The role of mitochondrial functionality in brewing yeast

Eoin Moynihan MSc.
Thesis submitted to the University of Nottingham
For the degree of Doctor of Philosophy

January 2019

Abstract

Within yeast cell cultures typically 1-3% of the total cells contain damaged mitochondrial DNA (mtDNA). Damaged mtDNA manifests in the production of respiratory deficient ‘petite’ yeast cells. In certain circumstances such as in response to stress-inducing conditions associated with the modern brewing process the number of petites can exceed 10-20% of the total cell count.

In order to provide insight into the formation of petites, this study aimed to analyse the mitochondria of several brewing yeast. The mitochondrial genotype was examined through sequencing and mtDNA fingerprints while the mitochondrial phenotype was examined using high resolution microscopy and flow cytometry. Interestingly, mtDNA fingerprinting revealed variation across *S. cerevisiae* strains, while conversely revealing little variation across *S. pastorianus* strains. Microscopy revealed some insight into the morphological variations of mitochondria, but flow cytometry yielded the greater insight into mitochondrial quantity and functionality. Additionally, changes in mitochondrial content throughout propagation, fermentation and storage were examined. With an understanding of the mitochondrial phenotype and genotype of respiratory competent ‘grande’, a comparative examination of the petite phenotype was undertaken. Genotypically and phenotypically the petites were shown to greatly differ to their grande counterparts, with less diverse mtDNA fingerprints and stunted mitochondrial morphology. Initial work was also undertaken to examine the impact of mitochondrial transfer from one species to another on yeast functionality.

Acknowledgements

Firstly, I would like to thank my supervisor Dr. Chris Powell, without whom this PhD would not be possible, for which I am forever grateful.

I would also like to acknowledge Dr. Sue James, and Prof. Katherine Smart for their input while working with SABMiller and Dr. Stuart Wilkinson while working with InBev

Additionally, I would like to acknowledge and thank all the technical staff who helped make this PhD possible in BABs Emily Fong, Mel Stuart and Dave Greening, David Quain, Robert Markus at CBS, David Onion and Nicola Croxall.

I would also like to thank all the past and present PhD students in BABs without whom this journey would have been truly boring, and a special thanks to James Mallett, Chris Alexander, Celina Duglin, Imogen Ramsey, Marit Nijman, Marcus Awoniyi and Stephanie Brindley for all the beers and laughs.

Thank you to my parents and family for tolerating me and offering their encouragement without truly knowing what I'm doing. Finally, and most importantly, I would like to thank Rebekah Tinney for her love, support and encouragement throughout my time here in Nottingham.

List of abbreviations

Bp	Base pairs
Cm	Centimetre
°C	Degree centigrade
G	Gram
Kb	Kilobase
L	Litre
m/m	Mass/mass concentration
w/v	Mass/volume concentration
µL	Microliter
µM	Micromolar
mL	Millilitre
mm	Millimetre
mM	Millimolar
min	Minute
M	Molar concentration
ng	Nanogram
nm	Nanometre
%	Percent
Rpm	Revolutions per minute
Sec	Second
TTC	Tetrazolium chloride
V	Volt
v/v	Volume/volume concentration
WLN	Wallerstein laboratory nutrient
YPD	Yeast extract peptone dextrose

Contents

CHAPTER 1 INTRODUCTION

1.1 Yeast and biotechnology

1.1.1 Yeast and human interactions

Yeast are microscopic unicellular fungi, found extensively throughout natural environments. Although not as wide-ranging as other microbes such as bacteria, yeast can be isolated from diverse terrestrial (Duarte *et al*., 2013), aquatic (Mitsuya *et al*., 2017) and atmospheric *(*Pohl *et al*., 2006) environments. With regard to interactions with mankind, yeast are often associated with certain diseases (Kobayashi, 1996), but are also recognised for their traditional role in producing fermented products, including foods and beverages (Hittinger *et al*., 2018). In addition, yeast are integral to the field of biotechnology, and play a role in biopharmaceuticals (Nielsen, 2013), bioremediation (Bahafid *et al*., 2017), probiotics (Hatoum *et al*., 2012), dairy-product production (Reed and Nagodawithana, 1990), and as a key organism in medical and biological systems research (Mustacchi *et al*., 2006; Abdulkhair, 2018). Indeed, a strain of the *Saccharomyces cerevisiae* yeast, S288C, was the first eukaryotic organism to have its entire genome sequenced (Goffeau, 1996; Foury *et al*., 1998; Engel *et al*., 2013). Since this event, it has been possible to use yeast as a model organism for investigating mammalian molecular biology and metabolic biochemistry (Feldmann, 2012). For example, analysis of yeast functionality and genomics has been important in cancer research and other areas related to understanding health in higher organisms (Guaragnella *et al*., 2014; Mohammadi *et al*., 2015).

Although many yeast species are beneficial and can be employed by humans for positive purposes, some yeast can be opportunistic pathogens, or act to spoil products. From the perspective of the food and beverage industry, the latter can have commercial and economic impacts. Commercial crops (for example barley, as well as wheat and other cereals) can be affected by forms of powdery mildew caused by *Blumeria graminis*, an ascomycetous fungal species that is pathogenic to plants (Gafni *et al*., 2015). Similarly, within the brewing industry, there are a range of wild yeast species that are able to spoil raw materials, process stages and the final product, with particularly serious implications when finally packaged and at the point of dispense (Shimotsu *et al*., 2015; Bokulich and Bamforth, 2013; Mallett *et al*., 2018).

While acknowledging some of the negative aspects of yeast as a group of organisms, their positive attributes are of arguably greater socio-economic significance; the use of yeast species in the production of alcoholic beverages is regularly referred to as one of the oldest forms of biotechnology (Verma *et al*., 2011). In the fermented beverage industry, the raw materials used broadly dictate the style and characteristics of a product. However, the yeast employed provides its own unique attributes; the production of ethanol, as well as the characteristic flavour and aromas of a beverage are imparted by the particular yeast involved. In the production of beer, two species belonging to the *Saccharomyces* genus are predominantly employed, known as *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*. Both species have distinctive genotypic/phenotypic traits and in brewing are used for the production of

ale and lager type beers, respectively (Pires *et al.,* 2014, Lodolo *et al*., 2008). *S. cerevisiae* strains generally produce aroma profiles rich in esters and higher alcohols (typically described as fruity notes), while *S. pastorianus* produces a product that is characteristically cleaner and crisp with fewer fruity aromas (Kerogus *et al*., 2017).

Both brewing yeast species are members of the *Saccharomyces sensu stricto* group which also encompasses some of the most important species used in the food industry, including the wine yeast *Saccharomyces bayanus* (Lodolo *et al*., 2008). *S. cerevisiae* exists naturally in the wild while *S. pastorianus* has only been isolated from man-made environments, and relies on humans for propagation (Chen *et al*., 2016). While often referred to as a 'domesticated' organism, *S. cerevisiae* has retained many of its wild traits; one such characteristic being the ability to produce aromatic compounds. It is thought that these may play a role in the attraction of flies, allowing for the dispersal of yeast throughout the natural environment (Verstrepen *et al*., 2003).

1.1.2. Genetic origins of brewing yeast

The phenotypic differences observed between the two primary brewing yeast types stems from their evolution. In addition to being distinct species, *S. cerevisiae* strains contain much greater genetic variation than those belonging to *S. pastorianus*. That is not to say that *S. pastorianus* is a genetically simple group of organisms; they are in fact interspecific allopolyploid hybrids derived from a *S. cerevisiae* parent and a non-*S. cerevisiae* species, initially believed to be *S. bayanus* but now widely

accepted to be *S. eubayanus* (Dunn and Sherlock, 2008; Kerogus *et al*., 2017). The broad genetic diversity found in *S. cerevisiae* strains explains its widespread use throughout biotechnology, however differences within *S. pastorianus* are more subtle, and strains can be divided into two distinct genetic subsets known as Saaz and Frohberg types. The presence of these subgroups suggests that at least two hybridisation events have occurred during the evolution of this species, since there are clear genetic differences between them (Dunn and Sherlock, 2008). Frohberg strains contain a greater complement of DNA derived from the *S. cerevisiae* parent, while Saaz strains contain a greater proportion of *S. eubayanus* DNA (Krogerus *et al*., 2015). Furthermore, Saaz strains are allotriploid while Frohberg strains are allotetraploid. (Chen *et al*., 2015). The group division correlates broadly with geographical/brewery groupings, with Saaz strains being found and used in companies originating in the Czech Republic and Denmark, and Frohberg strains generally associated with the Netherlands (Dunn and Sherlock, 2008).

1.1.3 Yeast identification

Although not used routinely in the brewery, there are many molecular methods which can be used in order to characterise yeast. PCR based methods include assessment of specific gene regions (Esteve-Zarzoso *et al*., 1999; Guillamón *et al*., 1998; Pham *et al*., 2011), analysis of repeat sequences such as mini- and micro-satellites (Schuller *et al*., 2004), or profiling based on random sequences. Other methods such as PFGE-CHEF for chromosome analysis (Casey *et al*., 1988), southern blotting

based on transposon distribution (Wightman *et al.*, 1996), CGH-array profiling (Dunn *et al*., 2005), and full genome sequencing are also available, but often cost prohibitive. However, the universally recognised means of identifying a yeast species is through analysis of the yeast internal transcribe spacer (ITS) region. In yeast the 5.8S rDNA gene is flanked by two variable regions ITS1 and ITS 2 (White *et al.,* 1990). This region can be amplified using PCR and the DNA can then be digested using restriction enzymes (Guillamón *et al*., 1998; Esteve-Zarzoso *et al*., 1999). Based on the size and number of the resulting DNA fragments it is possible to distinguish between yeast genus, species and the two groups of *S. pastorianus* i.e. Saaz and Frohberg.

For identification of yeast to the strain level, there are a huge number of methods that can be employed (Pincus *et al*., 2007). One widely recognised protocol is to amplify interdelta sequences by PCR in order to produce a DNA fingerprint (Legras and Karst, 2003; Xufre *et al*., 2011). Polymorphisms in the long terminal regions (LTRs) which flank the retrotransposons TY1 and TY2 result in the number and size of the resulting amplicons to vary according to strain (Legras *et al*., 2003; Xufre *et al*., 2010). While this method may lack the absolute sensitivity of techniques such as DNA sequencing, it is quick, cheap and robust, and more than sufficient for most applications.

1.2 Brewing

Brewing, along with baking and winemaking is amongst the oldest biotechnological processes employed by man. Since the production of the first fermented beverages, the process of brewing has undergone constant enhancement, influenced by years of practice and research. In conjunction with gradual improvements to standard practices, research into malting and brewing technology has resulted in the development of a variety of beer types and flavour profiles. In particular, understanding yeast biology has resulted in strains that can ferment vigorously, yield positive flavour profiles, and that are suitable for application in a variety of scenarios (for example, fermentation of high gravity worts). The once rather imprecise and dynamic process of brewing is thus comparatively standardised at the present time, and comprises a series of recognised steps as outlined in Figure 1.1. These key steps are unique to the brewing of beer; there are generally fewer, less involved, stages in producing other fermented products such as wine and cider. Adjustments at each stage of the process can have an impact on the product, either by directly determining its general characteristics, or by influencing quality.

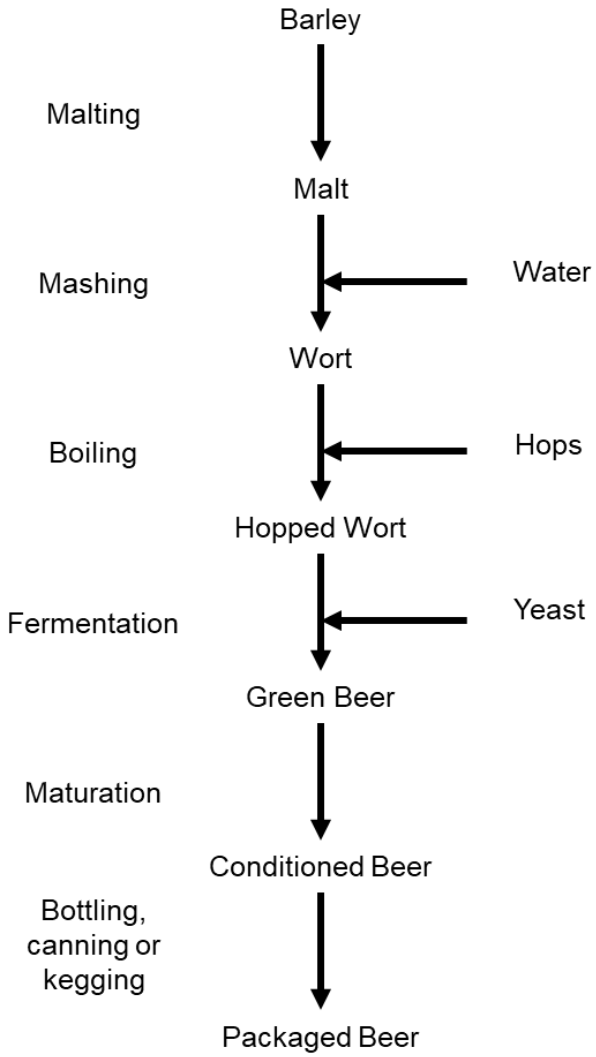


Figure 1.1 Overview of the brewing process

Malting is the process which supplies the fermentable sugars to be used by yeast for alcohol and CO_2 production, as well as the necessary nutritional factors for growth (Boulton and Quain, 2001). Typically, barley is used as the predominant nutrient source, although other grains such as wheat, rice and rye can also be employed. Malting can influence the flavour characteristics and appearance of the product, particularly in terms of colour, sweetness and mouthfeel (Gupta *et al*., 2010). Adjuncts (typically sugar syrups) can also be used in place of, or to supplement,

the use of malted barley. These are often used by large-scale producers of beer, as they can act as cheap sources of sugar, and allow operational flexibility within the brewery.

The process of mashing, in its simplest form, is the mixing of the grain with water; this process allows for amylases present within the grains to break down complex starches into simple sugars. Conducting mashing at different temperatures facilitates the activity of enzymes which in turn affects the final sugar concentration. The process of ‘lautering’ separates the spent grain from the liquid, containing fermentable sugars and known as ‘wort’. The elements of wort are generally consistent across the industry; however the ratios of individual components vary greatly and this has a significant impact on fermentation progression and the production of yeast metabolites (Boulton and Quain 2001). Irrespective, the wort provides yeast with an environment for growth and fermentation; while it is often considered to be the perfect environment for brewing yeast, it is also hospitable for many other microorganisms. Boiling the wort removes the possibility of contamination at this early stage of the process, but also allows for the addition of hops which provide bitterness, aroma and flavour to the beer.

As mentioned previously, the raw materials utilised largely dictate the style of the beer produced, however it is the fermentation stage during which the greatest and most desirable changes occur. Fermentation is conducted principally by yeast; the action of yeast influences the flavour and character of the product, and also gives rise to the production of

alcohol. Yeast are prepared for inoculation into the wort environment typically through a process called propagation (Section 1.3.2). The brewer selects the yeast strain based on specific requirements; different strains have varying characteristics with regard to sugar utilisation, flocculation, flavour profile production, and fermentative vigour.

After fermentation, conditioning (or maturation) allows the beer to develop its final character; at this stage the beer becomes stable with respect to desirable flavours and aromas. Filtering removes any remaining yeast sediment which may have built up while undergoing the maturing process. This step is often optional and is largely determined by the final product specifications. Some beer styles, such as wheat beers, often require some level of haze, while lagers must be clear or ‘bright’, with no evident haze at all. Once bottled the product is ready for consumption.

1.3 Yeast Management and Handling

1.3.1 Yeast Management

Since the isolation of the first pure lager strain in the Carlsberg laboratory in 1883 (Walther *et al*., 2014), it has become common to utilise a monoculture for each particular product. This practice differs to those employed by indigenous and eastern cultures, as well as some ‘traditional’ European breweries producing lambic style beers that use a diverse mixture of yeast, mould and bacterial species (Bokulich and Bamforth, 2013). However, the use of single cultures has been important

within the brewing industry in achieving consistency, leading to the development of established ‘brands’.

As a key biological agent in beer production, the yeast strain used impacts on two very important junctures in the brewing process: fermentation and conditioning/maturation. It is important to ensure that strains are well characterised, and that the quality of the yeast culture is maintained within the brewery as this can in turn play an integral role in determining the quality of the final product. To attain effective and consistent fermentation, breweries must employ proper yeast management and yeast handling practices. Yeast handling refers to the steps associated with the physical management of yeast (O’Connor-Cox, 1997; Lodolo *et al*., 2008), and encompasses the laboratory storage of cultures, propagation, pitching, fermentation, cropping, storage, and re-pitching of slurries. The life cycle of yeast within the brewery process is summarised in Figure 1.2.

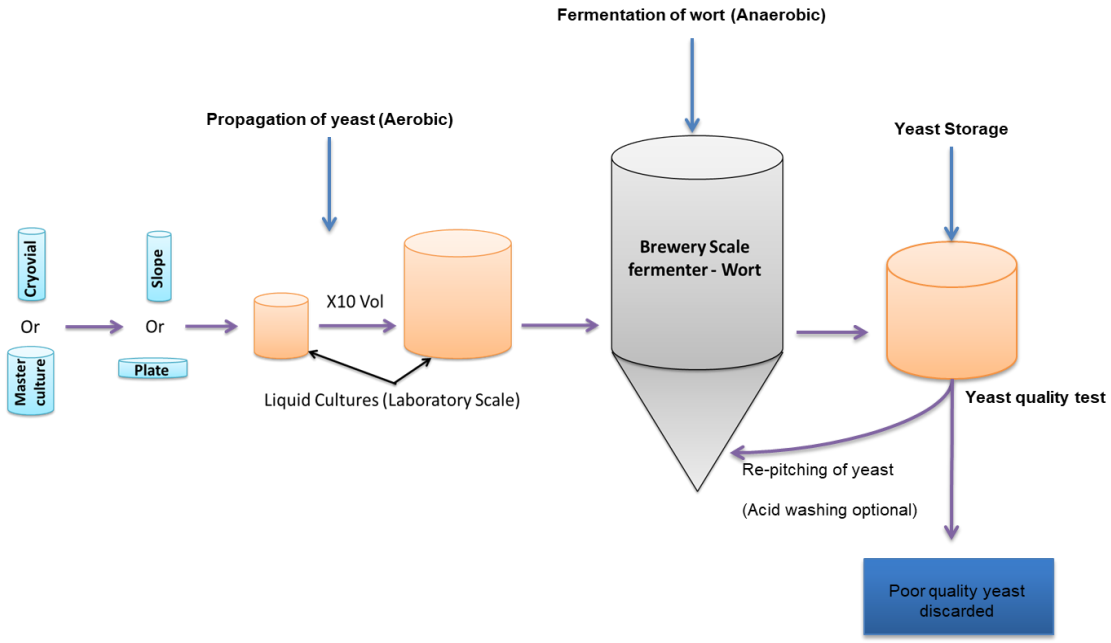


Figure 1.2 The life cycle of brewing yeast cultures

1.3.2 Propagation and pitching

The primary role of propagation is to produce sufficient yeast biomass to initiate fermentation (Vieira *et al*., 2013; Nielsen, 2005). During propagation, the cultivation and growth of yeast initially takes place in the laboratory, before being scaled-up to the plant. The first step of the process is to select and grow the appropriate strain of yeast from frozen or cryopreserved stocks. This is then cultivated in increasing volumes of liquid media within the laboratory until a specified biomass is achieved, sufficient to seed a plant propagator. From the lab stage, the yeast is transferred to the brewery and enters the propagation plant. This typically consists of a dedicated cylindroconical vessel which is equipped to provide gentle agitation (or alternatively pumped loop cycling of the medium), as well as continuous aeration or oxygenation (Boulton and Quain, 2001; Vieira *et al*., 2013). While in the propagation vessel, the yeast cells remain in log phase of growth, thus allowing biomass to be efficiently generated. The basic principles of yeast propagation are that by the end of the process there should be sufficient yeast to pitch a fermentation vessel, the yeast should be of good vitality and viability, and it should be free of contamination (Lodolo *et al*., 2008).

Once biomass has been generated, the yeast can be ‘pitched’ into the fermentation vessel; a term used to refer to the inoculation of wort with a known number of viable yeast cells. The yeast cell viability as well as the composition, gravity, temperature and dissolved oxygen content of the wort all have an impact on the final pitching rate (Stewart, 1996; Briggs

et al., 2009). However, a general 'rule of thumb' applied is to fine tune the amount of yeast required from a starting point based on $1x10^6$ cells / mL per °P (Boulton and Quain, 2001). Within the larger brewing companies, pitching is typically performed automatically, and the amount of live biomass introduced can be monitored on-line using devices such as the ABER compact yeast monitor (Boulton and Quain, 2001; Briggs, 2004).

1.3.3 Fermentation

Fermentation is the process during which the yeast consumes sugars present in the wort environment, producing ethanol, CO_2 and a range of flavour compounds. Because of its dependency on yeast metabolism, in many respects' beer can be considered to be merely a by-product of yeast growth (Boulton and Quain 2001). However, in reality this is an oversimplification, and the character of the beer is impacted by raw materials in conjunction with the genetic and physiological characteristics of the yeast strain employed.

Fermentation is typically conducted in large cylindroconical vessels (often greater than 5,000 hL in volume), which are equipped with cooling jackets and automated cleaning devices (Maule, 1985). The time required for fermentation can vary considerably based on the raw materials, the yeast, the fermentation temperature, and the final product specifications. The temperature at which lagers are fermented is typically ~14 °C while ales are fermented at ~20 °C. Typically ale fermentations are completed within 3-5 days, while lager fermentations can take 1-2

weeks or longer, depending on the conditioning process applied (Boulton and Quain, 2001; Briggs, 2004).

From the perspective of the yeast cell, fermentation essentially describes the means by which the cell produces energy from sugar. The wort environment contains a number of available carbohydrates including glucose, sucrose, fructose, maltose and maltotriose. The consumption of these sugars follows an orderly process, initially glucose, fructose and sucrose are assimilated, with maltose and maltotriose taken up more slowly (D'Amore *et al*., 1989; Boulton, 1991; Boulton and Quain, 2001). Maltose and maltotriose are broken down into glucose within the cell (Rautio and Londesborough, 2003; Alves *et al*., 2008), while sucrose is converted into glucose and fructose in the periplasmic space (Marques *et al*., 2016). Glucose and fructose are then broken down via glycolysis, a pathway which describes the conversion of these simple sugars into pyruvate (Feldmann, 2012; Wolfe, 2015). Pyruvate is subsequently decarboxylated to produce acetaldehyde, which is reduced through the action of alcohol dehydrogenases to produce ethanol (Pires *et al*., 2014; Wolfe 2015) (Figure 1.3).

Many of the important flavour compounds found at the end of fermentation appear at the early to mid-stages of fermentation, when the yeast are consuming available sugars and nutrients (Boulton and Quain 2001). The major flavour compounds include esters and higher alcohols, the majority of which are made either as a result of anabolic metabolism (i.e. production of components required for cellular growth and

homeostasis) (Hazelwood *et al*., 2008; Pires *et al*., 2014; Pires and Brányik, 2015), or during the breakdown and/or conversion of wort amino acids (Pires and Brányik, 2015). Yeast metabolism, while contributing to the formation of many positive aroma and flavour compounds, can also be involved in the production of compounds considered to be off flavours, including organic acids, sulphur compounds and aldehydes (Pires *et al*., 2014).

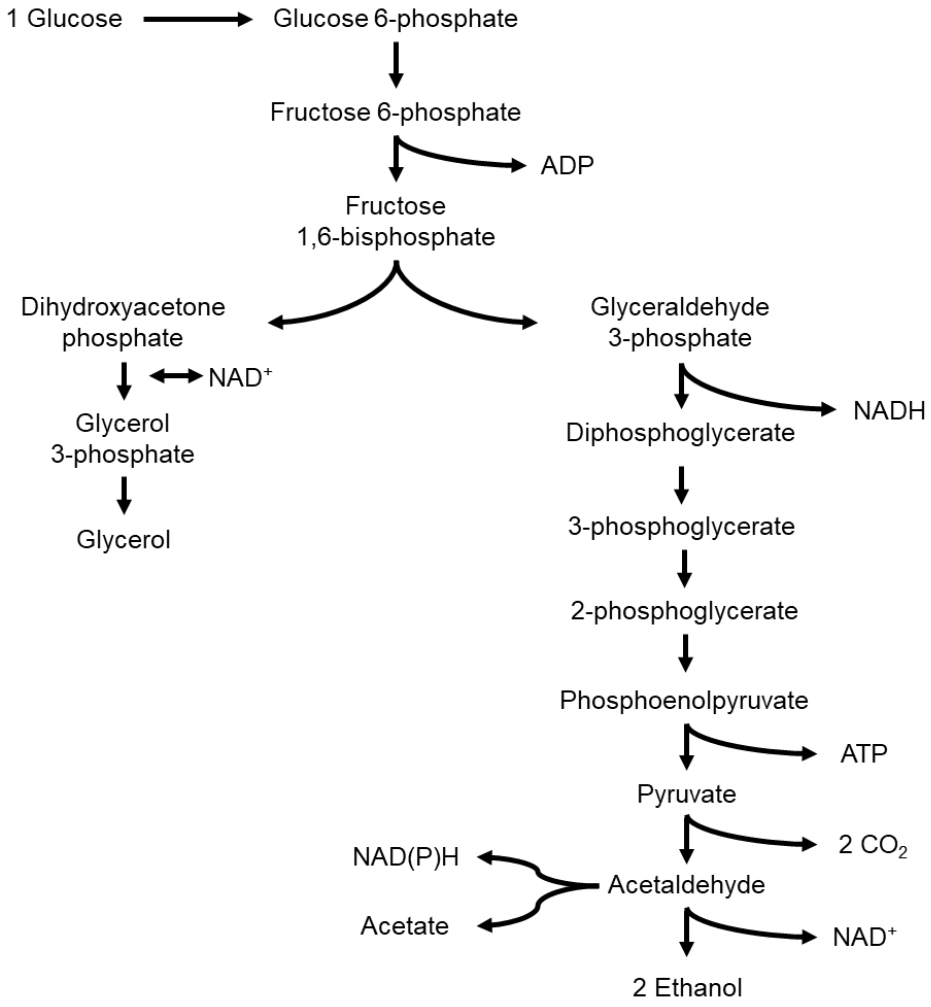


Figure 1.3 Glycolysis and the fermentation pathway

Although wort provides an excellent medium for yeast growth, the fermentation process can place yeast under stress. Stress factors

associated with brewing include osmotic, oxidative, temperature and nutritional (starvation), all of which can lead to a reduction in quality (Powell *et al*., 2000; Gibson *et al*., 2007). With the implementation of high gravity brewing, many of these stress factors are exacerbated which can result in complications. These include slow and sluggish fermentations which fail to complete, or those which exhibit aberrant flavour profiles in the product (Gibson *et al*., 2007; Lawrence *et al*., 2012). Mitigation of some of these issues can be achieved by ensuring adequate wort nutritional composition and yeast oxygenation, as well as making adjustments to yeast pitching rate (Debourg, 2010).

