeast. *Genome Research*.

Nakao, Y., Kanamori, T., Itoh, T., Kodama, Y., Rainieri, S., Nakamura, N., Shimonaga, T., Hattori, M. and Ashikari, T., 2009. Genome Sequence of the Lager Brewing Yeast, an Interspecies Hybrid. *DNA Research: An International Journal for Rapid Publication of Reports on Genes and Genomes*, 16(2), p.115.

New, A.M., Cerulus, B., Govers, S.K., Perez-Samper, G., Zhu, B., Boogmans, S., Xavier, J.B. and Verstrepen, K.J., 2014. Different levels of catabolite repression optimize growth in stable and variable environments. *PLoS Biology*, 12(1).

O'Connor-Cox, E., 1997. Improving yeast handling in the brewery, part 1: yeast cropping. *Brew. Guardian*, 126(12), p.26-34.

Ocampo, A., Liu, J., Schroeder, E.A., Shadel, G.S. and Barrientos, A., 2012. Mitochondrial respiratory thresholds regulate yeast chronological life span and its extension by caloric restriction. *Cell Metabolism*, 16(1), p.55-67.

Olaniran, A.O., Hiralal, L., Mokoena, M.P. and Pillay, B., 2017. Flavour-active volatile compounds in beer: production, regulation and control. *Journal of the Institute of Brewing*, 123(1), p.13-23.

Otero, J.M., Vongsangnak, W., Asadollahi, M.A., Olivares-Hernandes, R., Maury, J., Farinelli, L., Barlocher, L., Østerås, M., Schalk, M., Clark, A. and Nielsen, J., 2010. Whole genome sequencing of Saccharomyces cerevisiae: from genotype to phenotype for improved metabolic engineering applications. *BMC genomics*, 11(1), p.723.

Painting, K. and Kirsop, B., 1990. A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining. *World Journal of Microbiology and Biotechnology*, *6*(3), p.346-347.

Palkova, Z., Janderova, B., Gabriel, J., Zikanova, B., Pospíšek, M. and Forstová, J., 1997. Ammonia mediates communication between yeast colonies. *Nature*, 390(6659), p.532.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology advances*, *22*(3), p.189-259.

Paquin, C.E. and Adams, J., 1983. Relative fitness can decrease in evolving asexual populations of *S. cerevisiae*. *Nature*, 306(5941), p.368.

Pedersen, M.B., 1986. DNA sequence polymorphisms in the genus Saccharomyces III. Restriction endonuclease fragment patterns of chromosomal regions in brewing and other yeast strains. *Carlsberg research communications*, 51(3), p.163-183.

Pedersen, M.B., 1994. Molecular analyses of yeast DNA—Tools for pure yeast maintenance in the brewery. *Journal of the American Society of Brewing Chemists*, 52(1), p.23-27.

Peris Navarro, D., Sylvester, K., Libkind, D., Gonçalves, P., Sampaio, J.P., Alexander, W.G. and Hittinger, C.T., 2014. Population structure and reticulate evolution of Saccharomyces eubayanus and its lager-brewing hybrids. *Molecular Ecology,* 23(8), p.2031-2045.

Pfeiffer, T. and Morley, A., 2014. An evolutionary perspective on the Crabtree effect. *Frontiers in molecular biosciences*, *1*, p.17.

Pham, T., Wimalasena, T., Box, W.G., Koivuranta, K., Storgårds, E., Smart, K.A. and Gibson, B.R., 2011. Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery 'wild'yeast contaminants. *Journal of the Institute of Brewing*, 117(4), p.556-568.

Pickerell, A.T.W., Hwang, A. and Axcell, B.C., 1991. Impact of yeast-handling procedures on beer flavor development during fermentation. *Journal of the American Society of Brewing Chemists*, 49(2), p.87-92.

Pierce, J.S., 1970. Institute of Brewing: analysis committee measurement of yeast viability. *Journal of the Institute of Brewing*, 76(5), p.442-443.

Pillay, M. and Kenny, S.T., 1996. Random amplified polymorphic DNA (RAPD) markers in hop, Humulus lupulus: level of genetic variability and segregation in F1 progeny. *Theoretical and Applied Genetics*, 92(3-4), p.334-339.

Piper, P.W., 1993. Molecular events associated with acquisition of heat tolerance by the yeast Saccharomyces cerevisiae. *FEMS Microbiology Reviews*, 11(4), p.339-355.