1.3.4 Yeast cropping, storage and serial repitching:

Although many smaller breweries operate using a practice of 'pitch and ditch' (often using active dried yeast), most large companies recover yeast and re-use it. This procedure, known as serial repitching, is unique to the brewing industry and is a long standing practice; historically a brewer would save a portion of a successful or particularly flavoursome beer, and pitch it into a successive batch (Smart and Whisker, 1996; Powell *et al*., 2000). Although they did not know it at the time, these original brewers were in effect domesticating yeast by applying selective pressures.

The number of times a yeast culture can be used is influenced by the characteristics of the yeast strain and directed by company policy. Some breweries continue to re-use the same yeast over a huge number of generations (on rare occasions, indefinitely), while it is perhaps more

typical to limit the number of uses to between 3 and 15 times (Smart and Whisker, 1996). There are positive and negative aspects to repitching when compared to the single-use of slurries. The latter facilitates simplicity by minimising yeast handling; yeast storage vessels and measures to check the quality of yeast prior to repitching are not required. In contrast, collection of yeast requires capital expenditure and the implementation of laboratory and analytical practices. However, it does also ensure that the brewer has constant access to stocks, saves operational expense, and allows the yeast to be maintained in-house rather than relying on external suppliers.

Once a fermentation is complete, the yeast culture separates into clumps in a process known as flocculation. Flocculation is a reversible, asexual process that is calcium dependant and describes the process whereby yeast cells stick to one another forming large cell aggregates known as flocs (Verstrepen *et al*., 2003). The flocs either rise to the surface of the fermentation vessel (typically only in traditional 'open square' vessels used for ale production), or more commonly sink to the bottom of the vessel, where they collect in the 'cone'. Removal of the yeast from the vessel is termed 'cropping' and is typically conducted either 'warm' (prior to vessel chilling), or 'cold' (once the vessel has been chilled) (Boulton and Quain, 2001; Powell and Diacetis, 2007; Smart, 2008). The yeast obtained via cold-cropping is often of poorer quality than that of yeast cropped warm, primarily because the latter is removed earlier and can be placed in a more appropriate environment for storage (Quain and Tubb, 1982; McCaig and Bendiak, 1985; O'Connor-Cox, 1997; Lawrence *et al*.,

2013). Although ‘quality’ is typically defined by health (i.e. viability or vitality (Section 1.3.5), there is evidence to suggest that improper handling of yeast slurry, combined with cold cropping of yeast, can result in an increase in mitochondrial damage and petite frequency (Lawrence *et al*., 2013), a topic described in more detail in Section 5.2.5.

Once cropped, the yeast culture is transferred to a dedicated holding vessel, termed a yeast collection vessel, or ‘brink’. The precise conditions of storage may vary between companies, but often the yeast is stored under beer at cool temperatures (2 – 4 °C) with gentle agitation (Smart, 2008). Yeast are usually only stored for a short period of time before being re-pitched into the next brew. This time can vary between breweries, but the general consensus is no longer than 2-3 days (Smart, 2008).

1.3.5 Yeast vitality and viability

Before pitching, or re-pitching a yeast culture, an assessment of yeast quality is performed. Yeast quality is usually considered to encompass two parameters: yeast viability and yeast vitality. While yeast viability refers to the number of living yeast cells present in a yeast slurry, vitality is a measure of how effectively yeast are able to ferment. With this in mind, both factors are important considerations in understanding the yeast culture, although in practice typically only yeast viability is determined on a routine basis. There are a number of rapid methods which can be used to assess the viability of yeast cells. These often rely on the use of stains to assess yeast functionality as a means of

estimating the proportion of live cells present (Boulton and Quain, 2001). The most common dye is methylene blue, which is used in conjunction with visual observation through light microscopy (Pierce, 1970; Smart, 2008). The methylene blue assay relies on the capacity of living yeast to reduce the stain to a colourless form through an oxidative mitochondrial reaction, while dead cells cannot (Borzani and Vairo, 1958; Boulton and Quain, 2001; Boyd *et al*., 2003). While the methylene blue procedure remains the brewing standard, there are other methods available to assess yeast viability, often involving more advanced analytical techniques such as automated detection systems or flow cytometry. One of the most commonly used stains when examining yeast by flow cytometry is the DNA-binding fluorescent stain propidium iodide (PI); the stain cannot enter living cells but binds to DNA in deceased cells. The stain also forms the basis for many commercially available viability test kits such as those produced for the Nexcelom Cellometer and the Chemometec Nucleocounter.

Although examining populations for the proportions of living and dead yeast cells is a simple and effective method, analysis of vitality is a more complicated task. There are a number of different matrices which can be considered to define yeast vitality, including those listed in Table 1.1. Typically, these assess physiological characteristics related to activity, or involve the analysis of cell components known to be elevated when conditions are favourable (Bendiak and Fang, 2000). These are often assessed using a flow cytometry or an alternative automated device, enabling a large number of individual cells within a population to be

characterised. It should be noted that many of the methods available for viability and vitality analysis are indicative only; the ultimate proof of the quality of a brewing yeast population can only be achieved by conducting a fermentation. Although miniature fermentation systems exist, the issue with these is that they require time, which often precludes them as being used as a predictive tool.

Table 1.1 Common tests for yeast vitality

Metabolic activity tests	**Examples**
Monitoring energy levels	ATP level analysis / mitochondrial activity
Measuring cellular components	Ergosterol levels / glycogen levels
Fermentation capacity testing	Changes in pH / Metabolic changes during fermentation
Intracellular pH	Fluorescent analysis using flow cytometry

1.4 Yeast cell physiology

Yeast cell structure and appearance vary greatly with respect to cell size, shape and colour, even within the *Saccharomyces senso stricto* group (Torriani *et al*., 1999). These variations are often more evident when cells are grown in different environments and under different conditions. Irrespective, *S. cerevisiae* and *S. pastorianus* cells usually appear as ovoid or elliptical structures and occur singly, in pairs or sometimes as clusters (Boulton and Quain, 2001). When examined under a light microscope they can be seen to exhibit a diameter of between 7 – 10 µm

(Feldmann, 2012). Irrespective of size and shape, there are a number of basic components which make up a yeast cell, as shown in Figure 1.4.

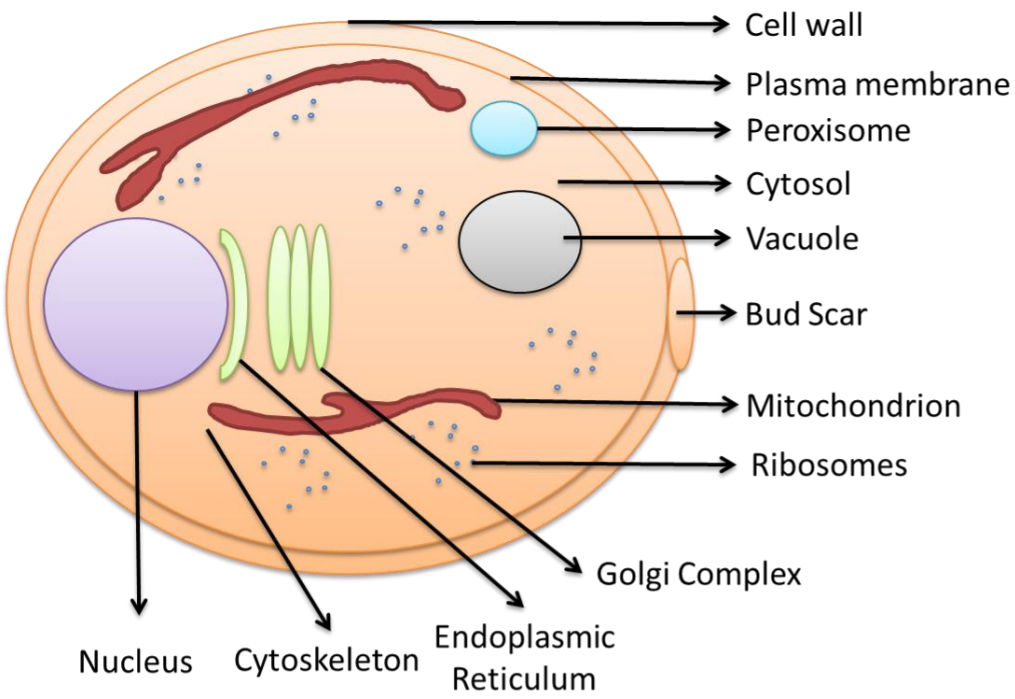


Figure 1.4 Cross section of a representative yeast cell.

As yeast cells are unicellular organisms, all of the internal components play an integral role in homeostasis and survival. The cell is surrounded by the cell envelope, comprising the cell wall, periplasmic space and the cell membrane. The cell wall is composed of a number of macro molecules and proteins and its primary function is to provide structural support for the yeast cell (Klis *et al*., 2002). However, binding proteins such as agglutinins and flocculins, which have a role in yeast cell mating (haploid and diploid yeast) and flocculation (Klis *et al*., 2002) can also be found associated with this structure. The periplasm is effectively the space between the inner surface of the cell wall and the outer surface of the plasma membrane. However, a number of important enzymes can be

found here, including those associated with cell wall maintenance and repair, and in the breakdown of sucrose (invertase) (Boulton and Quain, 2001).

Within the cell envelope, an aqueous solution known as the cytoplasm (which functions to support the organelles), and the cytoskeleton can be found. The latter is an actin-rich network consisting of microtubules and microfilaments which spans the cell and allows for movement of organelles, and for the assembly and disassembly of individual protein subunits (Feldmann, 2012). Yeast vacuoles act as storage and detoxification compartments for transition metals such as zinc and plays a key role in intracellular protein trafficking (Klionsky *et al*., 1990). The endoplasmic reticulum (ER) and golgi apparatus perform a variety of functions in all eukaryotic cells including protein folding, lipid synthesis and calcium homeostasis (Austriaco, 2012). While the former is associated with ribosomes and is responsible for protein and enzyme production, the latter is primarily involved in trafficking, including protein modification and transport.

The yeast cell also contains several types of DNA; the genetic information required for its existence. The nucleus houses the genomic DNA and comprises more than 6700 genes required for growth, reproduction, maintenance and repair (Taddei and Gasser, 2012). A much smaller compliment of DNA can also be found in the mitochondria, an organelle believed for many years to be almost exclusively associated with oxidative phosphorylation (OXPHOS). OXPHOS includes the TCA cycle

and the electron transport chain, the pathways involved in generating energy (ATP) from pyruvate using oxygen as the final electron acceptor, and in recycling the essential cofactor NAD^+. It should be noted that both of these pathways are repressed in brewing yeast due primarily to the Crabtree effect, often referred to as glucose repression (Trumbly, 1992; Kayikci and Nielsen, 2015). This metabolic regulatory mechanism prevents yeast from using OXPHOS pathways, even if oxygen is available. The caveat is that a threshold of fermentable sugar (typically, but not exclusively, glucose) must be present; in brewing yeast this can be as low as 0.2% (Kayikci and Nielsen, 2015). The significance of this from a brewing perspective is that during fermentation yeast generate energy from glycolysis, the result being that ethanol is formed from acetaldehyde as a means of regenerating NAD^+.

Because of the Crabtree effect and the inhibition of respiratory metabolism (the primary function of mitochondria in most eukaryotic organisms), for many years the relevance of the mitochondria to brewing fermentations was questioned. However, this attitude is perplexing, since it is equally well established that yeast without mitochondria (termed petites; Section 1.6) ferment poorly (Boulton and Quain, 2001). This conundrum has received little interest until recent years where the role of mitochondria in yeast under anaerobic conditions has begun to be fully investigated.

1.5 Mitochondrial structure

Yeast, like all eukaryotic organisms, have mitochondria comprised of two types of lipid bilayers: an outer membrane (MOM) and an inner membrane (MIM). These embody an intermembrane space (IMS) known as the mitochondrial matrix (Figure 1.4) (Westerman, 2002; Feldmann, 2012). The outer membrane protects the inner membrane and the matrix, and, also contains enzymes involved in the early stages of lipid metabolism (Feldmann, 2012; Pires *et al*., 2014). The inner membrane forms folds (invaginations) known as cristae, and contains cytochromes, ATP synthases and transport proteins involved in the electron transport chain (Foury *et al*., 1998; Lipinski *et al*., 2009; Albertin *et al*., 2013 and Osman *et al*., 2015). The four compartments within the mitochondria contain their distinct set of proteins, and it is a series of complex molecular mechanisms which recognise pre-proteins and localise them to their distinct areas within the mitochondria (Bohnert *et al*., 2007).

As described briefly above, the mitochondria also contain their own genome, which is maintained within the matrix, and is believed to be a vestige of the mitochondria's theorised endosymbiotic past (Jornayvaz and Shulman, 2010). There are multiple copies of the mitochondrial DNA (mtDNA) genome present within each mitochondrion, packaged in a nucleoprotein complex known as a nucleoid (Kaniak-Golik and Skoneczna, 2015).

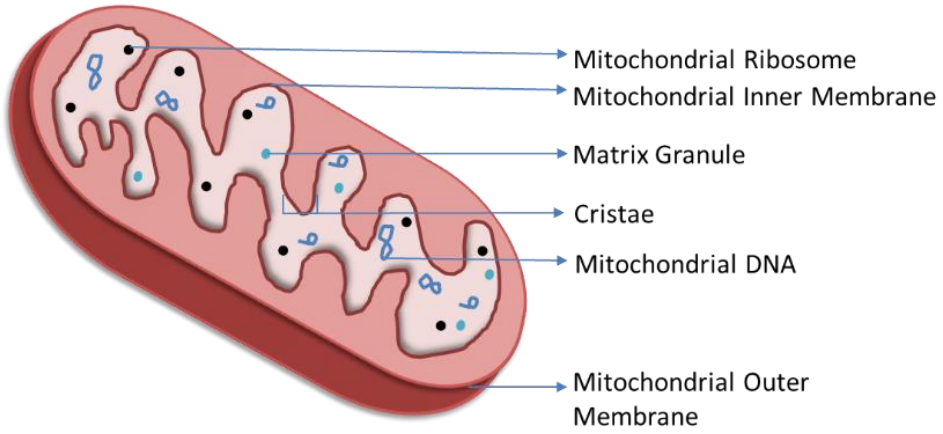


Figure 1.4 An overview of simplified mitochondrial physiology

Mitochondria are often depicted as singular free-floating ovoid shaped organelles within a cell. However, increasing evidence suggests that they are dynamic organelles which can appear in different shapes and confirmations (Visser *et al*., 1995; Otsuga *et al*., 1998; Dimmer *et al*., 2002). Analysis of haploid yeast strains has shown that under aerobic conditions (where yeast mitochondria are primarily producing ATP through the TCA cycle) mitochondria usually appear as large cylindrical/spherical organelles (Visser *et al*., 1995; Aung-Htut *et al*., 2013). Conversely, under anaerobic conditions (such as those associated with fermentation) mitochondria become smaller, since their main function (ATP production) is no longer taking place (Feldmann, 2012; Kitagaki *et al*., 2013). If sustained anaerobic conditions are encountered, pro-mitochondria, which are shrunken and stunted, with poorly formed cristae can develop (Rosenfeld *et al*., 2004).

Importantly, it is known that during replication and segregation of organelles, mitochondria become elongated and form a network-like structure within the cell (Simon *et al*., 1997; Shiota *et al*., 2015). It is therefore likely that mitochondrial fusion and fission events are integral to the inheritance and maintenance of the mitochondrial network (Section 1.6)(Nunnari *et al*., 1997; Bliek *et al*., 2013). In addition, it is increasingly recognised that division and fusion in mitochondria occur continuously; evidence suggests that mitochondria exist in their 'fused' form during exponential phase and fragment in stationary phase (Yaffe, 2003). In fact, current consensus is that the media on which the yeast cell is growing, growth phase, the respiratory state of the cell, and the stage of the cell cycle may all impact on mitochondrial morphology (Sesaki *et al*., 1999; Nunnari *et al*., 1997; Shaw *et al*., 2002; Osman *et al*., 2015). As such, the 'normal' state for a mitochondria is perhaps more difficult to define than previously thought.

1.6 Mitochondrial fusion and fission:

As described above, fusion and fission events are common in mitochondria, and serve a distinct purpose; morphological changes are related both to functionality and regulation (Dimmer *et al*., 2002*;* Picard *et al*., 2013*)*. Interestingly, fusion and fission events are controlled by three key GTPases encoded by nuclear genes: Fzo/mitofusin (MOM fusion), Mgm1 (MIM fusion) and Dnm1 (fission of MOM and MIM) (Sesaki *et al*.,1999; Shaw and Nunnari, 2002; De Vecchis *et al*., 2017). Since mitochondria are double membrane bound organelles, they require two

fusion events in order to be fully fused. The mitofusin Fzo1 is responsible for fusing of the MOM and is also involved in mitochondria-endoplasmic reticulum (ER) tethering. Close contact between mitochondria and the ER is also important for calcium transport to the mitochondrial matrix during cell signalling events (Zhan *et al.,* 2013; Williams *et al.,* 2013). In addition, the GTPase Mgm1 fuses to the MIM phospholipid bilayer and plays a role in maintaining the cristae (Shaw and Nunnari, 2002). Interestingly, studies involving Mgm1 mutants have shown that the mitochondrial outer membrane is able to fuse while the inner membrane of the two separate mitochondria remain compartmentalised. However, Mgm1 mutants also exhibit aggregated and fragmented mitochondria, which can result in a loss of mtDNA (Yaffe *et al*., 1999; Yoon *et al*., 2001; Shaw and Nunnari 2002). Conversely, yeast Dnm1 mutants yield mitochondria that are larger and interconnected, often described as being netlike, due to ineffective division (Yoon *et al*., 2010). However, double Mgm1 / Dnm1 mutants contain morphologically normal mitochondrial, suggesting that the default morphology reflects an intricate balance between fusion and fission events (Sesaki *et al*., 2003; Zuo *et al*., 2007).

As well as having a role in the morphology of mitochondria, it is apparent that the same enzymes are important in the transmission of mtDNA to daughter cells, a subject covered in greater detail in Section 1.8. During cell replication, mitochondrial division and movement must occur at specific times in order to reach the new daughter cell (Nunnari *et al*., 1997). Incorrect functioning of Mgm1 and Dnm1 causes aberrant fusion/fission events, leading to poor inheritance of mitochondria. In

addition, the complete absence of Mgm1 and Dnm1 can result in deleterious structural confirmations of mtDNA (Osman *et al*., 2015). If these 'DNA lesions' affect key mitochondrial genes then this results in daughter cells becoming respiratory deficient (Section 1.11).

1.7 Mitochondrial biogenesis and membrane functionality

The formation of mitochondria within a cell (mitochondrial biogenesis) occurs within the cytoplasm, and is controlled genetically as well as cytoplasmically (Jornayvaz and Shulman, 2010b; Shiota *et al*., 2015). With the unravelling of the mitochondrial genome, it appears that very little information is derived from the mitochondria itself, but that many of the key structural mitochondrial proteins are encoded in the nucleus (Fox, 2012; Battersby and Richter, 2013). Despite this, mitochondria cannot actually be synthesised *de novo*, but can only be replicated from existing mitochondria within the cell. Related to this, the transmission of mitochondria from a mother cell to its daughter requires that they have the ability to auto-replicate, while also sharing links with the yeast cell cycle (Figure 1.8). The fact that yeast cannot create mitochondria *de novo*, coupled with their ability to auto-replicate has given rise to the theory that mitochondria were originally self-sufficient prokaryotic organisms that became incorporated into an ancient cell by endocytosis leading to a symbiotic relationship that exists today (Nisoli *et al*., 2005; Jornayvaz and Shulman, 2010).

Regulation of an enzyme known as translocase, outer membrane (TOM) is required to induce mitochondrial biogenesis (Shiota *et al*., 2015). TOM

is required for sorting proteins produced by mt-ribosomes in the matrix as well as nu-DNA encoded proteins, allowing for their import and integration into the mitochondria and its various bilayers (Feldmann, 2012). The TOM complex acts as the gateway for protein import into the mitochondria, and increasing levels of TOM leads to more mitochondrial 'mass' (Shiota *et al*., 2014). After passing through the outer membrane, proteins are sorted by the translocase of the inner membrane (TIM). This is followed by a pre-sequence translocase-associated motor (PAM), which drives precursors to the matrix, before a sorting and assembly machinery (SAM) inserts β-Barrel proteins into the outer membrane (Höhr *et al*., 2015). β-Barrel proteins are essentially enclosed β-sheets (a common motif of regular secondary structure in proteins) which have hydrophobic and hydrophilic residues. These transmembrane proteins play a role in transporting metabolites and proteins, and are involved in regulatory and signalling processes (Paschen *et al*., 2005; Höhr *et al*., 2015). The process of mitochondrial biogenesis and assembly is illustrated in Figure1.5.

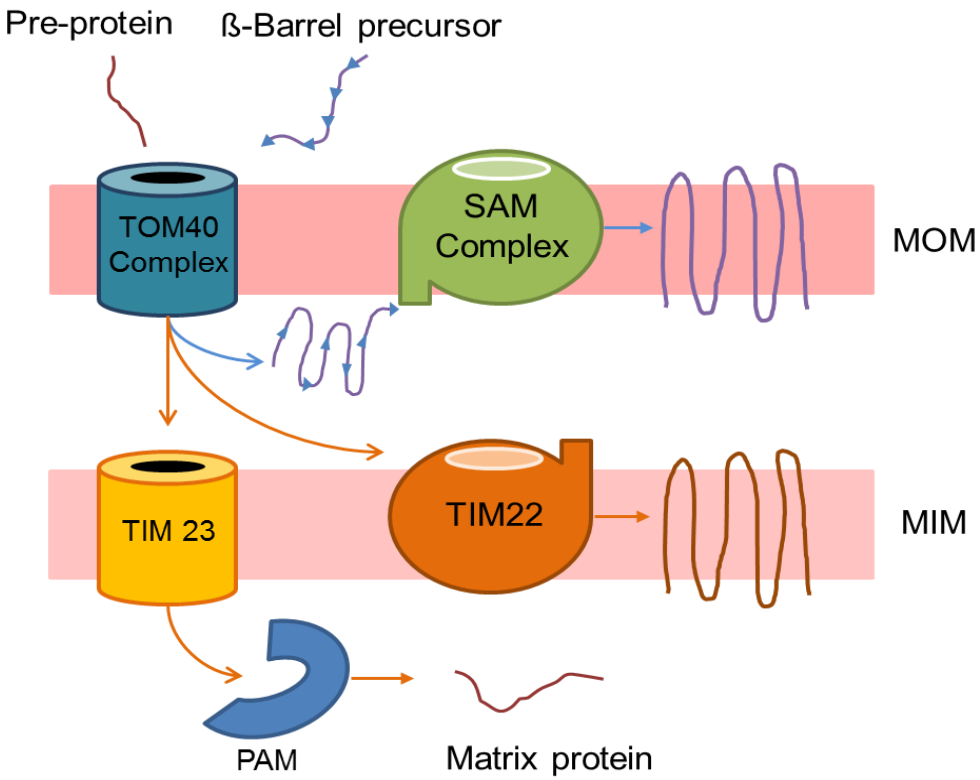


Figure 1.5 The mechanisms involved in importing mitochondrial pre-proteins. Virtually all pre-proteins are imported through the TOM complex. Once imported, the pre-proteins are sorted in the IMS where the proteins/precursors are incorporated into the MOM lipid bilayer, or sent through either the TIM complex (where proteins are integrated into the MIM), or sent to the matrix.

The inner membrane of the mitochondria is comprised of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, cardiolipin, phosphatidylserine and phosphatidic acid (Jornayvaz and Shulman, 2010; Tamura *et al*., 2012). Of these, cardiolipin is of great importance since its availability is integral to both mitochondrial biogenesis and function (Joshi *et al*., 2009; Samp *et al*., 2010; Paradies *et al*., 2014). Cardiolipin exists exclusively in bacterial and mitochondrial

membranes, and is closely linked with a number of proteins including those associated with the respiratory chain (Jacobson *et al*., 2002) and the mitochondrial apoptotic process (Lemasters *et al*., 1998; Guaragnella *et al*., 2012). Cardiolipin acts to produce an electrochemical gradient that allows for the production of ATP across the membrane (Paradies *et al*., 2014). The absence of this phospholipid (or any perturbations to the genes encoding for the phospholipid) can result in respiratory defects, issues with mitochondrial protein import, and cell wall biogenesis, causing cell death through apoptosis.

Mitochondrial content and dynamics are also impacted by the presence of cardiolipin and there is some evidence to suggest that lower levels of cardiolipin in brewing yeast result in the over production of dimethyl sulphide (DMS), an important flavour compound associated with lager (Samp *et al*., 2010). With increased cardiolipin content, the mitochondria have enhanced ATP availability, which supports the production of the sulphite reductase complex. This complex belongs to a family of iron-sulphur (FE-S) clusters (ISC), which are involved in an array of different cellular process, from cell homestasis to DNA replication and repair (Fuss *et al*., 2015), as well as being components of proteins important in the ET chain (Lill *et al*., 2012). The role of mitochondria in the reduction of sulphur compounds can be seen in Figure 1.6 (1), where the resulting cellular sulphur is used by the yeast cell primarily in cysteine and methionine biosynthesis pathways. In situations where there is a *reduction* in sulphite reductase enzyme activity (such as restriction of OXPHOS during fermentation), increased levels of SO_2 can be found.

The rationale for this is that yeast continue to release SO_2 derived intracellularly, and are also unable to reduce external SO_2 (Samp *et al*., 2010) (Figure 1.6 (2)). It has been suggested that this occurs to an even greater extent in respiratory deficient (petite) mutants (Section 1.11), which are unable to utilise extracellular sulphite as effectively as respiratory competent cells. Although the precise mechanism has yet to be fully elucidated, it is possible that the reduced functionality of mitochondria in petite cells results in a reduced cardiolipin content, which in turn yields more sulphite due to a decrease in overall activity of the sulphite reductase complex (Samp *et al*., 2010).