Piper, P.W., 1995. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiology Letters*, 134(2-3), p.121-127.

Pires, E.J., Teixeira, J.A., Brányik, T. and Vicente, A.A., 2014. Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology*, 98(5), p.1937-1949.

Piškur, J., Rozpędowska, E., Polakova, S., Merico, A. and Compagno, C., 2006. How did Saccharomyces evolve to become a good brewer? *TRENDS in Genetics*, 22(4), p.183-186.

Powell, C.D. and Diacetis, A.N., 2007. Long term serial repitching and the genetic and phenotypic stability of brewer's yeast. *Journal of the Institute of Brewing*, 113(1), p.67-74.

Powell, C.D., Quain, D.E. and Smart, K.A., 2000. The impact of media composition and petite mutation on the longevity of a polyploid brewing yeast strain. *Letters in Applied Microbiology*, 31(1), p.46-51.

Powell, C.D., Quain, D.E. and Smart, K.A., 2003. The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation. *FEMS Yeast Research*, 3(2), p.149-157.

Powell, C.D., Quain, D.E. and Smart, K.A., 2004. The impact of sedimentation on cone yeast heterogeneity. *Journal of the American Society of Brewing Chemists*, 62(1), p.8-17.

Powell, C.D., Van Zandycke, S.M., Quain, D.E. and Smart, K.A., 2000. Replicative ageing and senescence in Saccharomyces cerevisiae and the impact on brewing fermentations. *Microbiology*, 146(5), p.1023-1034.

Pratt, P.L., Bryce, J.H. and Stewart, G.G., 2003. The effects of osmotic pressure and ethanol on yeast viability and morphology. *Journal of the Institute of Brewing,* 109, p218-228.

Pruyne, D., Legesse-Miller, A., Gao, L., Dong, Y. and Bretscher, A., 2004. Mechanisms of polarized growth and organelle segregation in yeast. *Annual review of Cell and Developmental Biology*, 20, p.559.

Puligundla, P., Smogrovicova, D., Obulam, V.S.R. and Ko, S., 2011. Very high gravity (VHG) ethanolic brewing and fermentation: a research update. *Journal of Industrial Microbiology & Biotechnology*, 38(9), p.1133-1144.

Quain, D.E., Box, W.G. and Walton, E.F., 1985. Inexpensive and simple small-scale laboratory fermenter. *Laboratory Practice*, 34, p.84-5.

Quain, D.E., 1986. Differentiation of brewing yeast. *Journal of the Institute of Brewing*, 92(5), p.435-438.

Quain, D.E. and Boulton, C.A., 1987. Growth and metabolism of mannitol by strains of Saccharomyces cerevisiae. *Microbiology*, 133(7), p.1675-1684.

Quain, D.E., 1988. Studies on yeast physiology-Impact on fermentation performance and product quality. *Journal of the Institute of Brewing*, 94(5), p.315-323.

Quain, D.E., Powell, C.D., Hamilton, A., Ruddlesden, D. and Box, W., 2001. Why warm cropping is best. *Proc Congr Eur Brew Conv*, 28, p.388-395.

Quain, D.E., 2006. Yeast supply and propagation in brewing. *Brewing: New technologies*, p.167-182.

Quain, D.E., 2017. Yeast Supply, Fermentation and Handling–Insights, Best Practice and Consequences of Failure. *Brewing Microbiology*, p.53.

Rachidi, N., Barre, P. and Blondin, B., 1999. Multiple Ty-mediated chromosomal translocations lead to karyotype changes in a wine strain of Saccharomyces cerevisiae. *Molecular and General Genetics MGG*, 261(4-5), p.841-850.

Rainieri, S., Zambonelli, C. and Kaneko, Y., 2003. Saccharomyces sensu stricto: systematics, genetic diversity and evolution. *Journal of Bioscience and Bioengineering*, 96(1), p.1-9.

Rank, G.H., Casey, G. and Xiao, W., 1988. Gene transfer in industrial Saccharomyces yeasts. *Food Biotechnology*, *2*(1), p.1-41.

Ratledge, C., 1991. Yeast physiology—a micro-synopsis. *Bioprocess Engineering*, 6(5), p.195-203.

Reggiori, F. and Klionsky, D.J., 2013. Autophagic processes in yeast: mechanism, machinery and regulation. *Genetics*, 194(2), p.341-361.