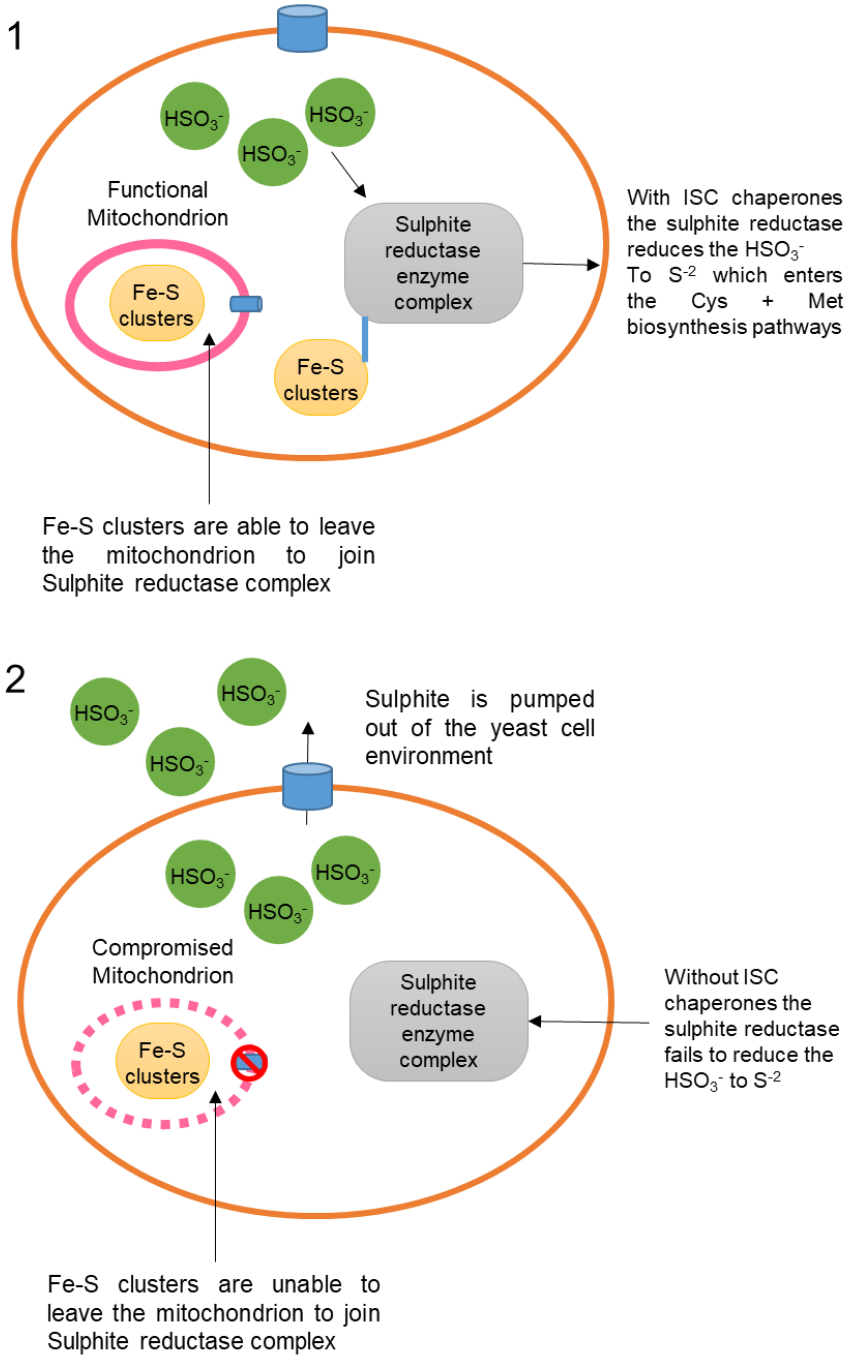


Fig 1.6 The impact of mitochondrial integrity on sulphur release. Iron-sulphur (Fe-S) clusters are required for efficient function of the sulphite reductase enzyme complex which acts to break down hydrogen sulphite (1) resulting in reduced sulphur. In compromised mitochondria (2), this does not occur leading to elevated sulphur which is pumped out of the cell (Samp *et al*., 2010).

1.8 Mitochondrial inheritance

Mitochondrial inheritance occurs bi-parentally with both mating cells donating their mitochondria to the new progeny (Christie *et al*., 2015). This bi-parental inheritance results in a level of heteroplasmy, the presence of more than one type of organellar genome. However after successive budding events the daughters soon become homoplasmic with no variation in the mitochondrial genome (Birky *et al* 1978; Christie *et al*., 2015). There are many factors involved in the inheritance of mitochondria, including mtDNA recombination, mtDNA expression, biogenesis, protein aggregation, morphology and metabolism (Nunnari *et al*., 1997; Sesaki *et al*., 1999; Zhou *et al*., 2014; MacAlpine *et al*., 2000). *Saccharomyces* yeast undergo asymmetric cell division, where mitochondrial segregation mechanisms are required in order to successfully deliver functional mitochondria to the budding daughter, while also ensuring that some are retained within the mother cell (Figure 1.7 and 1.8).

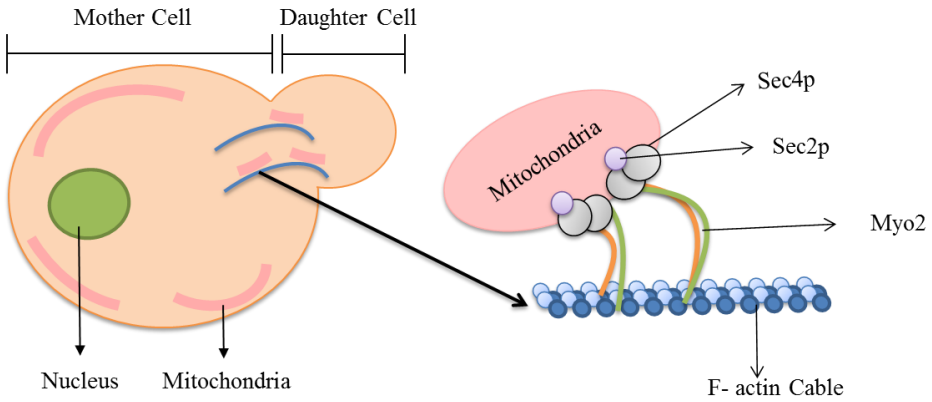


Figure 1.7 Segregation of mitochondria along F-actin cables in asymmetrically dividing yeast cell (Feldmann, 2012)

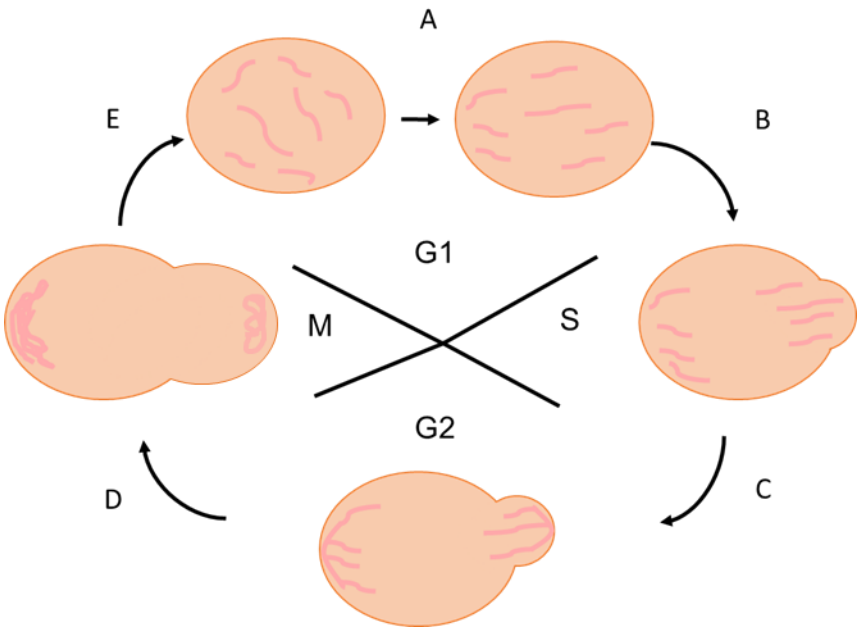


Figure 1.8 The yeast cell cycle and mitochondrial inheritance. A: Mitochondria are aligned along actin cables from mother to daughter; B: Movement towards polar ends of the cells; C: Mitochondria are retained at the polar ends; D: Accumulation at the polar regions; E: Release of mitochondria from polar ends (Vivea *et al*., 2013)

During the cell cycle, mechanisms are activated to control both the quality and the quantity of mitochondria to be distributed to the new cell (Vevea *et al*., 2014). Initially, as the bud begins to form, the production of actin fibres and re-orientation of the cytoskeleton occurs in order to actively transport mitochondria (and other organelles) to the new daughter cell (Fehrenbacher *et al*., 2003; Mishra and Chan 2014). Movement along the cytoskeleton is dictated by myosin proteins (Myo2), which are attached to their respective organelle by membrane-bound proteins known as Sec2p and Sec4p (Feldmann 2012). During the G1-S phases of cell division, the organelles align along the polarised actin cables towards the

future bud site (Simon *et al*., 1997; Vevea *et al*., 2013). From G2-S phase onwards they are then either retained within the mother cell or transferred to the daughter (Vevea *et al*., 2013).

Transfer of mitochondria from mother to daughter is not solely the responsibility of the cell; evidence suggests that damaged or poorly functioning mitochondria cannot be transferred. This is primarily because they demonstrate a reduced trans-membrane potential, required for movement along the actin filaments which constitute the cytoskeleton (Vevea *et al*., 2014). In addition, the presence of mitochondrial 'nucleoids' (partitioned mtDNA) are required for efficient and faithful transmission to daughter cells (Okamoto *et al.*, 1998). Unlike nuclear DNA, which is innately linked to the cell cycle, the replication and partitioning of mtDNA nucleoids is independent; the number of nucleoids varies depending on the needs of the cell (Altmann and Westermann, 2005). The processes which promote mtDNA sorting into nucleoids remains poorly defined, but there have been suggestions that it involves activation of the general amino acid response pathway, in turn triggered by deficiency in amino acids (MacAlpine, 2001). Hence, the nutritional status of the cell may also play a role in inheritance.

1.9 Mitochondrial DNA (mtDNA)

Although it is hypothesised that mitochondria may have originally been prokaryotic-like organisms that became incorporated into a host cell (Section 1.7), this occurred so long ago in evolutionary terms that mitochondria from different organisms are currently very different,

despite sharing common functionality. The mitochondrial genome of yeast comprises roughly 10% of the total cellular DNA (Briggs *et al*., 2004) and was first described structurally in 1986 (Zamaroczy and Bernardi, 1986). However, the full sequence was not confirmed until 1998 (Foury *et al*., 1998); prior to this the mitochondrial genome contained mistakes and was incomplete, initially being described as being ~10kbp shorter (Foury *et al*., 1998).

Analysis of MtDNA in *sensu stricto* yeast has shown that the *Saccharomyces cerevisiae* mitochondrial genome is larger than that of *S. pastorianus* and *S. eubayanus* (75kbp compared to 64kbp) (Nakao et al., 2009; Baker et al., 2015). However, the yeast mitochondrial genome is relatively large when compared to other eukaryotic organisms: around 60-85kbps in size compared to between 11kbp and 28kbp in many vertebrates (Kolesnikov and Gerasimov, 2012). In addition to size, the form of mtDNA is also believed to vary between organisms, although it is acknowledged that this remains the subject of much dispute. Some authors have suggested that the genome appears in a circular format (Kolesnikov and Gerasimov, 2012), while others report that there are no circular forms and that mtDNA only retains a linear confirmation (Gerhold *et al*., 2010). This is further complicated by proposed heterogeneity of the mtDNA molecule; some may be homogenously linear, or heterogeneously circular (Kolesnikov and Gerasimov, 2012). It should be noted that initial suggestions for a circular morphology came from examination of mammalian cells (Williamson, 2002). Subsequent analysis of yeast cells largely revealed only linear molecules, but many

authors attributed this to evidence of broken circular molecules (Williamson, 2002). Gerhold *et al*. (2010) also suggest that much of the earlier work on yeast mtDNA was focused predominately on respiratory deficient hyper-suppressive petites (Figure 1.9) which are not representative of typical mitochondria, and may have been misleading in terms of understanding the molecular structure of mtDNA. Interestingly, it seems that *S. cerevisiae* strains do typically contain linear copies of mtDNA, and that circular forms may be derived exclusively from petite mutants (Bendich, 2010). However, the debate over mtDNA structure does has major biological implications, since it impacts on the likely means of replication. For linear DNA, the main method of replication is recombinant driven replication (RDR), while the proposed mechanism for replication of circular mtDNA is via the rolling-circle replication (RCR) method; a more plasmid-like method of duplication (Figure 1.10). It should be noted that to some extent, the rolling-circle replication method may explain the presence of both linear and circular mtDNA molecules (Maleszka *et al*., 1991; Lewis *et al*., 2015).

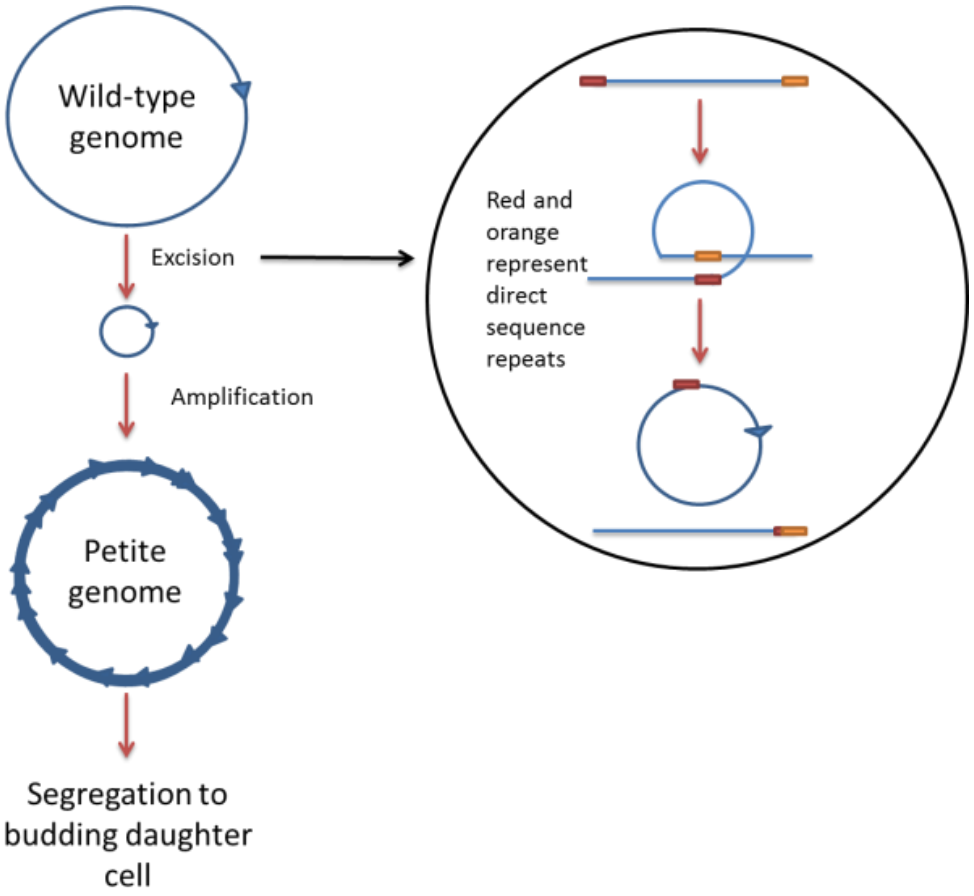


Figure 1.9 A schematic representation of the hyper-suppressive petite genome based on excision of ORI and amplification (Bernardi 1983).

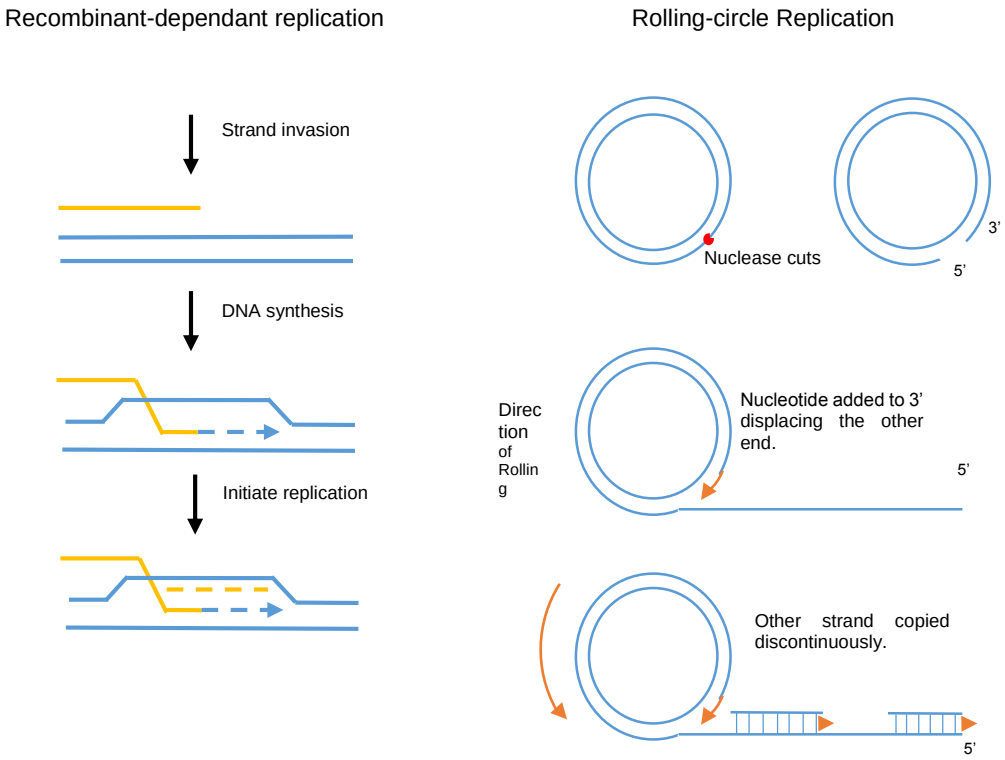


Figure 1.10 Possible mechanisms for mtDNA replication depending on the topology of the mtDNA. Linear DNA typically undergoes recombinant-dependent replication (RDR), while the mechanism for circular DNA is via rolling circle replication (RCR).

While there is still some debate over the topology of mtDNA, some matrices are known and accepted. *Saccharomyces sensu stricto* mtDNA content is A+T rich with a comparatively low G+C content, and the size of the genome varies from ~64 to ~85kbp in size (Piškur *et al*., 1998; Foury *et al*., 1998). Outside the *sensu stricto* group the total G+C content can be higher (Wolters *et al*., 2015), for example reports have shown that it can be as high as 52.7% in some *Candida* species (Fricova *et al*., 2010). Irrespective, within yeast as a group of organisms, the yeast mitochondrial genome is known to only encode for a limited number of

protein structures: cytochrome c oxidase subunits I, II and III (cox1, cox2 and cox3); ATP synthases subunits 6, 8 and 9 (atp6, atp8 and atp9); apocytochrome b (cytb); a ribosomal protein (var1); and open reading frames (ORFs) (Albertin *et al*., 2013; Foury *et al*., 1998; Lipinski *et al*., 2010; Osman *et al*., 2015)

Regulation of gene expression takes place at the post-transcriptional level and involves many general and gene-specific factors (Lipinski *et al*., 2009). Furthermore, as alluded to above, mitochondrial function depends strictly on the cell nucleus; many of the proteins required for biogenesis and activity are imported from the cytoplasm and derived from nuDNA (Scarpulla, 2012; Turk *et al*., 2013) (Section 1.7). This dependency on the nucleus is interesting and has led to the concept of 'crosstalk', an area of research which is expanding rapidly (Poyton and Dagsgaard, 2002; Kaniak-Golik and Skoneczna, 2015; Guaragnella *et al*., 2018).

1.9.1 Mitochondria and mtDNA repair

During oxidative metabolism, Reactive Oxygen Species (ROS) are generated, able to damage cellular structures including membranes and DNA (Zorov *et al*., 2014). Given the location of mtDNA and its proximity to the primary source of reactive oxygen species (ROS), it is not surprising that mtDNA is subject to damage (O'Rourke *et al*., 2002). Further compounding this issue is the fact that the mtDNA genome lacks histones, known to protect nuDNA against oxidative damage (Ljungman and Hanawalt, 1992; Alexeyev *et al*., 2013).

In order to counter these issues, mitochondria employ a number of mechanisms to repair or remove damaged DNA. The best described method is the base 'excision repair pathway' (Maclean *et al*., 2003; Stuart, 2005; Prakash and Doublié, 2015), which acts to remove bulky lesions which can occur spontaneously and accumulate over time. These lesions arise due to the reactivity of DNA, in conjunction with the presence of toxic chemicals (such as ROS) found naturally within the cell (Alexeyev *et al*., 2013). When lesions affect key mitochondrial genes this becomes detrimental to cell functionality and often results in cell death (Van Houten *et al*., 2016).

Another protective measure which enables the cell to withstand mitochondrial damage is through the relatively large mtDNA copy number. As well as there being multiple mitochondria per cell, the mtDNA genome is multi-copy; there have been reports of between 50 and 200 copies per mitochondrion (Solieri, 2010). The variation in mtDNA copy number is believed to occur in response to the physiological condition of the cells and the environment in which they find themselves (Hori *et al*., 2009). It is believed that the large copy number of mtDNA allows for the general dismissal of any damage which occurs naturally.

In addition to copy number, it is possible that there are specific pathways dedicated to maintaining mtDNA integrity, but considering the large copy number of mtDNA genomes they may be less effective in yeast than in other organisms (Ling, 2000). A number of studies have attempted to elucidate potential pathways, and to better understand which genes and

proteins are involved in mitochondrial functionality (Ling, 2000; Alexeyev *et al*., 2013; Wisnovsky *et al*., 2016; Zinovkina, 2018). Indeed, it is believed that the nuclear encoded genes MHR1, MGM101, MGT1 may be involved in maintaining mtDNA integrity, based on a comparative analysis of grande strains and the petites derived from gene knockout experiments (Zweifel and Fangman, 1990; Contamine and Picard, 2000).

1.10 Brewing Yeast Mitochondria and Fermentative Metabolism

Brewing yeast strains are facultative anaerobes and are able to grow in the presence and absence of molecular oxygen. While mitochondria may appear unnecessary in a predominantly anaerobic process, their role reaches far beyond that of respiration via oxidative phosphorylation. Evidence suggests that mitochondria also play a role in stress tolerance (Aguilera and Benítez, 1985; Grant *et al*., 1997; Baker *et al*., 2015, 2018), synthesis and desaturation of fatty acids (Verbelen *et al*., 2009; Sawada and Kitagaki, 2016), and the biosynthesis of amino acids (Zelenaya-Troitskaya *et al*., 1995). Consequently, even under anaerobic conditions the maintenance of mitochondria is essential, which further emphasises the physiological importance of mitochondria from a brewing perspective.

While the flavour profile of an alcoholic beverage is often dictated by the raw materials used to produce it, yeast metabolic products, intermediates and by-products produced during fermentation also play a major role in the final flavour and aroma of the product (Pires *et al*., 2014). When considering beer, arguably the most important products of fermentation include ethanol, fusel alcohols (higher alcohols), esters and VDKs. A

number of intermediary steps involved in the production of these by-products are influenced by mitochondria (Figure 1.11).

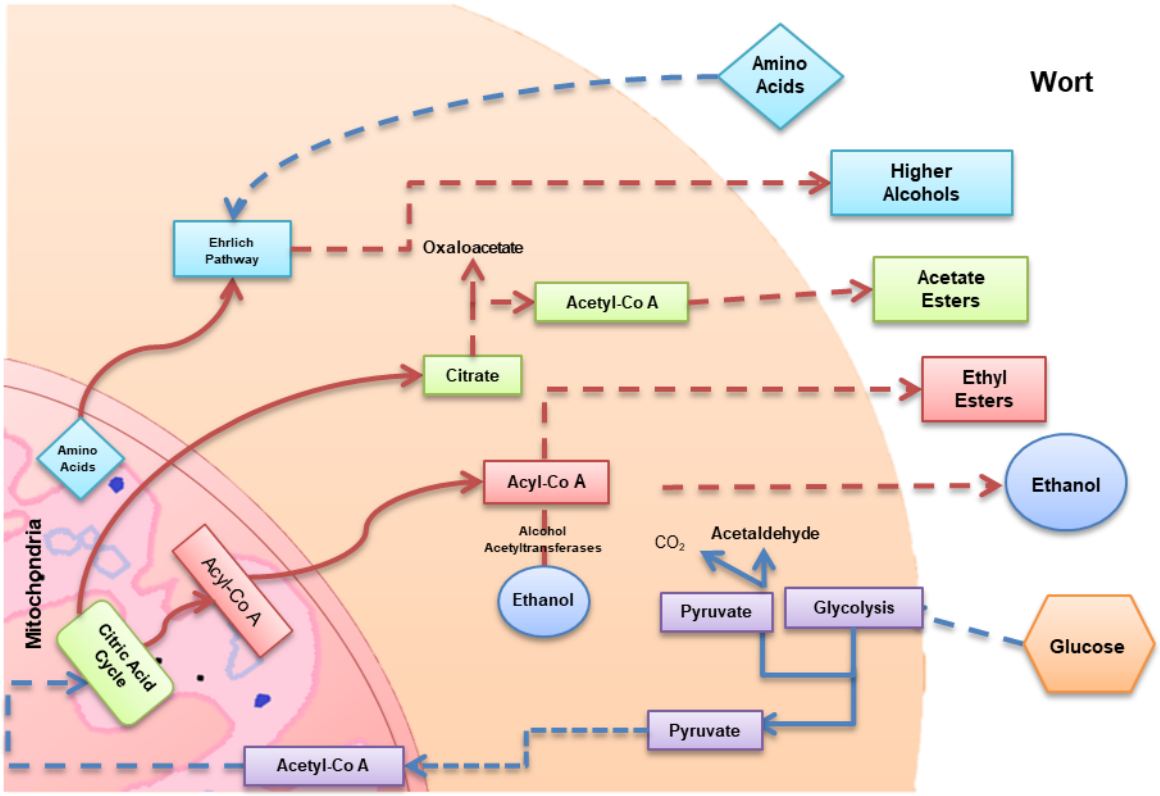


Figure 1.11 Sub-cellular locations of flavour development pathways within the yeast cell, indicating the role of mitochondria in the production of a number of organoleptic compounds.

Fusel alcohols are the most abundant organoleptic compounds present in beer. Brewing yeast absorb amino acids present in the wort and these undergo series of irreversible steps as part of the Ehrlich pathway, resulting in the production of fusel alcohols (Pires *et al*., 2014). The Ehrlich pathway has three steps: removal of the amino-group from the amino acid absorbed (transamination); decarboxylation to a fusel aldehyde; and reduction to form a fusel alcohol. Although the Ehrlich pathway itself takes place in the cytoplasm of the cell, enzymes

catalysing the synthesis of fusel alcohols (BAT1) have subcellular locations in the mitochondria (Takpho *et al*., 2018). A study by Avalos *et al.* (2013) employed a GM yeast where the Ehrlich pathway had been directed to the mitochondria, resulting in a 260% increase in the production of higher alcohols. The authors considered that this was evidence indicating that the mitochondrial matrix was actually a more favourable environment for the production of higher alcohols. In addition, the same study speculates that the increase in iso-butanol and iso-pentanol during fermentation is due to mitochondria consuming α-ketoisovalerate, preventing repression of the ILV gene linked to iso-pentanol production.

When compared to other yeast metabolites, esters are only present in trace amounts. Despite this, they represent one of the most important types of organoleptic compounds produced by yeast. This is because of their low flavour threshold in beer and their ability to dictate aroma in the final product. Esters are produced during the more vigorous early stages of fermentation, due to enzymatic condensation of organic acids and alcohols by alcohol acetyltransferase (AAT) (Saerens *et al*., 2010, Zhang *et al*., 2014, Pires *et al*., 2014). The role mitochondria play in the production of esters can be seen in the *de novo* biosynthesis of amino acids, where pyruvate produced from glycolysis enters the mitochondria and is oxidied to form acetyl coenzyme A (acetyl co-A), which can then be enzymatically condensed with a higher alcohol to produce acetate esters (Pires *et al*., 2014). Additionally, it is also important to note that amino acids produced by the mitochondrial *de novo* biosynthesis

pathway contribute to the production of higher alcohols through the Ehrlich pathway see Figure 1.11.

There are two important VDKs in beer: 2,3-butanedione (diacetyl) and the less significant 2,3-pentanedione (Pires *et al*., 2014). Diacetyl is by far the most examined and arguably the most important, due to its lower flavour threshold of ~0.1ppm compared to ~1ppm for 2,3-pentanedione. Although not an issue for all beer types, production of high levels of diacetyl creates a 'buttery' or 'butterscotch' flavour which is usually considered to be detrimental, especially in light lager beers. Yeast produces α-acetolactate (AAL) in the early stages of fermentation, primarily as a result of the valine synthesis pathway. AAL is subsequently converted to diacetyl by spontaneous de-carboxylation in the wort, before being re-absorbed and converted firstly into acetoin and then into the comparatively 'flavourless' 2,3-butanediol by the yeast cell as fermentation progresses. Reduction of diacetyl to below the threshold level largely dictates the length of time required for maturation, thus influencing the production capabilities of a brewery (Dasari and Kölling 2011; Pires *et al*., 2014).

Production of diacetyl is believed to be connected to import of the preprotein acetohydroxyacid synthase (Ahas), encoded for by the gene ILV2. This gene is upregulated in brewing yeast when starved of valine (and other amino acids), since it is under the control of the general amino acid control pathway. Acetohydroxyacid synthase (Ahas) plays a central role in diacetyl formation as it catalyses the first step in the multi-step

biosynthesis of branched chain amino acids: decarboxylation of pyruvate and condensation with a second molecule of pyruvate or 2-ketobutyrate (Duggleby *et al*., 2000; Feldmann, 2012; Pires *et al*., 2014). The second step involves conversion of α-acetolactate (AAL) into 2,3-dihyroxyisovalerate (Ilv2p), catalysed by acetohydroxyacid reductoisomerase (Ahar). Ilv2p is synthesised in the cytosol and has an N-terminal mitochondrial targeting sequence, which directs the import to the mitochondrial matrix (Dasari and Kölling, 2011). However, Ahas can also be internalized within the mitochondria (Pires *et al*., 2014) and when this occurs some AAL also becomes restricted within the double membrane of the mitochondria. Since there are three membranes to transpose (the plasma membrane, mitochondrial inner (MIM) and outer membrane (MOM)), AAL is prevented from reaching the extracellular environment. Consequently the mitochondrial membrane potential (MMP) plays an important role in diacetyl production since it can limit the extent to which AAL is excreted and thereafter de-carboxylated to diacetyl in the wort. It can be seen that mitochondria function is important in flavour production; the consequences of mitochondrial deficiency on flavour development are discussed further in Section 1.11.1.

1.11 The petite mutation in brewing yeast

Despite the importance of mitochondria for cell function, it is possible for individuals to survive with damaged mtDNA, or even when mitochondria are entirely absent. When this occurs, yeast is described as being 'respiratory deficient'; OXPHOS cannot be conducted and cells cannot grow on non-fermentable carbon sources such as glycerol or ethanol (Day, 2013). The original nomenclature, which still has widespread use, is the term 'petite' mutant, given due to the small size of colonies produced on agar plates (Ephrusi, 1949). Since the identification of respiratory deficient petite mutants, yeast have proven to be an extremely useful tool in unravelling extrachromosomal genetics in a host of eukaryotic organisms (Mell and Burgess, 2003).

In yeast, petites typically arise through cytoplasmically inherited mutations, i.e. the mutated genes are not located within the nucleus. However, despite being less common, there have been reported instances where respiratory deficiency can be induced due to mutations within the nuDNA (Solieri, 2010). Cytoplasmically inherited petites can be referred to as mit^- or ρ^-, while those produced by nuclear mutations are frequently termed pet^- (Coruzzi *et al*., 1979; Tzagoloff and Dieckmann, 1990; Solieri, 2010). Cytoplasmic mutants, can be further subdivided into Rho^- (ρ^-) and Rho^0 (ρ^0) types. Rho^- petites retain some mtDNA, while Rho^0 mutants are completely absent of mtDNA. The mtDNA complement of Rho^- mutants usually consists of amplified sequences of non-coding regions of DNA, which prevents mitochondria from functioning correctly.

The reason why there are two versions is currently unclear, however it is likely that the Rho^0 version may simply arise as a result of the overall physio-chemical instability of the Rho^- genome, or a failure of defective mitochondria to be transferred to daughter cells (Piskur, 1997; Osman *et al*., 2015) (Section 1.8). Of the two mutations, the Rho^- form is most frequently reported, although it should be noted that simple methods for differentiating between the two types are not readily available.

1.11.1The petite mutation in brewing

The petite mutation is arguably the most common yeast mutation observed in the brewing environment. Most brewing yeast slurries contain a number of respiratory deficient cells, at around 1-3% of the population, however there are frequently reported instances where petites accumulate, leading to a number of consequential impacts on performance and flavour development. As described previously (Section 1.11), the susceptibility of mtDNA to damage is much higher than that of the nuDNA. While the exact cause of the mutation(s) in brewing yeast remains unknown, they are likely to occur as a result of the physical, biological, and chemical stresses placed on yeast during the brewing process and during yeast storage (Jenkins *et al*., 2009; Lawrence et al., 2012; Smart, 2013). For example, even though brewing yeast are subject to the Crabtree effect (under which mitochondrial activity is supressed), ethanol accumulation during fermentation has been linked to the production of ROS within mitochondria through an overabundance of iron-sulphur cluster (ISC) associated proteins (Horowitz and

Greenamyre, 2010; Pérez-Gallardo *et al*., 2013; Gomez *et al*., 2014). This also means that respiratory deficient mutants can arise due to alterations in nuclear gene activity that result in mitochondria ISC chaperone proteins such as Atm1p, Hsp70p and Leu2p being under-produced. Interestingly, there is also evidence to suggest that deletions within the mtDNA genome (which results in mtDNA genetic instability), can also result in nuclear DNA instability (Veatch *et al*., 2009; Dirick *et al*., 2014; Kaniak-Golik and Skoneczna 2015). This suggests that in scenarios where cells contain compromised mitochondria there are reduced levels of ISC, and the proteins which mature within the mitochondria are not folded correctly (Veatch *et al*., 2009).

Irrespective of how they are formed, petites can play an important part in determining fermentation consistency and beer quality (Gibson *et al*., 2007; Jenkins *et al*., 2009). Petites are less active compared to their respiratory competent counterparts, divide more slowly, and may be seen to be physiologically weaker (Kominsky *et al*., 2002; Zubko and Zubko, 2014). It is important to note that although there is a lack of hard evidence, some yeast strains appear to be particularly prone to producing the petite mutation. The question remains whether this is an artefact of the strain itself, or the product/process employed for a particular fermentation type. Irrespective, there is much debate over what should be considered an acceptable percentage of petites within a brewing slurry varying. As mentioned above, it is accepted that a low level of petites are likely to exist at a given time, but these are typically at sufficiently small concentrations that their presence goes unnoticed. However there have

been reports of slurries containing much higher levels; Morrison and Suggett (1983) observed that up to 50% of stored yeast were respiratory deficient. If a threshold is exceeded, the primary option is to discard the yeast culture and implement a fresh yeast stream.

The presence of petites does not only impact on yeast growth, fermentation speed and consistency, but can also lead to inappropriate flavour production. In Section 1.10, the role of mitochondria in flavour generation was described. Suffice to say, when mitochondrial function is disrupted this can have a major bearing on flavour development, including esters and higher alcohols. However, of primary importance is the role of mitochondria in the diacetyl synthesis pathway. Under normal circumstances acetohydroxy acid synthase (Ahas) is localised within the mitochondria, causing α-acetetolactate (AAL) to be produced and maintained internally. However, defective (or absent) mitochondria with a reduced MMP do not allow Ahas to be actively sequestered internally. AAL is therefore created within the cytoplasm and more readily diffuses out of the cell where it is converted to diacetyl spontaneously through non-enzymatic oxidative decarboxylation (Duggleby *et al*., 2000; Dasari and Kölling, 2011; Pires *et al*., 2014). Hence it has been suggested that the higher concentrations of diacetyl associated with petite mutants is primarily due to increased excretion of AAL, due to deficient internalization of Ahas in mitochondria (Pires *et al*., 2014). This principle is explained further in Figure 1.12.

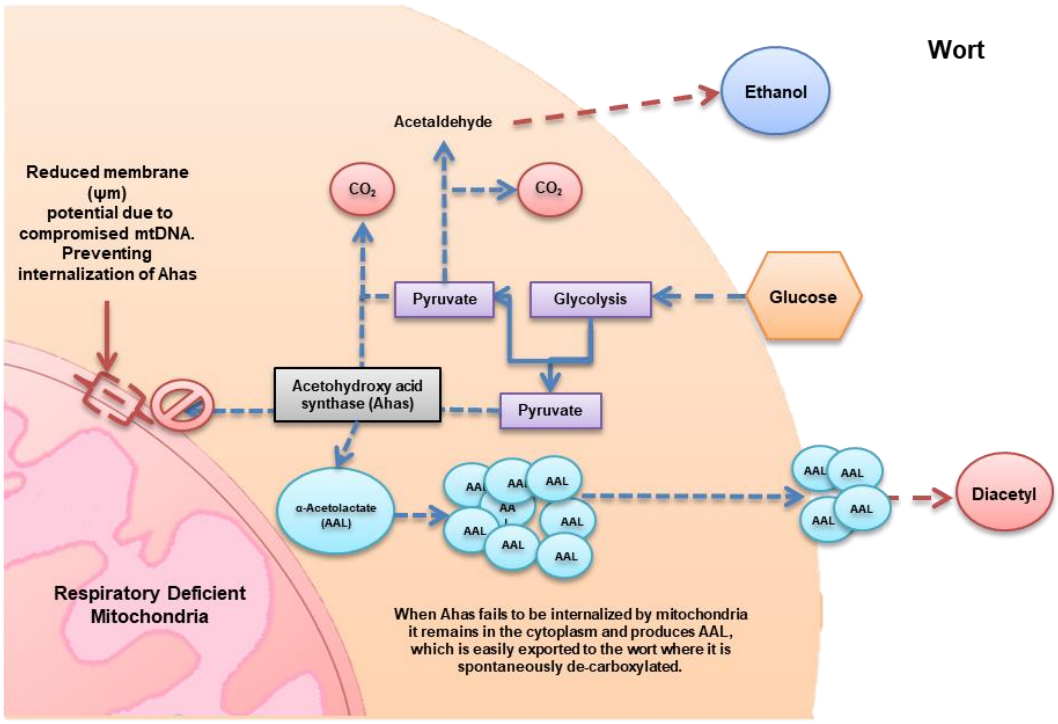


Figure 1.12 Mechanism for excess diacetyl production in petite yeast cells. Reduced membrane potential is associated with unhealthy or non-functioning mitochondria. This can have an impact on key enzyme localisation which, in this instance leads to the over production of diacteyl.

1.11.2 Detection and identification of petites

The 2, 3, 5-triphenyl tetrazolium chloride salt (TTC) overlay technique is the most commonly utilised method for detecting respiratory deficient mutants within the brewery. However, it should be noted that very few breweries employ testing on a routine basis, and the method is typically reserved for trouble shooting purposes. The TTC method requires that colonies of yeast have been cultivated on standard growth media. Following this, the TTC solution is poured over the surface of the plate.

TTC is a colourless redox indicator which is reduced to a red colour as yeast metabolises using the respiratory pathway. Since this does not occur in petite mutants, petite colonies remain white while respiratory competent cells turn pink/red, typically within 2-3 hours.

It is also possible to detect and identify petites based on analysis of the mtDNA. Due to the relatively small size of the yeast mtDNA genome (compared to the nuDNA), a fingerprint can be readily generated using restriction enzymes. This technique is known as restriction fragment length polymorphism (RFLP) and results in petite profiles which typically have less fragments, or smaller sized fragments than the wild-type strain. This method can be used for differentiating between Rho^{-} and Rho^{0} (if combined with other techniques for petite selection first), since theoretically Rho^{0} strains should contain no mtDNA. However, it is not entirely reliable for this purpose as there is a possibility that some fragments of nuDNA may be present at low concentrations. Other methods for detection of petite cells involve fluorescent stains (Chapter 4), however, many of these are not mtDNA specific, and those that are often rely on mitochondrial membrane potential in order to sequester the stain within the organelle. The DNA stain known as DAPI has been shown to be a particularly useful fluorescent dye for visualising mtDNA (Williamson and Fennell, 1979; Lawrence *et al*., 2012) and can be used as a means of detecting Rho^{0} cells. However, although it binds to mtDNA, this stain also attaches to nuclear DNA, which can result in a ‘sun and stars’ scenario where the brighter nuclear DNA bleaches out the less fluorescent mtDNA.

1.12 Aims and objectives

While the role mitochondria play in the survival of most cells/microorganisms is well documented, it is only recently that the role of yeast mitochondria has been more closely scrutinised. The current understanding is that yeast mitochondria are essential in several different cellular functions and for fermentation performance. While petites naturally occur at percentages of 1 – 3 % it is important to note that this not constant across all strains and that some strains are more susceptible to the mutation than others.

This study will firstly examine and characterise the mitochondria of grande and petites by examining their mtDNA profiles, with the aim of better understanding the grande strains mtDNA genomes. Building on this knowledge the rate at which the grandes produce petites will be assessed while also examining the petites impact on fermentation. Further comparative studies of grandes and petites will be undertaken specifically examining variation in mitochondrial content and functionality. Additionally this work will examine what impact the brewing yeast life cycle has on mitochondrial quantity and what changes in mtDNA content occur during fermentation. The primary goal of this work is to better understand the functional role mitochondria play in brewing yeast.

CHAPTER 2 MATERIALS AND METHODS

2.1 Yeast strains

The *S. pastorianus* lager yeast strains designated SMCC100, SMCC99, SMCC90 and SMCC57, and two *S. cerevisiae* ale-type strains referred to as D23 and D3 were obtained from the SABMiller culture collection (Table 2.1) as frozen glycerol stocks.

Table 2.1 Yeast strains used during experiments

Strain	Source	Information
SMCC100	SABMiller	Lager yeast
SMCC99	SABMiller	Lager yeast
SMCC90	SABMiller	Lager yeast
SMCC57	SABMiller	Lager yeast
D23	SABMiller research brewery	Ale yeast
D3	SABMiller research brewery	Ale yeast

2.2 Growth media and storage

2.2.1 YPD and YPGly media

All strains were grown and maintained on YPD media, comprising 2 % (w/v) D-glucose (Sigma, UK), 2 % (w/v) bacteriological peptone (Sigma, UK), and 1 % (w/v) yeast extract in RO (reverse osmosis treated) water). For storage on YPD plates/slopes, the YPD media was prepared with the addition of 1.2 % (w/v) agar (Sigma, UK). In order to assess the respiratory competency of samples (Section 2.3.4), yeast were grown on YPGly media, comprising a YP base supplemented with a non-

fermentable carbon source: glycerol at 2 % (w/v). Following preparation of media, sterilisation was achieved by autoclaving at 121 °C and 15 psi for 15 min.

2.2.2 Giant colony analysis (WLN media)

To examine the giant colony forming morphology of yeast strains, Wallerstein Laboratory Nutrient (WLN) media (ThermoFisher, UK) was prepared by suspending 75 g/L of media in RO-water, before sterilising by autoclaving at 121 °C and 15 psi for 15 min. The cells were diluted to produce only a small number of colony forming units per plate (10-20), and these were then grown at either 25 °C (lager strains) or 30 °C (ale strains) for two weeks.

2.2.3 Wort media

Hopped all-malt wort was obtained from the SABMiller research brewery at 15 °P unless directly specified in the text. Wort was collected immediately post-boil and transferred aseptically to (2 L) pre-sterilised vessels. Wort sugar concentration (degrees Plato) was calculated using a portable density meter (Anton Paar DMA 35, Austria). Once collected and cooled, the wort was frozen at -20 °C until required.

2.2.4 Cryogenic storage of yeast

For each strain, a yeast colony taken from a YPD agar plate was suspended in 1 mL YPD media, containing 25 % (v/v) glycerol as a cryo-protectant. Each of the resulting yeast suspensions was stored and

maintained in a freezer at -80 °C in bespoke cryovials (Sarstedt, UK) until required.

2.2.5 Preparation of working cultures

YPD-glycerol stocks were transferred to 10 mL sterile YPD in a 30 mL Universal bottle (Sterilin, ThermoFisher, UK). The culture was then incubated at 25 °C and 120 rpm for 48 hours in an orbital shaker (Braun Biotech, UK). The 10 mL culture was then added to 100 mL sterile YPD in a conical flask and incubated at 25 °C and 120 rpm for 48 hours.

Unless otherwise stated, working stocks of each strain were cultivated on YPD media comprising 2% w/v glucose (Sigma, UK), 2% w/v peptone (Sigma, UK), 1.5% w/v agar (Sigma, UK), and 1% w/v yeast extract (Sigma, UK). Each strain was cultivated by streaking onto solid YPD media and allowing growth at 25°C for 3-4 days prior to use. For experimental samples, colonies were taken from the agar plates at random, placed in 10 mL of liquid YPD, and shaken at 150 rpm for 3 days. This was then transferred after 3 days to a 250 mL conical flask containing 100 mL of liquid YPD and subsequently after another 3 days to a 2 L flask containing 1 L of YPD.

2.3 Analysis of yeast physiology characteristics

2.3.1 Cell enumeration

Yeast cell concentration was assessed according to ASBC Methods of Analysis Yeast-4 (1988). The yeast cells were washed and then diluted in RO water to reach a density of approximately 1.0×10^7 cells/mL, before

enumeration using a haemocytometer (Neubauer counting chamber; Weber Scientific International Ltd, UK) and a microscope at 400x magnification. The total number of yeast cells within the 1 mm^2 ruled area (25 squares) x 0.1 mm thickness were calculated following a standard protocol: cells which touching or resting on the top and right boundary lines were not counted; budding yeast cells were counted as one cell if the bus was less than half the size of the mother cell. If the bud was equal to or greater than half the size of the mother cells both cells were counted. At least 200 cells were counted to ensure statistical validity and cell density was calculated using the formula below (Equation 2.1).

Equation 2.1 Cell concentration calculation. The value 5 x 10^4 represents a constant based on the dimensions of the counting area.

$$Cell\ concentration\left(\frac{cells}{mL}\right)$$
$$= total\ cells\ in\ ruled\ area\ X\ dilution\ factor\ (if\ required)\ X\ 5x10^4$$

2.3.2 Yeast viability by methylene blue staining

Yeast viability was determined through staining with methylene blue and was conducted according to the method outlined in Pierce (1970). Methylene blue (Sigma, UK) was dissolved in 2 % (w/v) sodium citrate (Fisher Scientific, UK), producing a solution with a final concentration of 0.01 % (w/v). Yeast cell suspensions were diluted using RO water to reach a working concentration of approximated 1 x 10^7 cells/mL (Section 2.3.1). A 0.5 mL aliquot of yeast suspension was mixed with 0.5 mL methylene blue solution and gently agitated by hand. The solution was

then incubated for 5 min at room temperature and examined using a microscope at 400x magnification. Cell viability was measured using a haemocytometer as described previously (Section 2.3.1). Dark blue cells were counted as dead and those that remained unstained were considered viable. The number of live cells was calculated using Equation 2.2 and the number of viable cells expressed as a percentage of the total population.

Equation 2.2 Cell viability calculation

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

2.3.3 Statistical analysis of viability

The statistical significance of grande and petite viabilities was determined through methylene blue staining and data was assessed using an ANOVA in order to examine variance using IBM SPSS statistics software (version 24, IBM, USA). In this instance the null hypothesis was that there was no significant difference between petite and grande viabilities. If the P-value generated was less than 0.05 the null hypothesis of no significant difference was rejected.

2.3.4 Yeast species identification by rapid X-α-gal analysis

In order to characterise ale and lager yeast strains, cultures were grown in the presence of the melibiose analog: 5-bromo-4chloro-3indolyl-α-D-galactopyrandoside (X-α-gal) in liquid form (Box *et al*., 2012). The

principle of the method relies on the ability of lager strains to cleave X-α-gal through the use of melibiase, releasing a blue-green colour colouration (Tubb *et al*., 1985).

A stock solution of X-α-gal (Fisher Scientific, UK) was prepared, consisting of 62.5 mg X-α-gal dissolved in 10 mL sterile RO water containing 25 % (v/v)1, 2-propanediol (Fisher Scientific, UK). Yeast samples were grown in YPD media over 3 days in 10 mL volumes according to standard practice (Section 2.2.1). The yeast was separated from the media by centrifugation at 5,000 rpm for 5 min and washed in 5 mL sterile RO water. From this suspension 100 µL was added to an Eppendorf tube containing 10 µL X-α-gal stock solution. A negative control was also prepared in parallel, containing only the yeast suspension without X-α-gal. All samples were incubated statically at 25 °C for 30 min and then examined for a colour change. Samples with a blue-green hue (i.e. were able to cleave X-α-gal) were considered to be lager strains, while those that remained cream/white were deemed ale strains. Samples were re-examined after 24 hours to provide further assurance and to validate the designation of strains as lager or ale.

2.3.5 Analysis of respiratory deficient petite cells (TTC overlay and glycerol media)

The triphenyltetrazolium chloride (TTC) overlay technique of Ogur *et al* (1957), as described in the ASBC methods of analysis (ASBC, 1992a), was used to assess the level of respiratory deficient (petite) cells in each population. The method uses two solutions termed Solution A and B.

Solution A comprised double-strength phosphate buffer with agar (0.134 M PO_4 pH 7, 3 % agar): 5.04 g NaH_2PO_4 (anhydrous, MW 120.0) (Sigma, UK); 4.64 g Na_2HPO_4 (anhydrous MW 141.96) (Sigma, UK); 12 g agar (Sigma, UK) made to 400 mL with RO-water and sterilised by autoclaving. Solution B comprised 100 mL quadruple strength TTC solution. 0.4 g 2,3,5 triphenyl tetrazolium chloride solution (TTC). Both solutions were sterilised by passing through a 0.2 µm membrane filter and stored in a refrigerator, covered in aluminium foil to protect from the light, for a maximum of 3 months. Solutions A and B were mixed at 45 °C to make the overlay agar. Roughly, 20 mL of the overlay solution was poured onto yeast colonies pre-grown on YPD plates, and left for 3 hours. After this time, red colonies were deemed to be respiratory competent and white colonies were designated as petite mutants. To confirm the identity of petites, cells were streaked on YPGly plates as described previously (Section 2.2.1) and examined for any growth.

2.3.6 Quantification of cellular trehalose and glycogen

Trehalose and glycogen were quantified using a method previously described by Parrou and Francois (1997). In this protocol, glycogen and trehalose are digested to their constituent glucose molecules; glucose is then quantified and used as a measure of the quantity of the derivative complex storage sugars. To achieve this, yeast cells (5 x 10^8 cells/mL) were aliquoted into Eppendorf tubes, in triplicate. Samples were washed 3 times using 1 mL RO-water to ensure that all carbohydrates associated with the growth media had been removed; collection of cells was

achieved by centrifugation at 13,000 rpm for 5 min. After the final wash and removal of supernatant, the pellet was re-suspended in 250 µL of 0.25 M Na_2CO_3. The mixture was incubated for 2 hours at 95 °C in a waterbath, with occasional mixing of the samples. Consequently, 600 µL of 0.2 M sodium acetate pH 5.2 and 150 µL 1 M acetic acid were added to each tube. Each mixture was inverted a number of times to insure proper mixing. A 500 µL aliquot was transferred to a fresh Eppendorf tube and used for analysis of trehalose content, with the remaining 500 µL used for glycogen assessment. Glycogen and trehalose were digested to glucose by adding 10 µL of 10 mg/mL α-amyloglucosidase (from *Asperigillus niger* (Sigma-Aldrich, UK), or 10 µL of 3 mU trehalase (from porcine kidney (Sigma-Aldrich, UK) respectively. Samples were incubated at 57 °C (for glycogen) or 37 °C (for trehalose) for a minimum 8 hours (typically overnight) and collected by centrifugation at 3,500 rpm for 5mins. This enzymatic digestion resulted in the storage sugars being completely broken down into glucose, which was then assessed using a glucose quantification assay kit (GOPOD) (Megazyme, Ireland).

Prior to glucose determination, a ‘blank’ sample was generated by mixing 200 µL sterile filtered 0.45 µm RO-water with 3 mL of proprietary ‘chromogen’ reagent supplied with the GOPOD kit. A standard was also prepared using 100 µL of the Megazyme-supplied glucose reagent, by mixing it directly with 100 µL water and 3 mL of chromogen reagent. Once the standard and blank had been prepared, 100 µL of supernatant from the digested glycogen and trehalose samples were added to 15 mL tubes this was all completed in triplicate. To each of the 15 mL tubes, 100 µL

water and 3 mL of the chromogen reagent were also added and the solution was mixed by inversion. The resulting mixtures, along with the standard and the blank, were incubated at 40 °C for 20 min. The absorbance of each sample was read at 510 nm and the amount of glucose present in each sample (i.e. 100 µL of the original) was calculated using Equation 2.3. To determine the amount of glucose in 1 x 10^8 cells the value was multiplied by 10 (to determine the amount of glucose within 1 mL of sample) and divided by 5 to convert from the value per 5 x10^8 cells/mL to 1 x10^8 as indicated in Equation 2.4.

Equation 2.3. Glucose quantification

$$Amount\ of\ glucose\ (ug\ 0.1\ /\ mL) = \frac{OD\ sample}{OD\ standard}\ x\ 100$$

Equation 2.4. Quantification of glucose per 1X10^8 cells/mL

$$Amount\ of\ glucose\ per\ 1x10\text{^}8\ cells$$
$$= Amount\ of\ glucose\ in\ 0.1\ mL\ x\ 10/5$$

2.3.7 Determination of cell membrane potential

Cell membrane fluidity was measured using the staining methodology described by Hewitt and Nebe-Von-Caron (2000) and by Simonin *et al.* (2007), in conjunction with flow cytometry. Propidium iodide (PI) was utilised to provide an assessment of the overall integrity of the cell membrane; the mode of action requires that the cell membrane be permeable for effective staining of DNA by PI. Bis-oxonol (BOX) was

also used to evaluate changes in the cell membrane potential, since this stain is able to enter depolarised cells, where it can bind to proteins. This causes an increase in fluorescence and a shift from green/yellow to red (Simonin *et al.,* 2007), which can then be quantified. Stain details, excitation and emission spectra and binding locations are summarised in Table 2.2.