Rep, M., Krantz, M., Thevelein, J.M. and Hohmann, S., 2000. The transcriptional response of Saccharomyces cerevisiae to osmotic shock Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *Journal of Biological Chemistry*, 275(12), p.8290-8300.

Reynolds, T.B. and Fink, G.R., 2001. Bakers' yeast, a model for fungal biofilm formation. *Science*, 291(5505), p.878-881.

Richards, M., 1967. The use of giant-colony morphology for the differentiation of brewing yeasts. *Journal of the Institute of Brewing*, 73(2), p.162-166.

Ristow, H., Seyfarth, A. and Lochmann, E.R., 1995. Chromosomal damages by ethanol and acetaldehyde in Saccharomyces cerevisiae as studied by pulsed field gel electrophoresis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 326(2), p.165-170.

Robinow, C.F. and Johnson, B.F., 1991. Yeast cytology: an overview. *The Yeasts*, 4, p.7-120.

Rodgers, D.L., Kennedy, A.I., Hodgson, J.A. and Smart, K.A., 1999. Lager brewing yeast ageing and stress tolerance. *Proceedings of Congress-European Brewery Convention*, 27, p.671-678.

Rolland, F., Winderickx, J. and Thevelein, J.M., 2002. Glucose-sensing and-signalling mechanisms in yeast. *FEMS yeast research*, 2(2), p.183-201.

Ruis, H. and Schüller, C., 1995. Stress signaling in yeast. *Bioessays*, 17(11), p.959-965.

Ryall, B., Eydallin, G. and Ferenci, T., 2012. Culture history and population heterogeneity as determinants of bacterial adaptation: the adaptomics of a single environmental transition. *Microbiology and Molecular Biology Reviews*, 76(3), p.597-625.

Saison, D., De Schutter, D.P., Uyttenhove, B., Delvaux, F. and Delvaux, F.R., 2009. Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds. *Food Chemistry*, 114(4), p.1206-1215.

Sakamoto, K. and Konings, W.N., 2003. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology*, 2(89), p.105-124.

Sato, M., Watari, J. and Shinotsuka, K., 2001. Genetic instability in flocculation of bottom-fermenting yeast. *Journal of the American Society of Brewing Chemists*, *59*(3), p.130-134.

Scannell, D.R., Byrne, K.P., Gordon, J.L., Wong, S. and Wolfe, K.H., 2006. Multiple rounds of speciation associated with reciprocal gene loss in polyploid yeasts. *Nature*, 440(7082), p.341.

Scheda, R. and Yarrow, D., 1966. The instability of physiological properties used as criteria in the taxonomy of yeasts. *Archives of Microbiology*, 55(3), p.209-225.

Scherz, R., Shinder, V. and Engelberg, D., 2001. Anatomical analysis of Saccharomyces cerevisiaestalk-like structures reveals spatial organization and cell specialization. *Journal of Bacteriology*, 183(18), p.5402-5413.

Selmecki, A.M., Maruvka, Y.E., Richmond, P.A., Guillet, M., Shoresh, N., Sorenson, A.L., De, S., Kishony, R., Michor, F., Dowell, R. and Pellman, D., 2015. Polyploidy can drive rapid adaptation in yeast. *Nature*, 519(7543), p.349.

Serrano, R., Martín, H., Casamayor, A. and Ariño, J., 2006. Signaling alkaline pH stress in the yeast Saccharomyces cerevisiae through the Wsc1 cell surface sensor and the Slt2 MAPK pathway. *Journal of Biological Chemistry*, 281(52), p.39785-39795.

Sheltzer, J.M., Blank, H.M., Pfau, S.J., Tange, Y., George, B.M., Humpton, T.J., Brito, I.L., Hiraoka, Y., Niwa, O. and Amon, A., 2011. Aneuploidy drives genomic instability in yeast. *Science*, *333*(6045), p.1026-1030.

Singer, M.A. and Lindquist, S., 1998. Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose. *Trends in Biotechnology*, 16(11), p.460-468.

Skoneczna, A., Kaniak, A. and Skoneczny, M., 2015. Genetic instability in budding and fission yeast—sources and mechanisms. *FEMS Microbiology Reviews*, 39(6), p.917-967.

Slaughter, J.C., 2003. Biochemistry and physiology of yeast growth. *Brewing Microbiology,* p.19-66. Springer, Boston, MA.

Slutsky, B., Buffo, J. and Soll, D.R., 1985. High-frequency switching of colony morphology in Candida albicans. *Science*, 230(4726), p.666-669.

Smart, K.A. and Whisker, S., 1996. Effect of serial repitching on the fermentation properties and condition of brewing yeast. *Journal of the American Society of Brewing Chemists*, 54(1), p.41-44.

Smart, K.A., 2001. Biomarkers of yeast condition predicting fitness to ferment. *Proc. 8th Inst. Brew. Africa Sect. Congr*, p.138-149.

Soares, E.V., 2011. Flocculation in Saccharomyces cerevisiae: a review. *Journal of Applied Microbiology*, 110(1), p.1-18.

Soll, D., 2014. The role of phenotypic switching in the basic biology and pathogenesis of Candida albicans. *Journal of Oral Microbiology*, 6(1), p.22993.

Sombutyanuchit, P., Suphantharika, M. and Verduyn, C., 2001. Preparation of 5'-GMP-rich yeast extracts from spent brewer's yeast. *World Journal of Microbiology and Biotechnology*, 17(2), p.163-168.

Stanley, D., Bandara, A., Fraser, S., Chambers, P.J. and Stanley, G.A., 2010. The ethanol stress response and ethanol tolerance of Saccharomyces cerevisiae. *Journal of Applied Microbiology*, 109(1), p.13-24.

Stanley, D., Bandara, A., Fraser, S., Chambers, P.J. and Stanley, G.A., 2010. The ethanol stress response and ethanol tolerance of Saccharomyces cerevisiae. *Journal of Applied Microbiology*, 109(1), p.13-24.

statista.com. 2018. Lager share of total beer sales in the United Kingdom (UK) from 2002 to 2016. [ONLINE] Available at: https://www.statista.com/statistics/309006/lager-beer-sales-in-the-united-kingdom-uk/. [Accessed 25 March 2018].

Stewart, G.G. and Russell, I., 1986. One hundred years of yeast research and development in the brewing industry. *Journal of the Institute of Brewing*, 92(6), p.537-558.

Stewart, G.G., 2009. The Horace Brown Medal lecture: forty years of brewing research. *Journal of the Institute of Brewing*, 115(1), p.3-29.

Stewart, G.G., Hill, A.E. and Russell, I., 2013. 125th anniversary review: developments in brewing and distilling yeast strains. *Journal of the Institute of Brewing*, 119(4), p.202-220.

Stewart, G.G., 2016. Saccharomyces species in the production of beer. *Beverages*, 2(4), p.34.

Stratford, M., 1992. Yeast flocculation: a new perspective. *Advances in Microbial Physiology*, 33, p.1-71.

Stratford, M., Steels, H., Nebe-von-Caron, G., Avery, S.V., Novodvorska, M. and Archer, D.B., 2014. Population heterogeneity and dynamics in starter culture and lag phase adaptation of the spoilage yeast Zygosaccharomyces bailii to weak acid preservatives. *International Journal of Food Microbiology*, 181, p.40-47.

Sumner, E.R. and Avery, S.V., 2002. Phenotypic heterogeneity: differential stress resistance among individual cells of the yeast Saccharomyces cerevisiae. *Microbiology*, 148(2), p.345-351.

Sumner, E.R., Avery, A.M., Houghton, J.E., Robins, R.A. and Avery, S.V., 2003. Cell cycle-and age-dependent activation of Sod1p drives the formation of stress resistant cell subpopulations within clonal yeast cultures. *Molecular Microbiology*, 50(3), p.857-870.

Symmons, O. and Raj, A., 2016. What's luck got to do with it: single cells, multiple fates, and biological nondeterminism. *Molecular Cell*, 62(5), p.788-802.

Taidi, B., Kennedy, A.I., Hodgson, J.A., 2008. *Brewing Yeast Fermentation Performance*, Second Edition p.86–95. John Wiley & Sons.

Tenbült, P., De Vries, N.K., van Breukelen, G., Dreezens, E. and Martijn, C., 2008. Acceptance of genetically modified foods: the relation between technology and evaluation. *Appetite*, 51(1), p.129-136.

Teunissen, A.W.R.H. and Steensma, H.Y., 1995. The dominant flocculation genes of Saccharomyces cerevisiae constitute a new subtelomeric gene family. *Yeast*, 11(11), p.1001-1013.