Table 2.2 Fluorescent stains for examination of membrane fluidity

Stain	Ex (nm) / Em (nm)	Binding location
Propidium Iodide	535/617	Stains nucleic acid
Bis-oxonol (BOX)	530/560	Enters depolarised cells binding to intracellular proteins

To determine membrane fluidity, cells were first grown in YPD media for 3 days as described previously (Section 2.2.1.). Cells were then collected and washed three times using 1X phosphate buffered saline, pH 7.5 (PBS) (ThermoFisher, UK) in order to remove any residue from growth media. Cells were diluted to 1 X 10^7 cells/mL in a total volume of 10 mL, and a series of 1 mL aliquots were taken as control samples. The first was exposed to 70% ethanol for a period of 45 min, to kill cells prior to staining. Exposure to ethanol is required to 'fix' the cells so that when analysed using a flow cytometer dead cells can be 'gated', allowing only living cells to be analysed. A second sample was prepared by mixing a

50/50 solution of living and dead cells in an Eppendorf tube, also used for ‘gating’. A third aliquot, comprising only live cells was maintained on ice.

Once cells were prepared (control and experimental samples), they were washed in 1X, pH 7.5 (PBS) (ThermoFisher), and resuspended at a final concentration of 1 x 10^6 cells/mL. Aliquots of 1 mL were then labelled by dual staining with 10 µL of PI and 3 µL of BOX. In order to facilitate staining with BOX, 4 mM EDTA was also added to the samples. Cells were then incubated at 25 °C for 30 min and analysed using an Astrios cell sorter (Beckman Coulter, UK). The dead and 50/50 live/dead samples were run first to allow gating to be implemented for subsequent experimental cell samples. Once initial gates had been designated, experimental samples were stained and analysed using the same procedure described above.

2.4 Yeast growth and fermentation analysis

2.4.1 Yeast propagation

In order to produce sufficient yeast biomass to pitch into fermentations, yeast cultures were grown from pure isolated colonies taken from YPD agar. These were placed into 10 mL of sterile filtered wort (0.45 µL filter; Minisart, Fisher scientific, UK) and incubated at 25 °C, 150 rpm over 48 hours under aerobic conditions. The culture was then transferred to a sterile 250 mL conical flask sealed with a foam bung, containing 100 mL wort. After an additional 48 hours, the yeast was transferred to a sterile

2 L conical flask containing 1 L of wort and incubated over 48 hours under the same conditions.

2.4.2 Yeast pitching

After 48 hours growth in 1 L of wort (Section 2.2.4) the yeast slurry was transferred to pre-weighed vessels 50 mL and centrifuged at 5,000 rpm for 5 min. The resulting supernatant was removed and the vessel weighed again. The yeast concentrate was then re-suspended in a 50:50 mix with sterile RO-water. The number of viable cells was determined using methylene blue staining (Section 2.3.2) and cells were pitched into different volumes of wort at a rate of 1 x 10^6 cells/mL per °P.

2.4.3 Analysis of yeast growth characteristics

To establish the growth characteristics of yeast isolates under different growth conditions, yeast cultures were monitored using 96-well plates. Each well contained 15 °P hopped wort or YPD, unless otherwise stated, and cell concentration was tracked by optical density, determined by an automated microplate reader TECAN (Infinite ® 200 PRO series, TECAN, UK). The OD was measured at 600 nm wavelength every 15 minutes until stationary phase had been established. Data was analysed and collected using Magellan™ Data analysis software (TECAN, UK).

For stress tolerance determination, 96-well plates were supplemented with ethanol (Table 2.3) or hydrogen peroxide (Table 2.4). Cells were incubated for 46 hours and the data was analysed and collected using Magellan™ Data analysis software (TECAN, UK). When fermentation

performance analysis was required, the media detailed in Tables 2.3 and 2.4 were employed in conjunction with mini-fermenters as described in Section 2.4.6 below.

Table 2.3 Preparation of wort-ethanol mixture for determination of stress impact on growth

Wort - Ethanol			
Component	Wort + 0 %	Wort + 5 %	Wort + 10 %
15 °P Wort	197 µL	187 µL	177 µL
99.9 % Ethanol	-	10 µL	20 µL
Cell suspension	3 µL	3 µL	3 µL
Total Volume	200 µL	200 µL	200 µL

Table 2.4 Preparation of wort-H_2O_2 mixture for determination of stress impact on growth with control as seen above

Wort - H_2O_2			
Component	Wort + 1 mM	Wort + 3 mM	Wort + 6 mM
15 °P Wort	195 µL	192 µL	185 µL
100 mM H_2O_2	2 µL	6 µL	12 µL
Cell suspension	3 µL	3 µL	3 µL
Total Volume	200 µL	200 µL	200 µL

2.4.4 Oxygenated small scale (5 Litre) laboratory fermentations

Wort was produced and collected in the research brewery as described above (Section 2.2.4). Prior to use, wort was oxygenated with constant agitation (350 rpm) for 24 hours to maximise dissolved oxygen (DO) levels. Oxygen was applied through a HEPA filter (0.1 µm pore size) and sparged into the wort continuously (Figure 2.1). Simultaneously, the wort was brought to the temperature required for fermentation: 15 °C for lager and 18 °C for ale fermentations, by placing the bioreactor in a water bath (Grand, UK), and mixed using a waterproof stir plate (Variomag, USA).

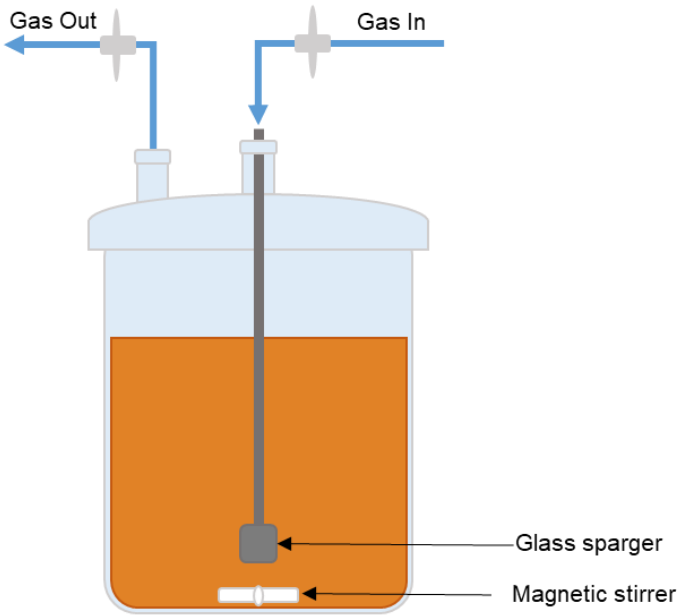


Figure 2.1 Oxygenation procedure used for wort preparation.

2.4.5 Small scale (100 mL) laboratory fermentations (mini fermentations)

Mini fermentations were performed using 150 mL glass hypovials according to the method outlined by Quain *et al*. (1989) and Powell *et al*. (2003). Vials containing a magnetic stirrer were autoclaved at 125°C and

16 psi prior to use. Once autoclaved, 100 mL of wort (Section 2.2.4.) was added to hypovials and an appropriate concentration of yeast was added; yeast was pitched at a rate of 1 x 10^6 cells/mL per °P. Hence for a 15 °P wort, a total of 1.5 x 10^7 cells/mL was used. Following pitching, vials were made air-proof using a rubber septum and sealed with a metallic crimp. In order to allow the escape of carbon dioxide produced during the fermentation a Bunsen valve was fashioned. This was achieved using a Durham tube placed within some silicon tubing with a cut slit opening, and a sterile needle piercing the rubber septum (Figure 2.2). Once prepared, mini fermentations were transferred an incubator set at 15 °C (for lager fermentations) or at 18 °C (ale fermentations). In all instances, vials were placed on stir plates set to 300 rpm to prevent settling of the yeast culture.

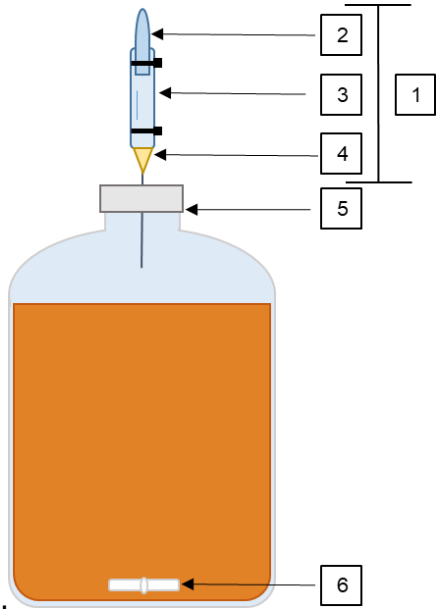


Figure 2.2 Mini fermentation vessel design (100mL working volume). 1: Bunsen valve; 2: Glass Durham tube; 3: Silicon tubing with opening; 4: Needle; 5: Metallic crimp covering rubber septum; 6: Magnetic stirrer.

Mini-fermentations were used primarily to assess strain performance under defined conditions. However, on occasion they were also used to determine the response of isolates to stress, using the 'stress media' described in Section 2.4.3, and compared to a base 15 °P wort as a control.

2.4.6 Small scale (10 L working volume) laboratory fermenters (Infors)

Fermentations were conducted using 15 L capacity stainless steel fermenters (Techfors-S, Infors-HT, Switzerland) with a working volume of 10 L (Figure 2.3). Infors fermenters were used when it was necessary to monitor and control pH, temperature, dissolved oxygen (DO), and stirring rate throughout the fermentation. For these fermentations, temperature was maintained at 15 °C (lager profile) and oxygen was adjusted to 1ppm per °Plato before pitching at 1.5 x 10^7 cells/mL. Conditions during fermentation were maintained using the Infors interface; pH and DO content were monitored remotely to ensure consistency across the fermentations. When required, samples were taken at regular intervals by withdrawing wort and yeast through a collection port at the bottom of the fermenter and stored at -80 °C until needed. The sample port was sterilised using 70 % ethanol before and after sampling. The samples were collected from the three Infors in triplicate.

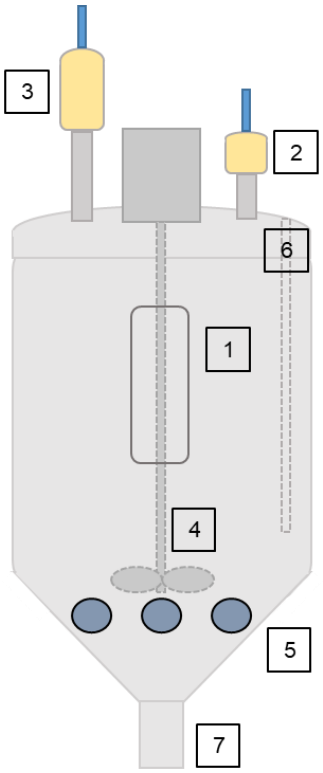


Figure 2.3 Infors fermenter configuration. 1: Viewing port; 2: Gas inflow passed through a HEPA filter; 3: Gas outlet, first through a condenser then a HEPA filter; 4: Impeller to achieve required mixing; 5: Ports for pH probe, and DO probe; 6: Temperature probe; 7: Sampling port, which can be steam sterilised or flushed with ethanol.

2.4.7 Quantification of volatile fermentation compounds using GC-MS

Fermentation samples were chilled to 4 °C and sonicated for 10 seconds in a sonicator bath (Fisher Scientific, UK), set to ‘degas’. After sonication 10 mL of each sample was filtered using a 0.45 µm and transferred to a GC vial (Fisher Scientific, UK) along with 50 µL of internal standard (1-butanol at a concentration of 1.6 mg/ L) and 3.5 g sodium chloride. Vials were sealed immediately by crimping with a GC vial cap (Sigma Aldrich, UK).

GC-MS beer volatile analysis was determined using the method described by Analytica-European Brewing Convention (EBC) (9.39) (2000). Lower boiling volatiles were analysed with a Scion 456-Gas Chromatograph (Scion Instruments, West Lothian, UK): samples (500 µL) were injected in splitless mode using a PAL Combi-XT autosampler (PAL System, Zwingen, Switzerland) onto a Zebron ZBWax column (60 m x 0.25 ID, Phenomenex Inc, Cheshire, UK). The GC carrier gas was helium, at a constant pressure of 15psi. The initial oven temperature was 85 °C for 10 mins, which was then increased to 110 °C for 13 mins at a rate of 25 °C/min, before finally being increased to 200 °C for 13.25 mins at a rate of 8 °C/min.

Table 2.5 Volatile compounds detected by GC-MS and their detection range

Volatiles	Detection Range
3-Methyl-1butanol	10 - 100 ppm
Isobutanol	5 - 50 ppm
Propanol	5 - 50 ppm
Acetaldehyde	10 - 100 ppm
Isoamyl acetate	0.5 - 5 ppm
Ethyl acetate	10 - 100 ppm
Ethyl Hexanoate	0.1 - 1 ppm
Ethyl Octanoate	0.1 - 1 ppm

When examining VDKs, the method used was broadly based on the Analytica-European Brewing Convention (EBC) (9.24.2) (1999). Lower boiling volatiles were analysed with a Scion 456-Gas Chromatograph (Scion Instruments, West Lothian, UK). Samples (500 µL) were injected in splitless mode using a PAL Combi-XT autosampler (PAL System, Zwingen, Switzerland) onto a Restek Rtx-5MS (Restek, US) (Crossbound 5 % diphenyl/95 % dimethyl polysiloxane) 30 m, 0.25 mm ID, 0.8 µm df column. The GC carrier gas was helium, at a constant pressure of 50 psi. The initial oven temperature was 30 °C for 2 mins, which was then increased to 120 °C for 2 mins at a rate of 70 °C/min, before finally being increased to 200 °C for 13.25mins at a rate of 8 °C/min. The sample preparation was identical to that described for volatile analysis, with the exception that the internal standard comprised 5 mg/L 2, 3 hexanedione. All samples were examined in triplicate.

2.5 Petite mutation collection, analysis and induction

A number of naturally occurring petites were isolated from brewery yeast samples taken from fermentations and propagation using TTC media, as described in Section 2.3.3. In addition, laboratory-grown cultures were assessed in order to determine the 'natural' rate of petite production in non-stressed samples (Section 2.5.4). Finally, petite mutants were also obtained by actively inducing their production using starvation media (Section 2.2.2), and by applying the mutagen ethidium bromide (Section 2.5.3).

2.5.1 Assessment of petite production rate

In order to assess the rates at which different brewing strains 'naturally' yielded respiratory deficient mutants, each yeast strain was first grown in YPD under optimum conditions (Section 2.4.1). Cells were then plated on YPD media in triplicate and left to grow at 25 °C for 3 days. When sufficient colony growth had occurred (typically ~1000 colonies for each strain), the plates were examined for petite production using the TTC overlay method described in Section 2.3.4. The number of petites was expressed as a percentage of the entire population.

2.5.2 Induction of petites through glucose starvation

Starvation is a stress that is commonly experienced by yeast during the brewing process (Wallis *et al*., 1972) and is also known to lead to the generation of petite cells (Wallis and Whittaker, 1974). This principle was applied in order to generate petites in the laboratory. Yeast cells were grown in 10 mL YPD media in a shaking incubator at 25 °C and 120 rpm for 48 hours. The cells were then washed three times using RO-water and enumerated using the method described in Section 2.3.1. An aliquot of 2 x 10^6 viable cells was then incubated in 25 mL of STAR media (Section 2.2.2) under the same conditions for 48h. Cells were then cultivated on YPD agar at 25 °C until colonies were observed and the plate was then overlaid with TTC in order to identify petites (Section 2.3.4). Any petite colonies present were isolated and their respiratory status was confirmed by monitoring their inability to grow on YPGly agar.

Since glycerol is a non-fermentable carbon source, petites were unable to grow on this media.

2.5.3 Induction of petites through exposure to ethidium bromide

Application of the mutagen ethidium bromide to induce respiratory deficiency in yeast cells is a well-established procedure. The method applied here was based on those described previously by Rickwood (1991) and Schneider-Berlin *et al.* (2005). Yeast suspensions were pre-grown in YPD as described in Section 2.2.1 and cells were washed in sterile RO water. Viability was examined using the procedure described in Section 2.3.1 and 2 x 10^6 viable cells/mL were inoculated into 25 mL YPD medium containing 100 µg/mL ethidium bromide (Invitrogen, UK). The cultures were protected from light by covering the flasks in aluminium foil, and incubated at 25 °C, with agitation at 120 rpm over 5 hours. The cultures were washed in sterile RO water and cells were grown on YPD agar for TTC overlay analysis, and on YPGly for respiratory status confirmation in an identical fashion to that described in Section 2.3.4.

2.5.4 Induction of petites through exposure to ethanol

Ethanol has been shown previously to damage mtDNA (Ibeas and Jimenez, 1997). With this in mind, yeast slurries were pre-grown in YPD media as described in 2.2.1 and cells were then washed using RO water. The viability was determined using the procedure described in Section 2.3.1 and and 2 x 10^6 viable cells/mL were inoculated into 25 mL YPD containing 5 % ethanol and 10 % ethanol. These were then incubated at 25 °C, with agitation at 120 rpm over 24 hours. The cultures were washed

in sterile RO water and cells were grown on YPD agar for TTC overlay analysis, described in Section 2.3.4.

2.6 Genotypic examination of yeast

2.6.1 Nuclear DNA extraction

To obtain genomic DNA, the nuDNA extraction method described by Legras and Karst (2003) was employed. Yeast was propagated in 10 mL YPD (Section 2.2.1) for 24-48 hours at 25 °C. A 2 mL aliquot was removed and placed in a screw cap cryotube (Sarstedt, UK) rather than an Eppendorf as the sample will be later vortexed with phenol. The sample was then centrifuged at 5,000 rpm for 5 min. The pellet was resuspended in 400 µL lysis buffer (10 mM Tris pH 7.6, 1 mM EDTA, 100 mM NaCl, 2 % (w/v) Triton X-100, 1 % (w/v) sodium dodecyl sulphate (SDS). To the cell suspension, 400 µL of phenol/chloroform/isoamayl alcohol 25/24/1 (v/v) (Sigma-Aldrich, UK) was added along with 0.5 g glass beads (0.45 – 0.55 mm diameter, acid washed) (Sigma-Aldrich, UK). The cell suspension was then vortexed for 4 mins. Once mixed, 200 µL of Tris EDTA (10 mM Tris, 1 mM EDTA) pH 7.6 buffer (TE buffer) was added before centrifuging for 5 min at 6,000 rpm. The resulting upper phase was carefully pipetted and transferred into a fresh Eppendorf tube, and 500 µL of chloroform/isoamyl 98/2 (v/v) was added. The samples were then inverted gently and the mixture was centrifuged at °, 000 rpm for 2 min. The aqueous phase was removed and transferred to a fresh Eppendorf on ice, where two volumes of absolute ethanol were added in order to precipitate DNA. The resulting mixture was then centrifuged at

14,000 rpm for 5 min. The supernatant was discarded leaving a DNA pellet, which was air-dried for 15 min. The pellet was dissolved in 50 µL 10 mM TE buffer pH 8.0 (Fisher Scientific, UK) and either used immediately or stored at -20 °C until required.

2.6.2 Internal transcribed spacer region (ITS) PCR for species identification

In order to identify yeast to the species level, nuclear DNA was extracted as described above (Section 2.6.1) and the ITS regions characterised by PCR-RFLP (restriction fragment length polymorphism) using the primers detailed in Table 2.6 (White *et al*., 1990) and the restriction enzymes shown in Table 2.7 (Guillamón *et al*., 1998). PCR reactions were conducted in a final volume of 50 µL, comprising 31 µL molecular grade water, 10 µL 5X Phusion HF buffer (NEB, UK), 1.5 µL 50 mM $MgCl_2$, 1 µL of 10 µM dNTPs, 0.5 µL of each primer (0.5 µM), 5 µL of extracted nuDNA, and 0.5 µL Phusion DNA polymerase (NEB, UK). The DNA was then amplified using a thermocycler Techne TC-512 (Cole-Parmer, UK) with the following conditions: Initial denaturation 95 °C for 15 min, 35 cycles of denaturing at 95 °C for 1 min, annealing at 55 °C for 2 min, elongation at 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. The thermocycler was set to hold indefinitely at 4 °C on completion.

Table 2.6 Primer sequences used for ITS PCR

Primer Name	Primer sequence
ITS1	5'–TCCGTAGGTGAACCTGCGG–3'
ITS4	5'–TCCTCCGCTTATTTGATATGC–3'

A portion of the amplified ITS region was retained, while samples were also digested using the restriction enzymes outlined in Table 2.7, each in separate reactions. The RFLP mix consisted of 12 µL of amplified DNA product, 1.5 µL restriction enzymes as supplied and 1.5 µL of the relevant reaction buffer (NEB, UK). In each instance the ITS product was incubated with the restriction enzyme at 37 °C for 1 hour. Restriction digests were resolved, along with the retained PCR product, on agarose gel (1.5 % (w/v) (Sigma-Aldrich, UK)) pre-stained with 0.2 µg/mL ethidium bromide (Invitrogen, UK) using gel electrophoresis at 80mV for 80 minutes. The DNA fragments were visualised under UV light using a GelDoc-IT®2 imaging system (UVP LLC, USA) and the sizes (base pairs) of resulting bands were determined against a 1 kb and a 100 bp ladder (Promega, UK). Species identification was confirmed by comparison to the literature, and specifically to the expected banding profiles reported by Esteve Zarzoso *et al*. (1999) and Guillamon *et al*. (1998).

Table 2.7 Restriction enzymes used for digestion of the yeast ITS region

RFLP	
Enyzme	**Restriciton sequence**
Cfo I	5'-GCG/C-3'
Hae III	5'-GG/CC-3'
Hinf I	5'-G/ANTC-3'

2.6.3 Inter-delta PCR for strain differentiation

Yeast strains were identified using inter-delta PCR according to the method of Legras and Karst (2003). Nuclear DNA was extracted using the method outlined in Section 2.6.1 and inter-delta regions of yeast DNA were amplified using the primers specified in Table 2.8. PCR reactions were conducted in a final volume of 50 µL, comprising 31 µL molecular grade water, 10 µL 5X Phusion HF buffer (NEB, UK), 1.5 µL 50 mM$MgCl_2$, 1 µL of 10 µM dNTPs, 0.5 µL of each primer (0.5 µM), 5 µL of extracted nuDNA, and 0.5 µL Phusion DNA polymerase (NEB, UK). DNA was amplified using an thermocycler Techne TC-512 (Cole-Parmer, UK) using the following conditions: Initial denaturation 95 °C for 4 min, 35 cycles of denaturing step 95°C for 60 sec and annealing at 55 °C for 30 sec, elongation at 72°C for 120 sec with a final elongation step at 72 °C for 10 min. The thermocycler was set to hold indefinitely at 4 °C.

Table 2.8 Primer sequences used for inter-delta PCR

Primer Name	Primer sequence
Delta12	5'–TCAACAATGGAATCCCCAC–3'
Delta21	5'–CATCTTAACACCGTATATGA–3'

PCR products were resolved on a 1.5 % w/v agarose gel (Sigma-Aldrich, UK), pre-stained with 0.2 µg/mL ethidium bromide (Invitrogen, UK), at 80 mV for 80 minutes. DNA fragments were visualised under a UV light using a GelDoc-IT®2 imaging system (UVP LLC, USA) and the sizes (base pairs) of each DNA band were determined against a 1 kb and 100 bp ladder (Promega, UK).

2.6.4 Mitochondrial DNA extraction

Pure mtDNA was extracted using a modified method based on Defontaine *et al.* (1991) and Nguyen, (2000). Cells were grown in YPD media and collected by centrifugation at 3,000 rpm. The resultant yeast pellet was washed 3 times with sterile RO water and pelleted by centrifugation (3,000 rpm for 5 min). Cells were then re-suspended in 500 µL of solution A (1.2 M sorbitol and 50 mM EDTA, both from Fisher Scientific, UK) and 10 µL of β-mercaptoethanol (Sigma-Aldrich, UK.) prior to incubation at 37°C for 10 min. The cells were pelleted by centrifugation (3,000 rpm for 5 min) and re-suspended in 500 µL solution B (0.5 M sorbitol, 10 mM EDTA and 50 mM tris-HCL, all Fisher Scientific, UK) with 20 µL of 10 mg/mL lyticase enzyme (Sigma-Aldrich, UK). Cell

suspensions were then incubated at 37°C for 1 hr before being sonicated using a Soniprep 150 plus (MSE, UK) at 19 KHz for a total of 30 sec, with a 15 sec interval (i.e. 15 sec sonication, 15 sec pause, and 15 sec sonication). Cell matter was pelleted by centrifugation (2,000 rpm for 5 min) and the supernatant, this fraction contained the isolated mitochondria, and was retained. This fraction was then centrifuged again (13,000 rpm at 4 °C for 10 min) and the supernatant discarded, leaving the isolated mitochondria. The mitochondrial pellet was re-suspended in 175 µL solution B, supplemented with 5 µL DNAase, and 20 µL DNAse buffer (NEB UK). The solution was incubated at room temperature for 10 min and the suspension was washed with solution B to remove DNAase and any residual genomic DNA. Mitochondria were collected by centrifugation (15,000 rpm at 4 °C for 10 min) and the supernatant discarded. The pellet was re-suspended in 5 µL RNAse and 500 µL lysis buffer (100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, 4 mM Sodium lauroyl sarcosinate, all from Fisher Scientific, UK) to which 500 µL phenol-chloroform (Sigma-Aldrich, UK) was added, and mixed by gentle pipetting. The suspension was centrifuged (15,000 rpm at 4 °C for 5 min) and the supernatant retained, to which 600 µL chloroform was added. The suspension was centrifuged (16,000 rpm at 4 °C for 5 min) and the aqueous phase retained. 25 µL of 5 M NaCl and 1 volume of Isopropyl-alcohol were added to the solution and incubated at room temperature for 30 min. The precipitated DNA was then pelleted (16,000 rpm at 4 °C for 30 min) and subsequently washed with 600 µL of 75 % ethanol, before finally being re-suspended in 40 µL RO water. The mtDNA was then

either examined directly after extraction using RFLP or stored overnight at 4 °C.

2.6.5 Mitochondrial DNA RFLP

A NanoDrop ND-1000 spectrophotometer (ThermoFisher, UK), was used to determine the initial extracted mtDNA concentration and this was then adjusted to 500 µg/µL using RO water. The mtDNA was then cut, in separate reactions, using HaeIII, Hinfi or DdeI restriction enzymes using the protocol described in Section 2.6.3. The enzymes chosen to digest whole mtDNA genomes were selected since they show a preference for cutting around G/C sequences in Table 2.9. These are known to be relatively rare within the mtDNA, limiting the total number of fragments obtained (Dieckmann and Gandy, 1987). The digestion reaction comprised 12 µL of mtDNA, 1.5 µL of restriction enzyme as supplied and 1.5 µL of the relevant reaction buffer supplied with each enzyme. The mixtures were incubated at 37 °C for 30 min and digested fragments were separated on a 1.5 % (w/v) agarose gel. Gels were run at 80 mV for 2.5 hours and visualised under UV light using light using a GelDoc-IT®2 imaging system (UVP LLC, USA). MtDNA restriction fragment sizes were determined against a 1 kb ladder (NEB, UK).

Table 2.9 RFLP enzymes used to examine mtDNA fingerprints

RFLP	
Enyzme	Restriciton sequence
Hae III	5'-GG/CC-3'
Hinf I	5'-G/ANTC-3'
Dde I	5'-C/TNAG-3'

2.6.6 Mitochondrial genome sequencing

MtDNA was extracted from strains SMCC100, SMCC99, SMCC90, SRB3 and D23 using the method outlined above (Section 2.4). 5 µg of mtDNA was subsequently sent for sequencing at DEEPSeq, University of Nottingham. Analysis at DEEPSeq included library preparation of samples, as well as reconstruction and examination of mtDNA genomes for single nucleotide polymorphisms (SNPs), and insertions and deletions when aligned against the sequences described in Table 10. Although the mtDNA extraction method applied resulted in mtDNA which was pure enough for sequencing, it should be noted that some samples occasionally contained DNA which did not match the 'mitochondrial' reference. This was therefore presumed to be contaminating nuDNA and the data was excluded; all results shown were mapped against the previously sequenced *S. pastorianus and S. cerevisiae* mtDNA genomes (Table 2.10). The resulting data was examined using Unipro UGene software. Genome annotation was completed using GeSeq, a web-based

interface using Javascript developed for annotation of organelle genomes (Tillich *et al.,* 2017).

Table 2.10 Reference genomes for *S. pastorianus* and *S. cerevisiae*

S. pastorianus	Nakao *et al.,* 2009	http://www.ncbi.nlm.nih.gov/nuccore/EU852811.1
S. cerevisiae	Foury *et al.,* 1998	https://www.ncbi.nlm.nih.gov/nuccore/NC_001224.1

In order to achieve greater insight into the extent of this variation, each sequence was examined for insertions/deletions (INDELs), and single nucleotide polymorphisms (SNPs) using SnpEff an open source tool which annotates variants.

2.7 Enumeration and examination of mitochondria

2.7.1 Mitochondria staining

Typically cells were imaged live and not fixed, unless specifically stated. However, to create fixed cells, populations were exposed to 4 % paraformaldehyde (Sigma, UK) for 30 min at room temperature. Once fixed the cells were washed using flow cytometry grade 1X PBS (ThermoFisher, UK) and stained as for live cells using the procedure below.

The stains Rhodamine B hexyl ester and MitoTracker FM were used to provide information regarding the total mitochondrial content of yeast cells. Rhodamine 123 and $DiOC_6(3)$ were used to examine mitochondrial

membrane potential, where an increase in fluorescence represented an increase in the mitochondrial membrane potential (MMP) within the cell population. All fluorescent stains were obtained from ThermoFisher – Molecular Probes Invitrogen™ (UK) as part of the 'Probes for Yeast Mitochondria' kit. This kit comprised a number of different stains, which were optimised prior to use as detailed in Table 2.11. Briefly, the optimum working concentration of Rhodamine123 was found to be 45 µM, while Rodamine B hexyl ester and MitoTracker FM were found to produce more consistent staining at a concentration of 200 nM. All stains with the exception of $DiOC_6(3)$ were dissolved in DMSO; $DiOC_6(3)$ was re-suspended in ethanol according to the manufacturer's recommendation.

Table 2.11 Stain info and manufacturers recommended working concentrations for stains used

Stain	Ex (nm) / Em (nm)	Recommended Concentration	Working Concentration	Binding location
Rhodamine123	505/534	30-50 µM	45 µM	Mitochondrial membrane
Rhodamine B hexyl ester	555/579	100 nM	200 nM	Mitochondrial membrane
MitoTracker Green FM	490/516	100 nM	200 nM	Mitochondrial membrane
DiOC6(3)	484/501	200 µM	10µM	Mitochondrial membrane
Syto18	468/533	10 nM	200nM	Mitochondrial DNA

Prior to staining, yeast populations were enumerated and aliquots comprising 1x 10^6 cells/mL were prepared in either 10 mM HEPES buffer solution, pH 7.4, with 5 % glucose (all stains except Rhodamine 123), or 50 mM sodium citrate buffer, pH 5, with 5 % glucose (Rhodamine 123 only). Both sets of buffer were sterilised by autoclaving at 121 °C and 15 psi for 15 min and filtered using a 0.45 µm filter (Sarstedt, UK) prior to use. Filtering was performed to remove as many potential artefacts as possible before visualisation. Regardless of the stain, cells were incubated for 30 min at 25 °C with gentle shaking at 120 rpm. Once the cells had been sufficiently stained, they were centrifuged at 4,000 rpm for 5 min in a swing rotor centrifuge ALC PK120 (ALC, UK). A swing rotor centrifuge was used in order to reduce any potential impact a fixed motor might have on yeast cell shape.

2.7.2 Mitochondria visualisation using structured illumination microscopy (SIM)

Following staining (Section 2.7.1), 5 µL of yeast cells were washed using 2 µm filtered RO-water (0.45 µm filter (Sarsteadt, UK) and placed on a glass slide (75 x 25 mm pre-cleaned, Corning, US), covered with a cover slip (18 x 18 mm, Ziess, Switzerland), and sealed using Covergrip™ (Biotium, UK). Yeast mitochondria and mtDNA were observed within living or fixed cells using a Zeiss Elyra Super Resolution Microscope (Zeiss, Switzerland). In order to visualise mitochondria in living cells a x63 objective (water) was used and for fixed cells a x100 (oil-immersion) lens was employed. Image processing software (ZenBlack, Zeiss)

allowed creation of 3D images of yeast mitochondria and mtDNA. The open source software known as FIJI (image processing package based on ImageJ, available from the Git repository) was also used to further examine the mitochondrial content of yeast strains, using the images produced using the ZenBlack software (Zeiss, Switzerland).

2.7.3 Analysis of mitochondria within yeast populations using flow cytometry

Mitochondrial dynamics within populations of yeast cells were assessed using flow cytometry. This was performed in conjunction with the stains Rhodamine B hexyl ester and MitoTracker Green FM to determine the mitochondrial mass of individual yeast cells, while mtDNA content was assessed using Syto18. All staining was conducted as described previously Section 2.7.1. and cells were subsequently washed with PBS buffer and diluted to $5x10^6$ in PBS. Samples were examined using a Beckman Coulter: Astrios EQ cell sorter (Beckman Coulter, UK). The Astrios EQ cell sorted has 7 laser/ 7 pinholes which are configurable between wavelengths 355 nm, 405 nm, 488 nm, 532 nm, 560 nm, 592 nm, and 645 nm. The number of cells to be examined per sample was set to 20,000 cells and Kaluza software (Beckman Coulter, UK) was used to analyse the data collected. In order to examine variance the data was subjected to an ANOVA using IBM SPSS statistics software (version 24, IBM, USA).

2.8 Production of cybrid yeast strains

2.8.1 Protoplast production and mitochondrial isolation.

To generate cybrid yeast cells, containing genomic DNA from the designated 'parental' strain and mitochondria from a donor strain, a method adapted from Sulo *et al.* (1989) was applied. The lager yeast SMCC57 was used as the parental 'receiver', and the ale strain D23 acted as the donor yeast. Each strain was grown to stationary phase in YPD (Section 2.2.1) and approximately 1 $X10^9$ cells were collected by centrifugation at 3,000 rpm in 50 mL centrifuge tubes for 5 min at room temperature. The supernatant was removed and each pellet was re-suspended in 10 mL 1 M sorbitol, 25 mM EDTA pH8, 50 mM dithiothreitol (DTT) and pelleted again by centrifugation 3,000 rpm for 5 min, discarding the supernatant. The cells were then washed with 10 mL 1 M sorbitol twice and centrifuged for 5 min at 3,000 rpm. The pellet was re-suspended in sorbitol/citrate buffer (1 M sorbitol, 1 mM EDTA, 10 mM sodium citrate, pH 5.8). From this suspension, 1 mL aliquots were prepared and, to each, 15 µL 10 mg/mL lyticase enzyme from *Arthrobacter luteus* was added. The resulting suspension was incubated for 70 min at 30 °C.

The receiver strain was centrifuged at 3,000 rpm for 10min, and the pellet re-suspended in TSC buffer (10 mM Tris-HCL pH 7.5, 1 M sorbitol, 10 mM $CaCl_2$) and incubated at 30 °C for 10 min. The donor strain protoplasts were centrifuged at 3,000 rpm for 5 min. After removing the supernatant, the pellet was lysed using a lysis buffer (100 mM NaCl, 10

mM EDTA, 50 mM Tris-HCl, 4 mM sodium lauroyl sarcosinate, all from Fisher Scientific, UK). To insure all of the protoplasts had lysed, this was performed for 30 min at room temperature. The cell suspensions were then sonicated using a Soniprep 150 plus (MSE, UK) at 19 KHz for a total of 15 sec. The resulting mixture of mitochondria and cell debris was pelleted by centrifugation (2,000 rpm for 5 min) and the supernatant containing the mitochondria was retained, while cell debris and larger organelles (for example, the nucleus) within the pellet were discarded. The supernatant (mitochondrial fraction) was then centrifuged at 13,000 rpm at 4 °C for 10 min and resulting supernatant was then discarded, leaving the isolated mitochondria.

At this point, the receiver strain was centrifuged at 3,000 rpm for 10 min, re-suspended in 200 µL fresh TSC buffer, and incubated with the mitochondrial pellet for 30 min at 30 °C. After this initial incubation, 2 mL of polyethylene glycol (PEG) solution (40 % w/v PEG 4000, 10mM Tris-HCL pH7.5, 10 mM $CaCl_2$) was added, and further incubated at 30 °C for 30 min. Cells were then centrifuged at 3,000 rpm for 10 min and re-suspended in 2 mL 1 M sorbitol. From this suspension, aliquots of 200 µL were plated on selective glycerol media (YPGly; Section 2.2.2), osmotically stabilised by adding 0.6 M KCL to the agar. This media was used to select cells based on their capacity to respire (i.e. to select respiratory competent cells with mitochondrial function). Fixation agar (3.5% agar) was immediately added by gently pouring it over the plates, which consisted of YPGly media supplemented with 10 mM Na-pyruvate, 1 % ethanol, and osmotically stabilised to a final concentration of 0.4 M

$CaCl_2$. The plates were then stored at 25 °C and checked over a period of several weeks for colony growth. Isolated cybrids were stored cryogenically (Section 2.2.5) and working stocks were prepared for analysis as for standard yeast cultures (Section 2.2.6).

2.8.2 Confirmation of cybridisation

In order to confirm that the cybrid strains contained nuclear DNA from the parental lager strain and mitochondria from the donor ale strain, two types of PCR analysis were performed. The inter-delta PCR method for strain identification described previously (Section 2.6.2) was employed to ensure the correct genomic DNA contribution. To confirm transfer of mitochondrial, amplification of COXI primers was performed, using the primers detailed in Table 2.12. Using this method, the origin of the mtDNA present could be determined; *S. cerevisiae* COX1 has a size of ~150 bp, while the *S. pastorianus* copy of COX1 is ~500 bp. Hence an inter-delta PCR product matching the parental lager strain in conjunction with a COX1 PCR product of ~150bp indicated a successful cybridisation event.

Table 2.12 Primer sequences for COXI gene

Primer Name	**Primer sequence**
COXI-F	5’-CTACAGATACAGCATTTCCAAGA-3’
COXI-R	5’-GTGCCTGAATAGATGATAATGGT-3’

For COX1 PCR, DNA was extracted using the method outlined previously (Section 2.6.1); although primarily designed for obtaining nuDNA, this

method also yields small amounts of mtDNA which can be analysed using primers specific to regions of the mtDNA genome. The reaction volume for the PCR was 20 µL , containing 7 µL molecular grade water, 10 µL 2X Phusion master mix, and 1 µL of each primer at a concentration of 20 µM. The mtDNA was amplified using a thermocycler with the following conditions: Initial denaturation 95 °C for 30 sec, with 35 cycles of denaturing at 95 °C for 10 sec, annealing at 66 °C for 30 sec, elongation at 72 °C for 45 sec and a final extension at 72 °C for 5 minutes. The thermocycler was set to hold indefinitely at 4 °C on completion.

CHAPTER 3

CHARACTERISATION OF BREWING YEAST STRAINS

3.1 Introduction

The production of beer-type beverages via fermentation has been performed for many centuries, however understanding the science behind the process is a comparatively recent concept (Lodolo *et al*., 2008). Although the origins of beer can be traced to Egyptian civilisation and beyond (Boulton and Quain, 2001), identification of yeast as the primary organism behind the process was only unequivocally confirmed between 1855 and 1875 by Louis Pasteur (Barnett, 2000). Furthermore, it is only in recent years that the origins and diversity of brewing strains has begun to be fully investigated (Berlowska *et al*., 2014; Gallone *et al*., 2018). Despite this, ale and lager strains are known to be distinct, belonging to the species *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* respectively. *S. pastorianus* yeasts are hybrid organisms, derived from a *S. cerevisiae* parent and another species, most likely *S. eubayanus* (Dunn and Sherlock, 2008; Kerogus *et al*., 2017) (Section 1.1.2.). Although lager strains are not as genetically diverse as ale strains, they can be further divided into two groups, known as Saaz and Frohberg types (Dunn and Sherlock, 2008) (Section 1.1.2.). This division is based on genetic differences; Saaz strains share more similarity to the *S. eubayanus* parent and exhibit a higher degree of cold tolerance than Frohberg strains, which share a larger portion of DNA in common with *S. cerevisiae* (Dunn and Sherlock 2008; Gibson *et al*., 2013; Chen *et al*., 2015). Despite these fundamental differences in nuDNA, it is believed that all lager strains contain mitochondria that originated from the *S. eubayanus* parent (Baker *et al*., 2015, 2018), and since *S. pastorianus*

yeast are a relatively ‘recent’ species, it is possible there is little variation in the genetic make-up of the mtDNA. Conversely, reports have indicated that there is considerably more variation in mtDNA content within the *S. cerevisiae* species (Wolters *et al*., 2015). Despite this, to date there has been only limited comparative analysis of mtDNA sequences between brewing strains.

In order to fully characterise the yeast strains under investigation, a combination of standard and molecular techniques were applied. Giant colony morphology and the X-α-gal method were used as simplistic methods for differentiating brewing strains, and for classifying them as lager/ale respectively. In addition, genetic analysis was performed to identify each strain to the species level. ITS PCR, a method recently proposed for adoption as the primary fungal identification system (Schoch *et al*., 2012), was used to identify each strain to the species level, while interdelta PCR, a technique known to be useful for strain identification (Legras and Karst, 2003; Xufre *et al*., 2011) was applied to fingerprint each yeast. This genetic characterisation was necessary to ensure that strains were correctly classified, and that they were sufficiently distinct from one another to provide useful information in subsequent tests. In addition, yeast mitochondrial DNA (mtDNA) was analysed by restriction digest as a means of identifying and differentiating strains, and finally by sequencing to allow for a more detailed characterisation of mtDNA composition. It is anticipated that this analysis will act as a foundation for further studies into yeast mitochondria structure and function in subsequent chapters.

Experimental approach

Four proprietary lager yeasts designated SMCC100, SMCC99, SMCC90 and SMCC57, and two ale yeasts termed D3 and D23 were used for all studies. Each yeast was investigated using a combination of traditional techniques targeting key phenotypes, as well as a series of genetic-based methods as a means of classification, differentiation and, in the instance of mtDNA analysis, characterisation.

3.2 Results

3.2.1 Determination of strain brewing classification by X-α gal analysis

Although a brewing designation (ale/lager) was provided by SABMiller for each strain, this was confirmed by analysing the response of each yeast to the melibiose homolog X-α-gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) as described in Section 2.2.3. This test determines the ability of yeast to cleave the X-α-gal molecule using the enzyme melibiase, resulting in the development of a blue coloration derived from indol. Since only *Saccharomyces pastorianus* (lager) yeast strains are able to produce melibiase (Box *et al*., 2012; Bokulich and Bamforth, 2013), this method allows for their differentiation by simple visual analysis. It can be seen from Figure 3.1 that strains SMCC100, SMCC99, SMCC90, and SMCC57, all produced a blue/green pigment and could be confirmed as lager strains. Strains D23 and D3 remained colourless and were consequently considered to be ale-type strains.

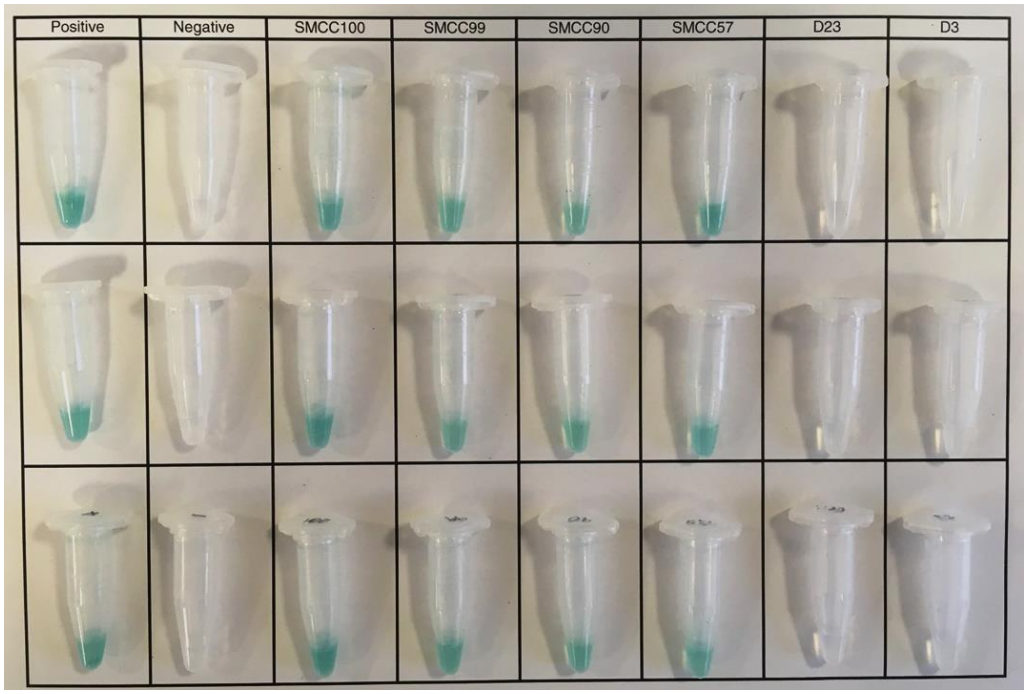


Figure 3.1 Categorisation of yeast strains as lager or ale based on melibiase activity. Blue colouration indicates lager strains, while ale strains remain colourless. In this instance, the common lager yeast strain W34/70 was utilised as a 'control', while the negative sample comprised a water blank.

3.2.2 Determination of yeast genus and species using ITS-PCR analysis

In order to classify each yeast to the genus and species level, DNA from each strain was subjected to PCR analysis of the internal transcribed spacer (ITS) region, as described in Section 2.6.2. This region of the ribosomal DNA is particularly useful for identification of yeasts and is currently used as the primary fungal barcode marker for species identification (Schoch *et al*., 2012). Analysis of the ITS region is a two stage process: the ITS region is PCR amplified before being digested into

smaller fragments using restriction enzymes in order to allow for greater discrimination (Section 2.6.2).

Using primers specific to the ITS region, PCR amplification of DNA from each strain revealed that this section of rDNA was identical in size; all yeast yielded an amplicon of approximately 880bp in size (Figure 3.2). This was not unexpected, since both *S. cerevisiae* yeast and *S. pastorianus* share a similar ITS region which is identical in length (Pham *et al*., 2011).

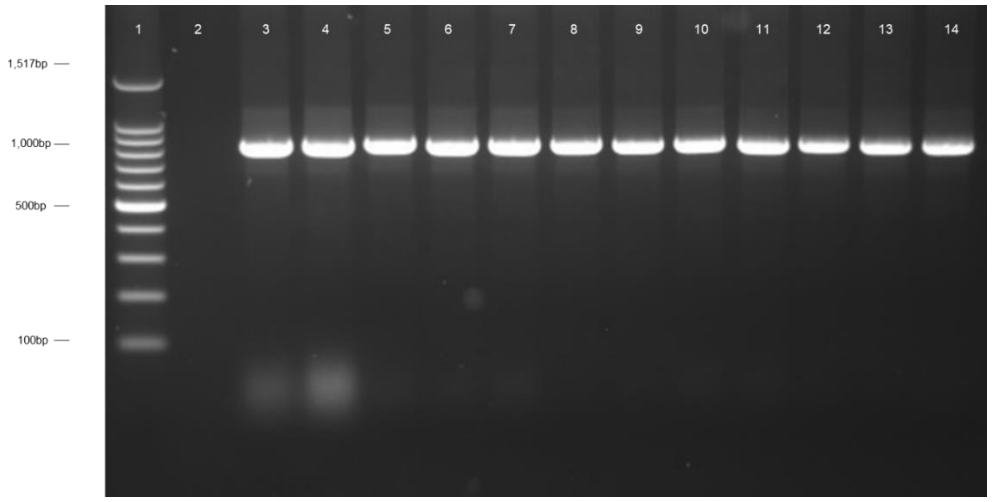


Figure 3.2 Amplification of the ITS region of yeast ribosomal DNA. Lane 1: 100bp ladder (NEB); lane 2: Blank; lane 3 & 4: SMCC100; lane 5 & 6: SMCC99; lane 7 & 8: SMCC90; lane 9 & 10: SMCC57; lane 11 & 12: D23; lane 13 & 14: D3.

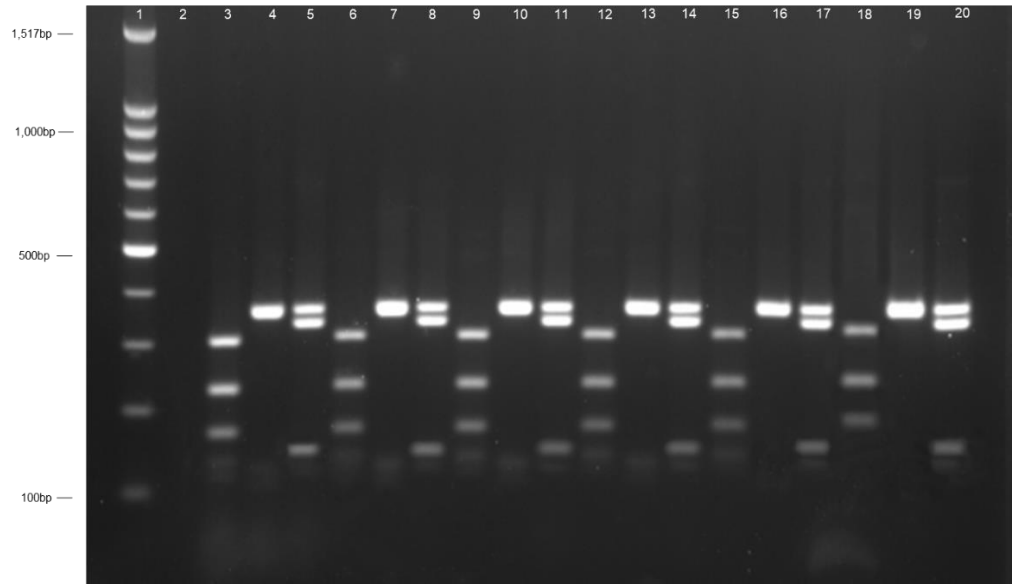


Figure 3.3 RFLP analysis of the ITS region obtained from brewing yeast strains. Lane 1: 100bp ladder (NEB); lane 2: Blank; lanes 3-5: SMCC100 digested with HaeIII, HinfI and CfoI respectively; the order of enzymes is repeated for subsequent yeast strains. Lanes 6-8: SMCC99; lanes 9-11: SMCC90; lanes 12-14: SMCC57; lanes 15-17: D23; lanes 18-20: D3.

In order to increase the sensitivity of the analysis, each ITS amplicon was digested using three restriction enzymes (HaeIII, HinfI and CfoI) in separate reactions. It can be seen from Figure 3.3 that all the yeast strains produced amplicons of 320, 220, 180, and 140 bp using enzyme HaeIII; 380 bp using enzyme HinfI; and 380 and 340 bp when using enzyme CfoI. Comparison of these profiles to those expected based on the literature (Guillamón *et al*., 1998; Esteve-Zarzoso *et al*., 1999; Granchi *et al.*, 1999; Pham *et al*., 2011), indicated that these strains should be classified as *S. cerevisiae* yeast, in contrast to data obtained from X-α-gal analysis (Section 3.2.1). This discrepancy can be explained since it is known that Frohberg type lager yeast strains produce an ITS-

RFLP profile which corresponds to *S. cerevisiae* (Pham *et al*., 2011). This is likely due to the origins of this group of lager yeast, which share a greater proportion of DNA with *S. cerevisiae* than the *S. eubayanus* parental strain (Krogerus *et al*., 2015). The suggested *S. pastorianus* profiles originally published by Esteve-Zarzoso (1999) and Guillamon (1998) evidently refer to Frohberg type lager yeast strains. Consequently, it can be seen that the application of a combination of methods (in this case ITS-PCR and X-α-gal analysis) is required for accurate species identification. However, using these methods in parallel it was possible to categorise brewing strains into as two categories: ale, and lager (Frohberg).

3.2.3 Differentiation of yeast strains

Once the genus/species and brewing type of each yeast had been confirmed, differences between strains were investigated using a combination of giant colony morphology analysis (Section 2.2.3) and interdelta PCR (Section 2.6.3).

3.2.3.1 Strain differentiation based on giant colony analysis

The use of giant colony morphology analysis for the characterisation of brewing yeasts is a well-established technique (Richards, 1967). While the methodology may appear rather crude when compared to more modern methods such as genotyping, it has been proven to be consistent and effective in differentiating brewing strains (Boulton and Quain, 2001). The principle of this method is that individual colonies produce a specific topography when cultivated for an extended period of time on solid

media. In this study WLN agar was employed; this media contains a bromocresol green dye which causes colonies to take on specific colours which can also be used as an aid to differentiation (Hall, 1971).

To examine giant colony type, cells were grown for 3 weeks on WLN agar and the morphology of the resulting colonies were examined. It can be seen from Figure 3.4 that there was a significant degree of variation in colony size and colouration between each strain. Strain SMCC100 produced colonies that were entirely light green with a smooth topology, while strain SMCC99 yielded colonies that were a distinctive white colouration. Strain SMCC 90 formed a colony with a white base and pale green centre and a rough looking topology, and SMM57 produced a colony which was a greenish shade of white with a similar rough looking topology. Strains D23 and D3 produced colonies with white bases and green centres, but the shade of green was far deeper in D3 colonies, which also appeared to have a ‘smoother’ looking topology.