Thevelein, J.M., 1994. Signal transduction in yeast. *Yeast*, 10(13), p.1753-1790.

Timmins, É.M., Quain, D.E. and Goodacre, R., 1998. Differentiation of brewing yeast strains by pyrolysis mass spectrometry and Fourier transform infrared spectroscopy. *Yeast*, 14(10), p.885-893.

Tokuriki, N., Stricher, F., Serrano, L. and Tawfik, D.S., 2008. How protein stability and new functions trade off. *PLoS Computational Biology*, 4(2).

Toone, W.M. and Jones, N., 1998. Stress-activated signalling pathways in yeast. *Genes to Cells*, 3(8), p.485-498.

Váchová, L. and Palková, Z., 2005. Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. *The Journal of Cell Biology*, 169(5), p.711-717.

Váchová, L., Čáp, M. and Palková, Z., 2012. Yeast colonies: a model for studies of aging, environmental adaptation, and longevity. *Oxidative Medicine and Cellular Longevity*.

Váchová, L., Kučerová, H., Devaux, F., Úlehlová, M. and Palková, Z., 2009. Metabolic diversification of cells during the development of yeast colonies. *Environmental Microbiology*, 11(2), p.494-504.

Váchová, L., Šťovíček, V., Hlaváček, O., Chernyavskiy, O., Štěpánek, L., Kubínová, L. and Palková, Z., 2011. Flo11p, drug efflux pumps, and the extracellular matrix cooperate to form biofilm yeast colonies. *The Journal of Cell Biology*, 194(5), p.679-687.

Van den Broek, M., Bolat, I., Nijkamp, J.F., Ramos, E., Luttik, M.A., Koopman, F., Geertman, J.M., De Ridder, D., Pronk, J.T. and Daran, J.M., 2015. Chromosomal copy number variation in Saccharomyces pastorianus is evidence for extensive genome dynamics in industrial lager brewing strains. *Appl. Environ. Microbiol.*, *81*(18), p.6253-6267.

Vanderhaegen, B., Neven, H., Verachtert, H. and Derdelinckx, G., 2006. The chemistry of beer aging–a critical review. *Food Chemistry*, 95(3), p.357-381.

van Dijken, J.P. and Scheffers, W.A., 1986. Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiology Letters*, 32(3-4), p.199-224.

Varon, M. and Choder, M., 2000. Organization and cell-cell interaction in starved Saccharomyces cerevisiae colonies. *Journal of Bacteriology*, 182(13), p.3877-3880.

Vasicova, P., Lejskova, R., Malcova, I. and Hasek, J., 2015. The stationary phase cells of *S. cerevisiae* display dynamic actin filaments required for processes extending chronological life span. *Molecular and Cellular Biology*.

Veroli, G.Y.D, Fornari, C., Goldlust, I., Mills, G., Koh, S.B., Bramhall, J.L., Richards, F.M. and Jodrell, D.I., 2015. An automated fitting procedure and software for dose-response curves with multiphasic features. *Scientific Reports*, 5, p.14701.

Verstrepen, K.J., Derdelinckx, G., Dufour, J.P., Winderickx, J., Thevelein, J.M., Pretorius, I.S. and Delvaux, F.R., 2003. Flavor-active esters: adding fruitiness to beer. *Journal of Bioscience and Bioengineering*, 96(2), p.110-118.

Verstrepen, K.J., Derdelinckx, G., Verachtert, H. and Delvaux, F.R., 2003. Yeast flocculation: what brewers should know. *Applied Microbiology and Biotechnology*, 61(3), p.197-205.

Verstrepen, K.J., Iserentant, D., Malcorps, P., Derdelinckx, G., Van Dijck, P., Winderickx, J., Pretorius, I.S., Thevelein, J.M. and Delvaux, F.R., 2004. Glucose and sucrose: hazardous fast-food for industrial yeast? *Trends in Biotechnology*, 22(10), p.531-537.

Verstrepen, K.J., Jansen, A., Lewitter, F. and Fink, G.R., 2005. Intragenic tandem repeats generate functional variability. *Nature Genetics*, 37(9), p.986.

Verstrepen, K.J. and Klis, F.M., 2006. Flocculation, adhesion and biofilm formation in yeasts. *Molecular Microbiology*, 60(1), p.5-15.