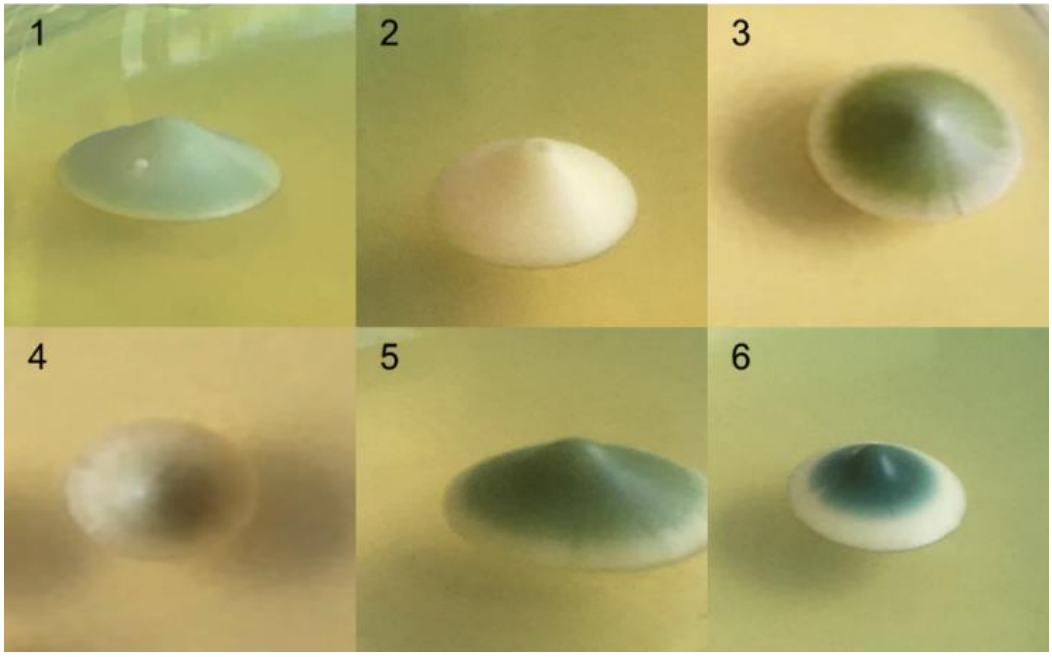


Figure 3.4 Photographs of giant colonies produced by different strains grown on WLN agar over a period of 3 weeks. Image 1: SMCC100; image 2: SMCC99; image 3: SMCC90; image 4: SMCC57; image 5: D23; image 6: D3.

3.2.3.2 Differentiation of brewing yeast strains using interdelta PCR

In order to further characterise and differentiate strains, a PCR protocol was applied to amplify interdelta regions of yeast strains (Section 2.6.3.). Delta regions are pieces of DNA that flank yeast transposons and have been shown be highly discriminatory in yeast (Legras and Karst, 2003). Amplification of DNA from in-between these delta regions exploits this diversity, allowing for strain identification by producing a series of fragments based on the frequency and distribution of delta sequences throughout the genome. From interdelta fingerprint analysis, it can be seen that there were differences between each of the lager strains analysed (Figure 3.5). Although the profiles were similar, when compared

to SMCC99 it can be seen that strain SMCC100 is missing a band of ~450 bps in size. Additionally, SMCC90 could be differentiated based on an amplicon present at ~750 bps. The yeast strain SMCC57 was arguably the most easy to identify since this strain is missing any DNA fragments between 1000 bp and 500 bp in size.

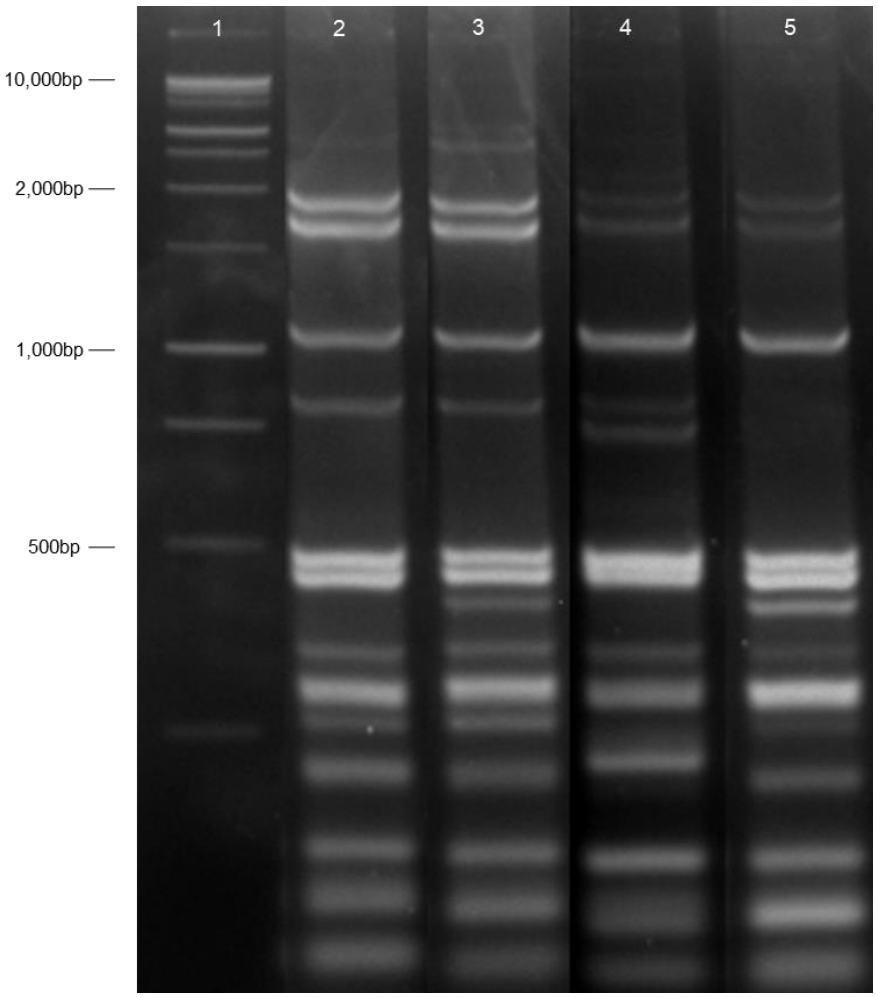


Figure 3.5 DNA fingerprint analysis of lager strains based on PCR of interdelta regions within the genome. Lane 1: 1kb ladder; lane 2: SMCC100; lane 3; SMCC99; lane 4: SMCC90; lane 5 SMCC57.

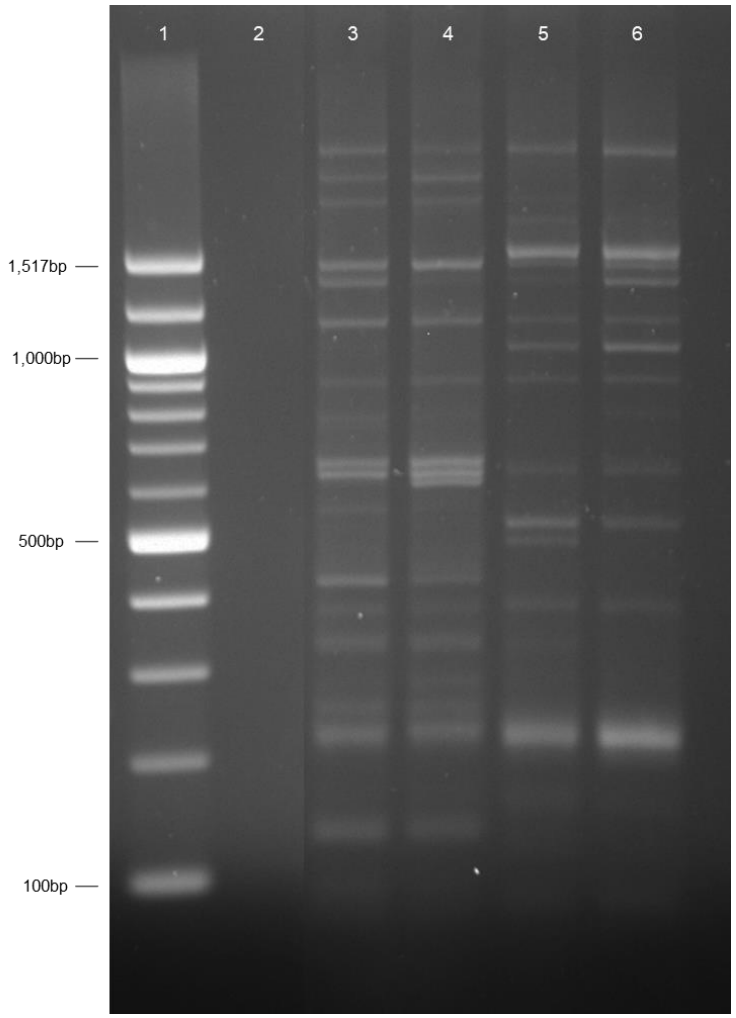


Figure 3.6 DNA fingerprint analysis of ale strains based on PCR of interdelta regions within the genome. Lane 1: 1kb ladder; lane 2: Blank; lane 3: D23; lane 4: D23; lane 5: D3; lane 6: D3.

In contrast to the analysis of lager strains, the ale yeasts were more readily identified. It can be seen from Figure 3.6 that there are clear differences between D23 and D3. For example strain D23 shows bands at ~2500 bp, ~2000 bp and 450 bp, whereas strain D3 does not. Conversely, strain D3 shows bands at 1000 bp, and 500 bp, whereas these are absent in strain D23. It is important to note that although differences in band intensities can be observed between strains, these

cannot be reliably used for differentiation since they can be impacted by the efficiency of the PCR reaction and the quality of the template DNA. Irrespective, the data shown indicates that the strains were indeed different. Based on this analysis, along with data from giant colony morphology, it can be seen that each strain was unique. This provides confidence that the strains under investigation were sufficiently different as to be useful in subsequent physiological and genetic analysis.

3.2.4 Examination of brewing yeast mtDNA

Although it is evident that brewing yeast mitochondria are important for cell functionality (Section 1.5), very little is known about their precise genetic makeup. Furthermore, there have been limited studies into the genetic diversity of mitochondria in brewing yeast as a group of organisms. In this section, we aimed to investigate the differences which occur between the strains in terms of their mtDNA profiles. In order to do this, mtDNA from each strain was profiled by DNA fingerprinting (Section 2.6.4) and by full sequencing (Section 2.6.6).

3.2.4.1 mtDNA fingerprinting using restriction enzymes

In order to produce fingerprints for each yeast strain, mtDNA was extracted and cut into fragments using the restriction enzymes HaeIII, HinfI and DdeI. These enzymes were selected based on their recognition sites, which are targeted towards GC regions (Section 2.6.5). Since *S. cerevisiae* mitochondrial sequences are generally characterised as having a relatively low G+C content (Langkjær *et al*., 2003), this causes

the number of bands produced to be restricted to a manageable number, allowing for visual discrimination between strains.

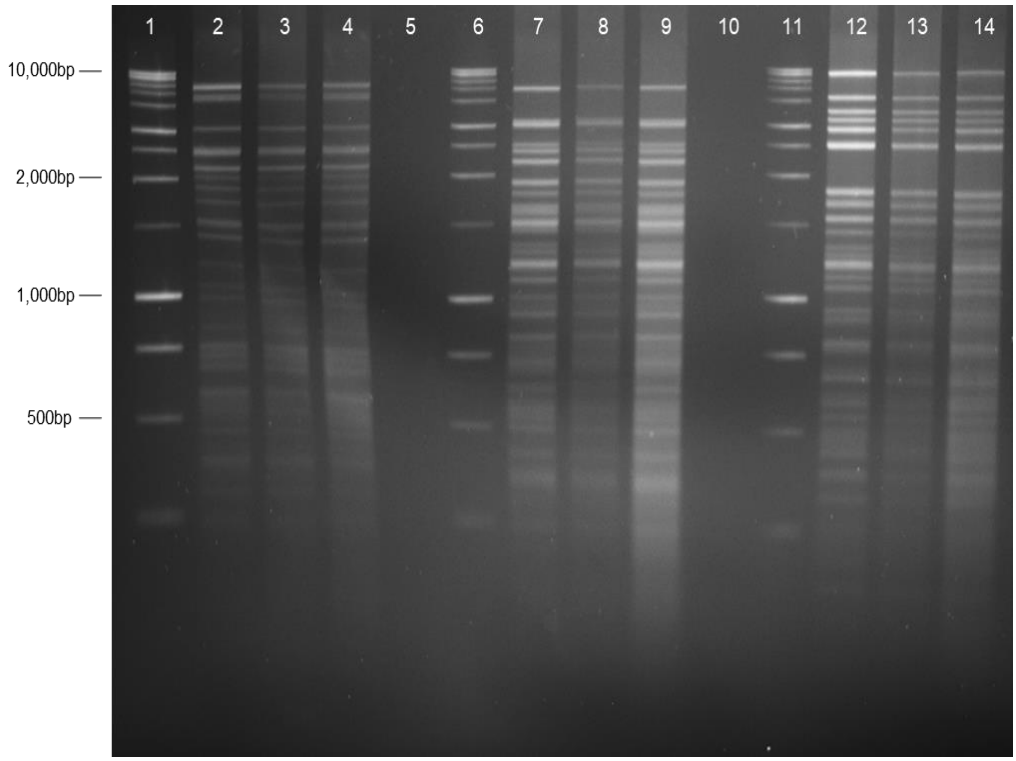


Figure 3.7 mtDNA fingerprints of brewing yeast strains. Lanes 1: 1kb ladder; lane 2 - 4 strains SMCC100, SMCC99, SMCC90 digested by HaeIII; lane 5: Blank; lane 6: 1kb ladder (Promega); lane 7 - 9 strains SMCC100, SMCC99, SMCC90 digested by HinfI; lane 10: Blank; lane 11: 1kb ladder (Promega); lane 12 - 14 strains SMCC100, SMCC99, SMCC90 digested by DdeI.

The resulting fingerprints showed that there were no obvious differences between the lager strains; all strains showed identical profiles (Figure 3.7). However, the ales strains analysed produced patterns of restriction fragments which were clearly different from one another, and from the lager strains tested (Figure 3.7). The lager yeast SMCC100, SMCC99,

SMCC90 could be differentiated from ale strains since they yielded unique bands at 3000 bp, while strain D23 and D3 could be identified due to the presence of a number of fragments between 2000 bp and 3000 bp. It should be noted that when examining the ale strains with enzymes HinfI and DdeI, the differences in profile between the two strains were less distinct, indicating that HaeIII was the most discriminatory enzyme for the production of mitochondrial DNA fingerprints based on restriction sites.

The data presented supports previous suggestions that there is a lack of diversity within the *S. pastorianus* mtDNA genome (Rainieri *et al*., 2008). The reason for this may be related to the history of lager yeast strains; reduced mtDNA diversity may be a consequence of hybridisation events during the evolution of the *S. pastorianus* species. *In vitro* studies of interspecific hybrids have indicated that the inherited mitochondria can be derived from either of the parental strains (De Vero *et al.,* 2003, Rainieri *et al.,* 2008 Versphol *et al.,* 2008). However, to date, only mitochondria derived from the *S. eubayanus* parental strain have been identified in lager brewing yeasts. The reasons for this are currently unknown, however this may be due to selective pressures placed on the yeast strain at the point of hybridisation, resulting in the *S. eubayanus* mtDNA genotype becoming established (Magalhães *et al.,* 2017, Baker *et al.,* 2018). In contrast, the variation observed within the *S. cerevisiae* strains may simply be due to the overall genetic complexity of the species which is an evolutionary 'older' species, which has evolved as a group to become phenotypically and genotypically diverse.

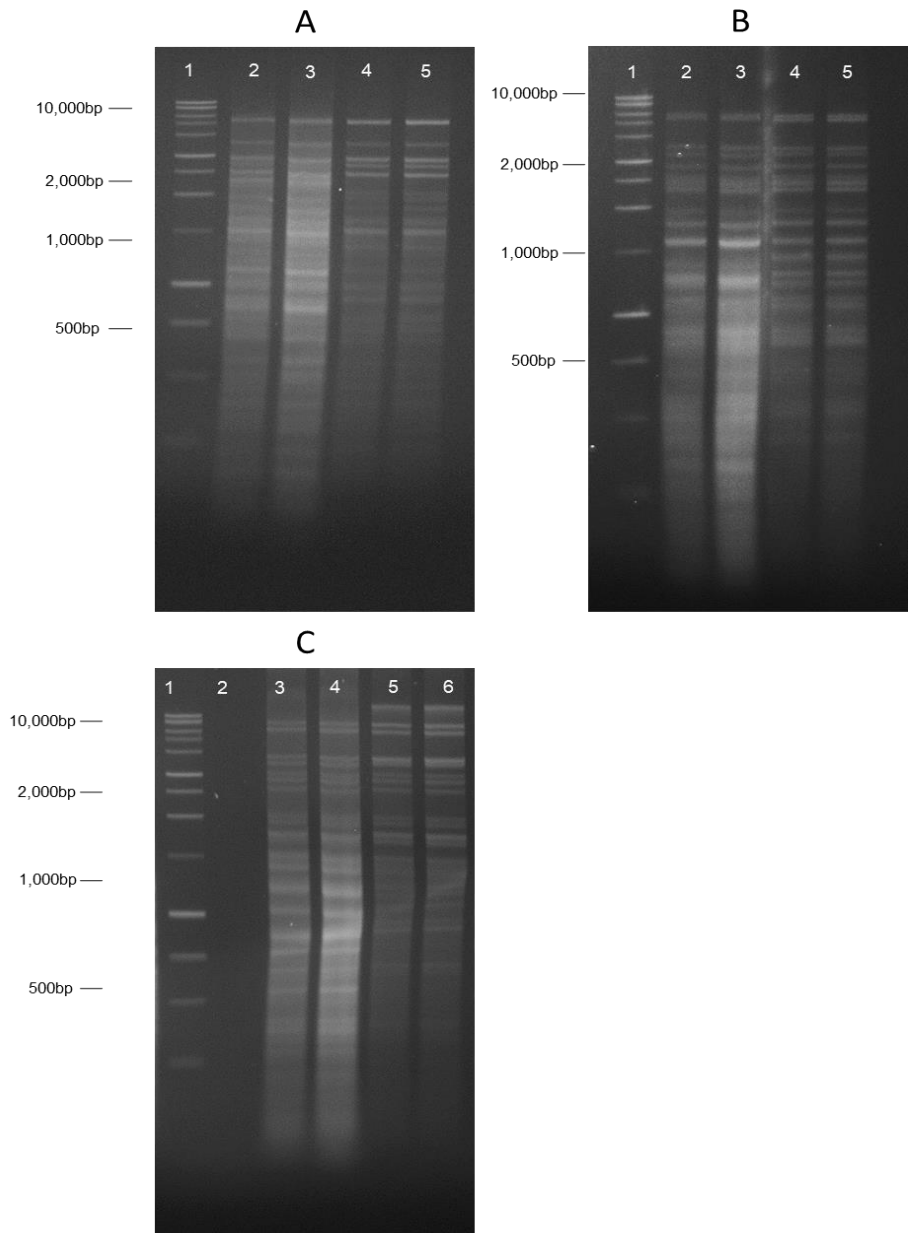


Figure 3.8 Analysis of mtDNA using restriction digest enzymes. (A) mtDNA digested using HaeIII. Lanes 1: 1kb ladder (Promega); lane 2: D23; lane 3: D23; lane 4: D3; lane 5: D3; (B) mtDNA digested using HinfI. Lanes 1: 1kb ladder (Promega); lane 2: D23; lane 3: D23; lane 4: D3; lane 5: D3; and (C) mtDNA digested using DdeI Lanes 1: 1kb ladder (Promega); lane 2: Blank; lane 3: D23; lane 4: D23; lane 5: D3; lane 6: D3.

3.2.4.2 Gene mapping of brewing yeast mtDNA

In order to gain a better understanding of the extent of variation within the mtDNA of brewing yeast strains, full mitochondrial genome sequencing was performed as described in Section 2.6.6. Results from SMCC100, SMC99, SMCC90, D23 AND D3 were compared with additional data obtained from the National Center for Biotechnology Information (NCBI) for strains W34/70 (*S. pastorianus*, a widely used strain, derivative of many industrial lager strains), FM1318 (*S. eubayanus* type strain), S288c (haploid laboratory strain) and GLYBRCY22-3 (a *S. cerevisiae* strain used in biofuels). This data was used to build annotated mtDNA genome maps, determine phylogeny, and examine for single nucleotide polymorphisms using GeSeq as described in Section 2.6.6. mtDNA genome maps were produced for: *S. cerevisiae* strains S288c (Figure 3.9) and GLYBRCY22-3 (Figure 3.10); S*. eubayanus* type strain FM1318 (Figure 3.11); reference lager yeast W34/70 (Figure 3.12); the proprietary lager yeast strains SMCC100 (Figure 3.13), SMCC99 (Figure 3.14) and SMCC90 (Figure 3.15); the proprietary ale strains D23 (Figure 3.16) and D3 (Figure 3.17).

Analysis of the data allowed a visual representation of the mtDNA to be created, depicting locations of different genomic components (genes, ribosomal RNAs and transfer RNAs), as well as the important complexes which are required for aerobic respiration (Figures 3.9-3.17). These complexes and their locations within the genomes varied across the different species, but remained similar within the *S. pastorianus* strains, as expected based on fingerprint analysis. It should be noted that the

reference strain for *S. pastorianus* W34/70 yielded some additional information missing in the other lager strains analysed; specifically within regions marked as ‘other’ genes, for example the coding region ACI60 which is an open reading frame sequence. However, in general the location of genes and complexes in the sequences obtained from brewing yeast strains corresponded to those of the reference strains. Despite this, it was also apparent that each of the strains were different, even if the degree of variation was very small in some instances.

Analysis of mtDNA sequence data derived from the *S. cerevisiae* ale strains indicated that there was diversity in terms of the location of the annotated genes, as well as in the size of the synthases and complexes. One example is the VAR1 gene fragment, which was observed to be in different locations in D3 and D23, and the Complex IV (cytochrome c oxidase) which was noted to be short in strain D3. In addition, there were genes present in D23 that were not found in either the reference SC288c genome or the D3 mitochondrial genome sequence. For example, there appeared to be two VAR1 gene fragments in D23 which appear sequentially, while in D3 and SC288c the VAR1 fragments were much smaller. Despite this variation, the location of many complexes were conserved across the *S. cerevisiae* species; specifically the Complex III (ubichinol cytochrome c reductase), which appeared across all *S. cerevisiae* strains with only small differences in overall size.

When comparing the industrial brewing strains, the immediate difference observed was the total size of the mtDNA genomes, with *S. pastorianus*

lager strains being consistently smaller than *S. cerevisiae* ale yeast (70kbp compared to 82-85 kbp), as shown in Table 3.1. Interestingly, the genome size of *S. eubayanus* strain FM1318 was smaller (63 kbp) than for the lager strains analysed, indicating that some changes to the mtDNA had evidently occurred since the original hybridisation event. In addition to genome size, the locations of genes and other components such as ribosomal RNAs, transfer RNAs and other complexes were very different in *S. eubayanus* compared to the *S. pastorianus* and *S. cerevisiae* brewing yeast strains.

Table 3.1 Size of the mitochondrial genome in industrial and laboratory strains.

Strain	Species	Type	mtDNA genome size
SMCC100	*S. pastorianus*	Lager	70 kbp
SMCC99	*S. pastorianus*	Lager	70 kbp
SMCC90	*S. pastorianus*	Lager	70 kbp
D23	*S. cerevisiae*	Ale	85 kbp
D3	*S. cerevisiae*	Ale	85 kbp
W34/70	*S. pastorianus*	Lager (database)	70 kbp
FM1318	*S. eubayanus*	Species type strain	63 kbp
SC288c	*S. cerevisiae*	Haploid laboratory	85 kbp
Glybrcy22-3	*S. cerevisiae*	Wild yeast strain	82 kbp

Furthermore, it can be seen that there were differences in gene coding regions between the brewing strains; *S. cerevisiae* strains appeared to have a larger diversity in coding sequences with many of the complexes being conserved (as mentioned previously), and many additional ribosomal components when compared to *S. pastorianus* sequences. From analysis of the *S. pastorianus* strains it can be seen that the

complex regions (i.e. Complex III and Complex IV) remained constant in size and location. This was also true of the ribosomal units including proteins and RNAs. However, when comparing the *S. pastorianus* lager strains with the ale production yeast, it is clear that the complex regions in *S. cerevisiae* strains were larger and contained a greater array of ribosomal units.