Vevea, J.D., Swayne, T.C., Boldogh, I.R. and Pon, L.A., 2014. Inheritance of the fittest mitochondria in yeast. *Trends in Cell Biology*, 24(1), p.53-60.

Voigt, J.C. and Walla, G.A., 1995. A novel yeast propagation system. *Proc Inst Brew (C & SA Sect)*, 5, p.173-178.

Voordeckers, K., De Maeyer, D., van der Zande, E., Vinces, M.D., Meert, W., Cloots, L., Ryan, O., Marchal, K. and Verstrepen, K.J., 2012. Identification of a

complex genetic network underlying Saccharomyces cerevisiae colony morphology. *Molecular Microbiology*, 86(1), p.225-239.

Voordeckers, K., Kominek, J., Das, A., Espinosa-Cantu, A., De Maeyer, D., Arslan, A., Van Pee, M., van der Zande, E., Meert, W., Yang, Y. and Zhu, B., 2015. Adaptation to high ethanol reveals complex evolutionary pathways. *PLoS genetics*, *11*(11), p.e1005635

Wainwright, T., 1973. Diacetyl—a review: Part I—analytical and biochemical considerations: Part II—brewing experience. *Journal of the Institute of Brewing*, 79(6), p.451-470.

Wang, J., Shen, N., Yin, H., Liu, C., Li, Y. and Li, Q., 2013. Development of industrial brewing yeast with low acetaldehyde production and improved flavor stability. *Applied Biochemistry and Biotechnology*, 169(3), p.1016-1025.

Weigert, C., Steffler, F., Kurz, T., Shellhammer, T.H. and Methner, F.J., 2009. Application of a short intracellular pH method to flow cytometry for determining *Saccharomyces cerevisiae* vitality. *Appl. Environ. Microbiol.*, 75(17), p.5615-5620.

Wellman, A.M. and Stewart, G.G., 1973. Storage of brewing yeasts by liquid nitrogen refrigeration. *Applied Microbiology*, 26(4), p.577-583.

Wendland, J., 2014. Lager Yeast Comes of Age. *Eukaryotic Cell*, 13(10), p.1256-1265.

White, P.A., Kennedy, A.I., Smart, K.A., 2008. *Brewing yeast fermentation performance*. John Wiley & Sons.

White, T.J., Bruns, T., Lee, S.J.W.T. and Taylor, J.L., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18(1), p.315-322.

Wiemken, A., 1990. Trehalose in yeast, stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek*, 58(3), p.209-217.

Wightman, P., Quain, D.E. and Meaden, P.G., 1996. Analysis of production brewing strains of yeast by DNA fingerprinting. *Letters in Applied Microbiology*, 22(1), p.90-94.

Wilke, C.M. and Adams, J., 1992. Fitness effects of Ty transposition in Saccharomyces cerevisiae. *Genetics*, 131(1), p.31-42.

Wojda, I., Alonso-Monge, R., Bebelman, J.P., Mager, W.H. and Siderius, M., 2003. Response to high osmotic conditions and elevated temperature in *Saccharomyces cerevisiae* is controlled by intracellular glycerol and involves coordinate activity of MAP kinase pathways. *Microbiology,* 149, p1193-1204.

Wolfe, K.H. and Shields, D.C., 1997. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature*, *387*(6634), p.708.

Xiao, Y., Bowen, C.H., Liu, D. and Zhang, F., 2016. Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis. *Nature Chemical Biology*, 12(5), p.339.

Yadav, A. and Sinha, H., 2018. Gene–gene and gene–environment interactions in complex traits in yeast. *Yeast*.

Zhu, Y.O., Siegal, M.L., Hall, D.W. and Petrov, D.A., 2014. Precise estimates of mutation rate and spectrum in yeast. *Proceedings of the National Academy of Sciences*.

Zhuang, S., Smart, K. and Powell, C., 2017. Impact of Extracellular Osmolality on Saccharomyces Yeast Populations during Brewing Fermentations. *Journal of the American Society of Brewing Chemists*, 75(3), p.244-254.

Zyrina, A.N., Smirnova, E.A., Markova, O.V., Severin, F.F. and Knorre, D.A., 2017. Mitochondrial superoxide dismutase and Yap1p act as a signaling module contributing to ethanol tolerance of the yeast Saccharomyces cerevisiae. *Applied and Environmental Microbiology*, 83(3).