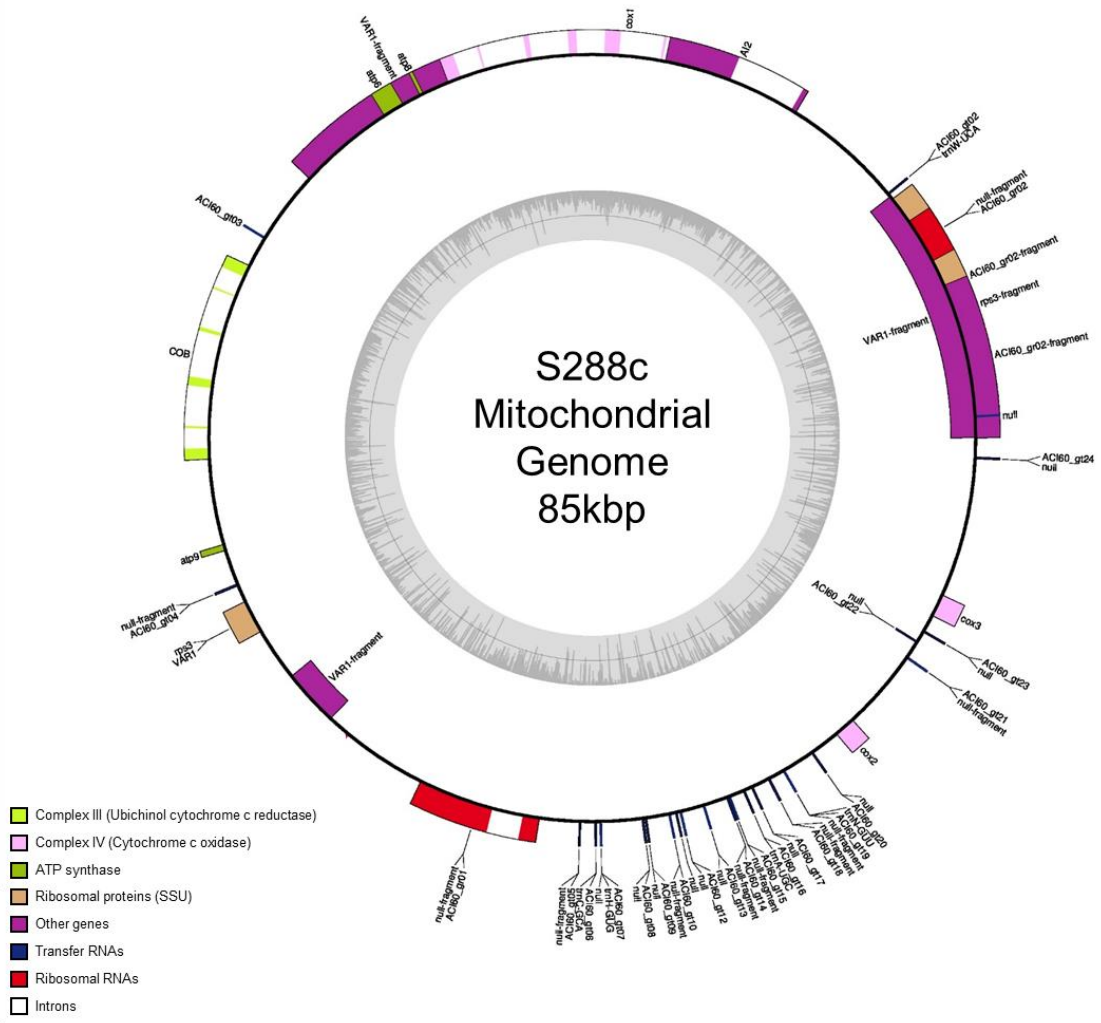


Figure 3.9 mtDNA genome map of the yeast reference strain S288c (*S. cerevisiae*). Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

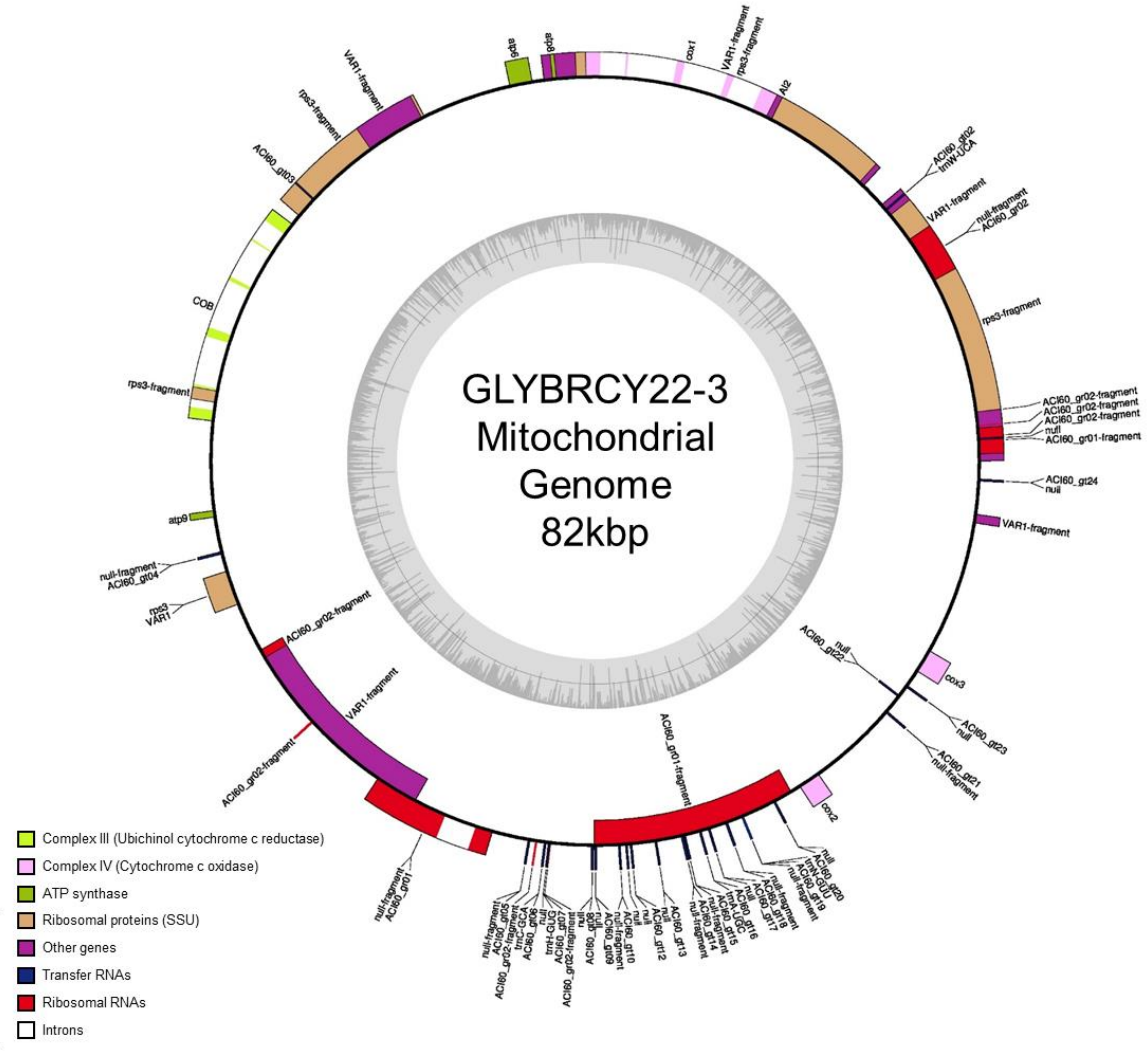


Figure 3.10 mtDNA genome map of the yeast strain Glybrcy22-3 (*S. cerevisiae*). Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

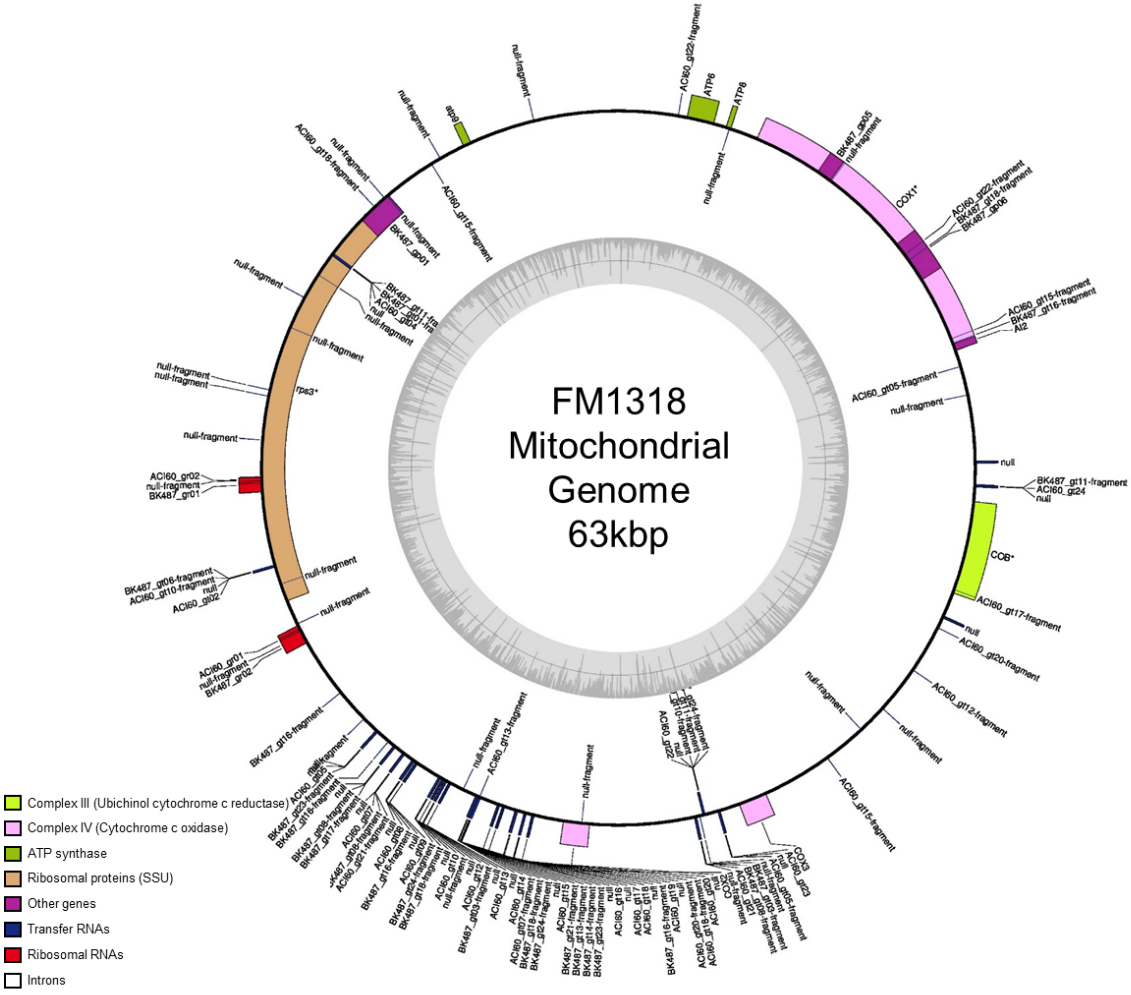


Figure 3.11 mtDNA genome map of the yeast strain FM1318 (*S. eubayanus*). Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

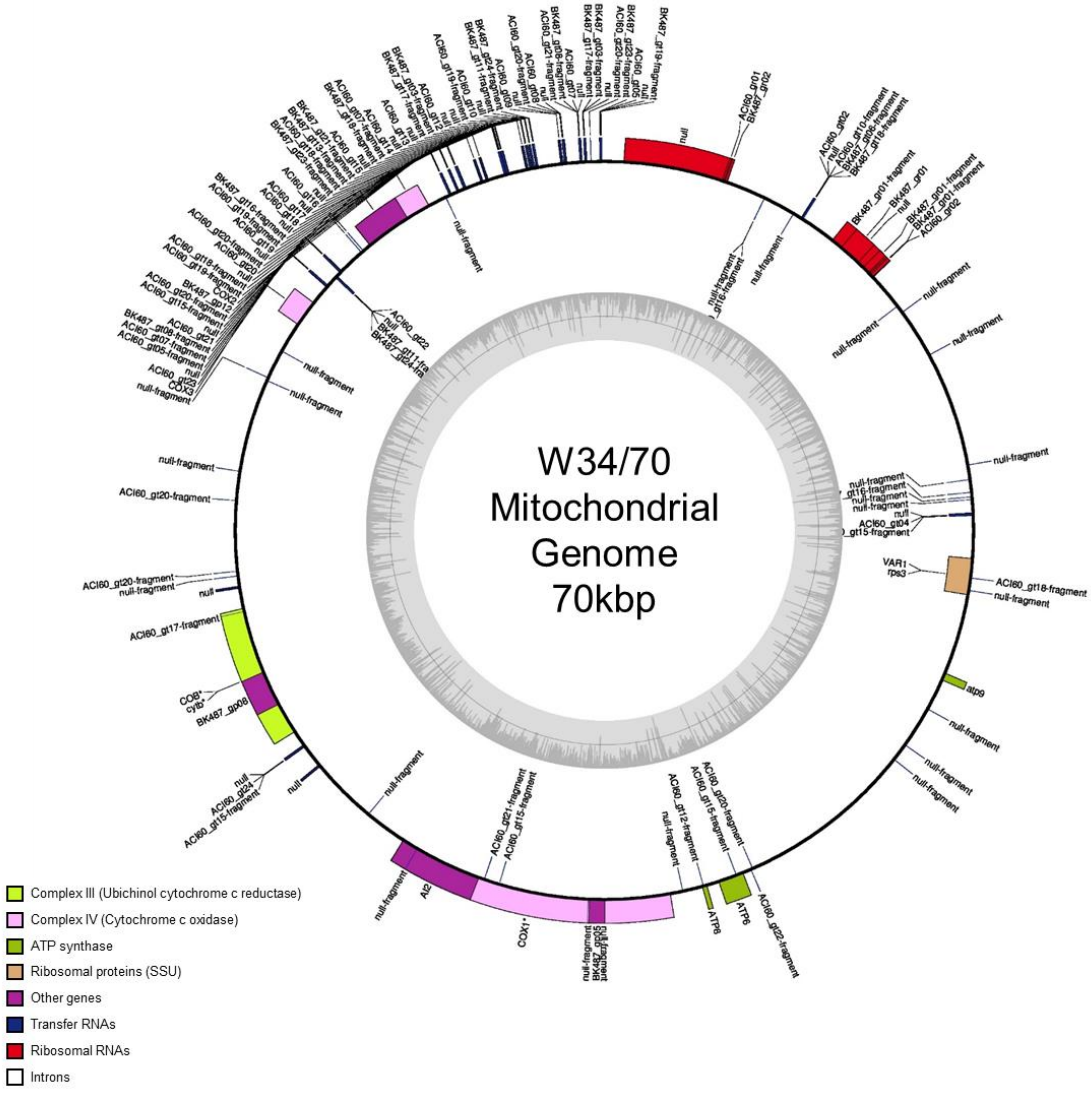


Figure 3.12 mtDNA genome map of the lager yeast reference strain W34/70. Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

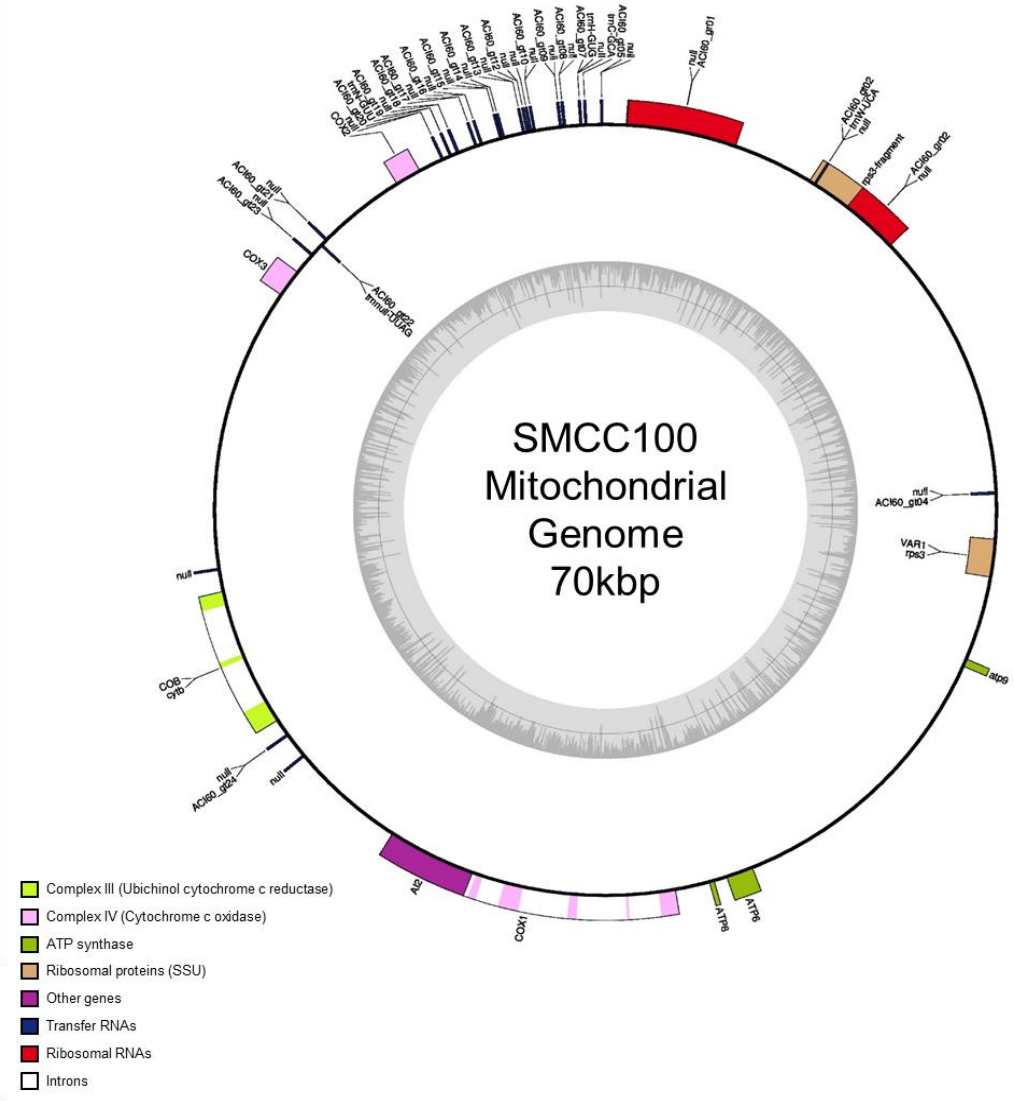


Figure 3.13 mtDNA genome map of the lager yeast strain SMCC100. Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

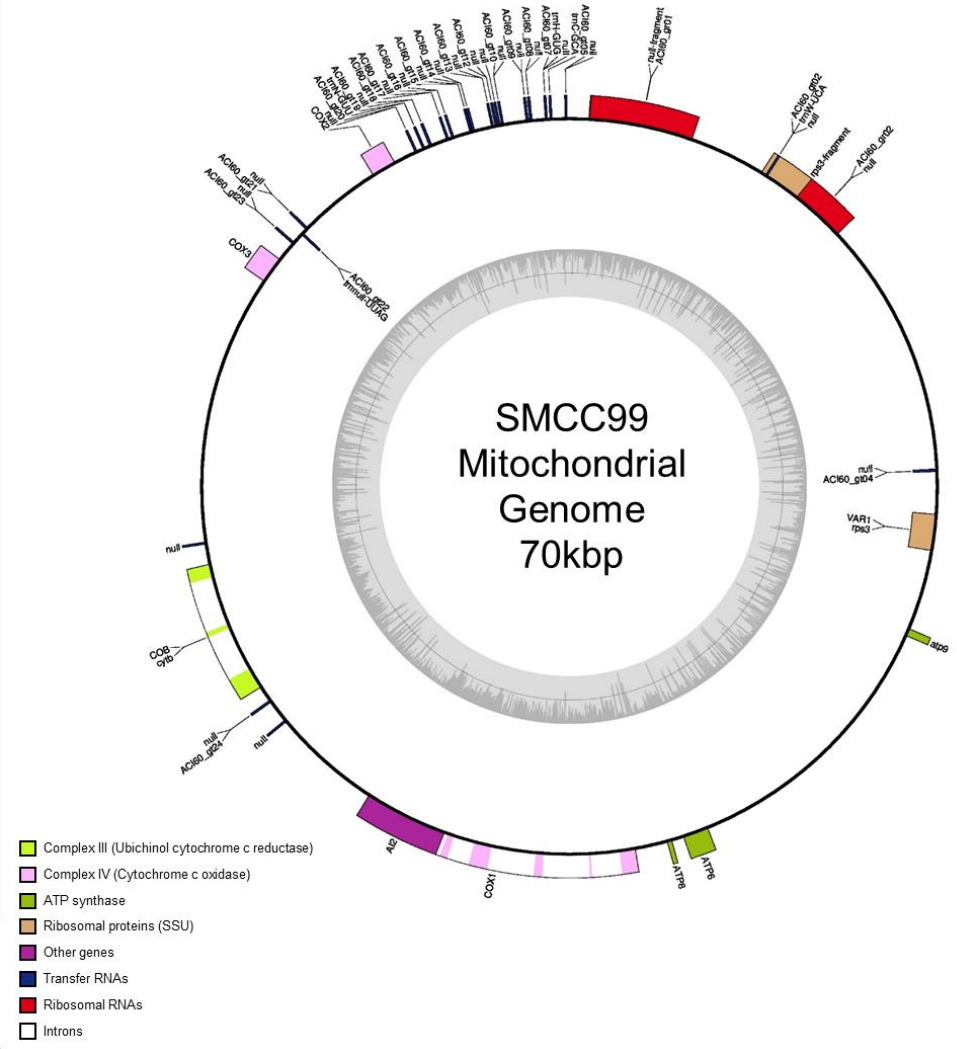


Figure 3.14 mtDNA genome map of the lager yeast strain SMCC99. Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

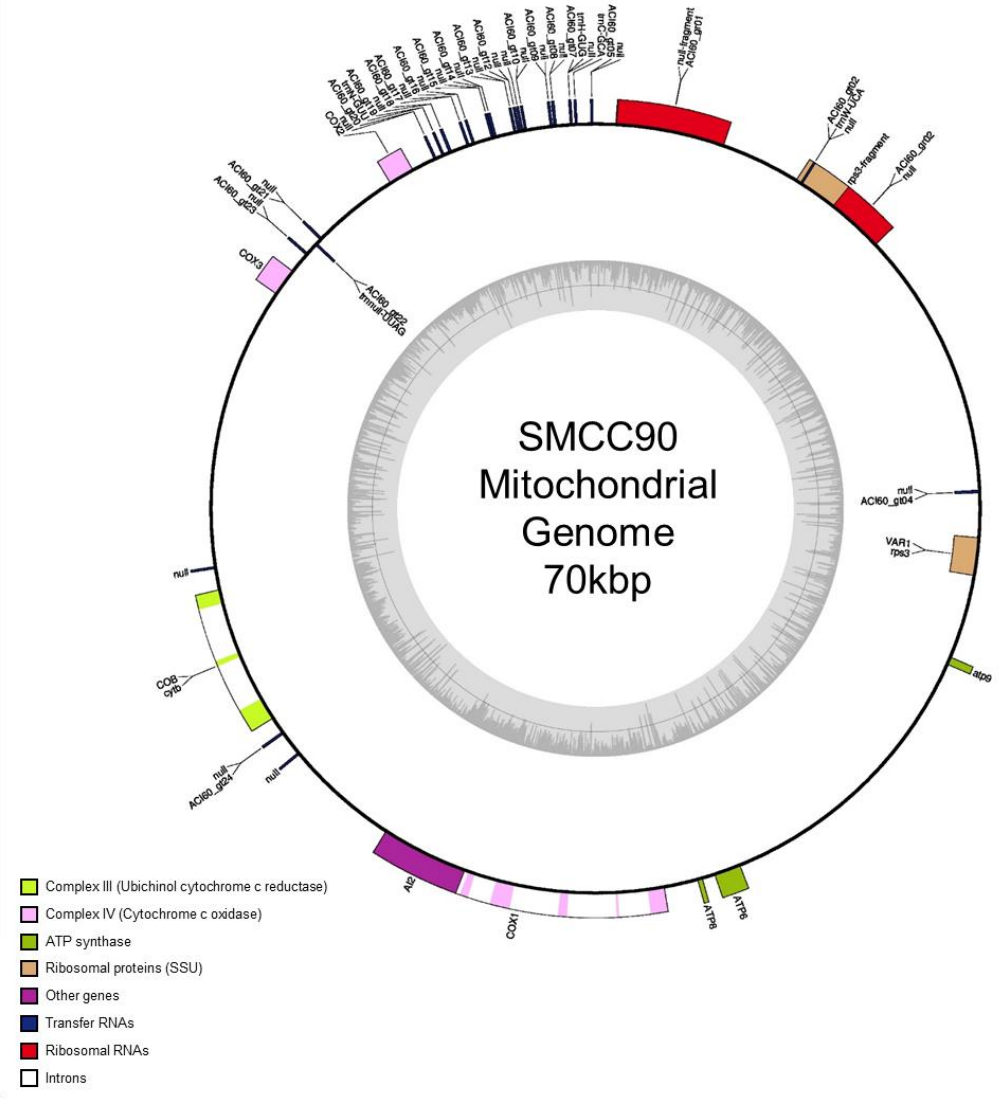


Figure 3.15 mtDNA genome map of the lager yeast strain SMCC90. Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

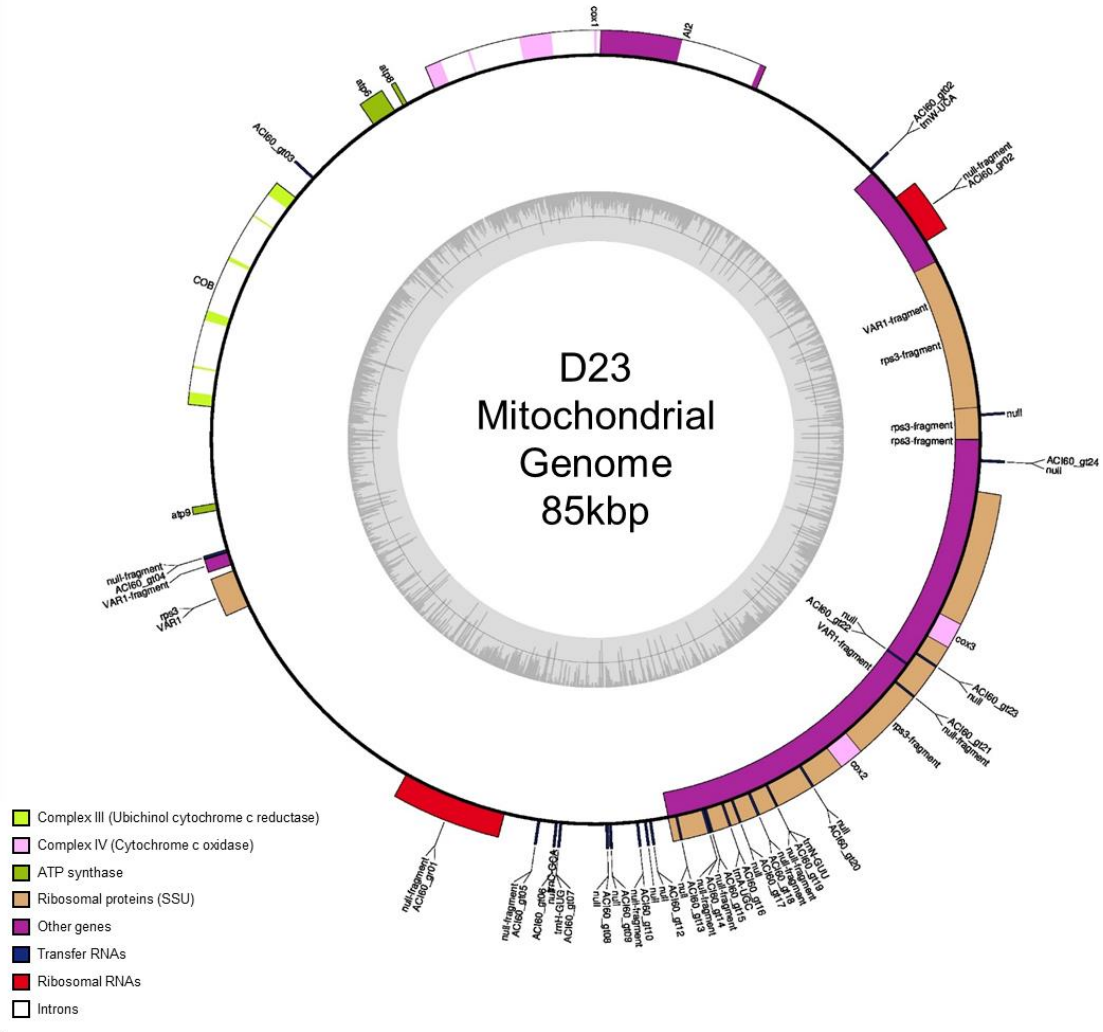


Figure 3.16 mtDNA genome map of the ale yeast strain D23. Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

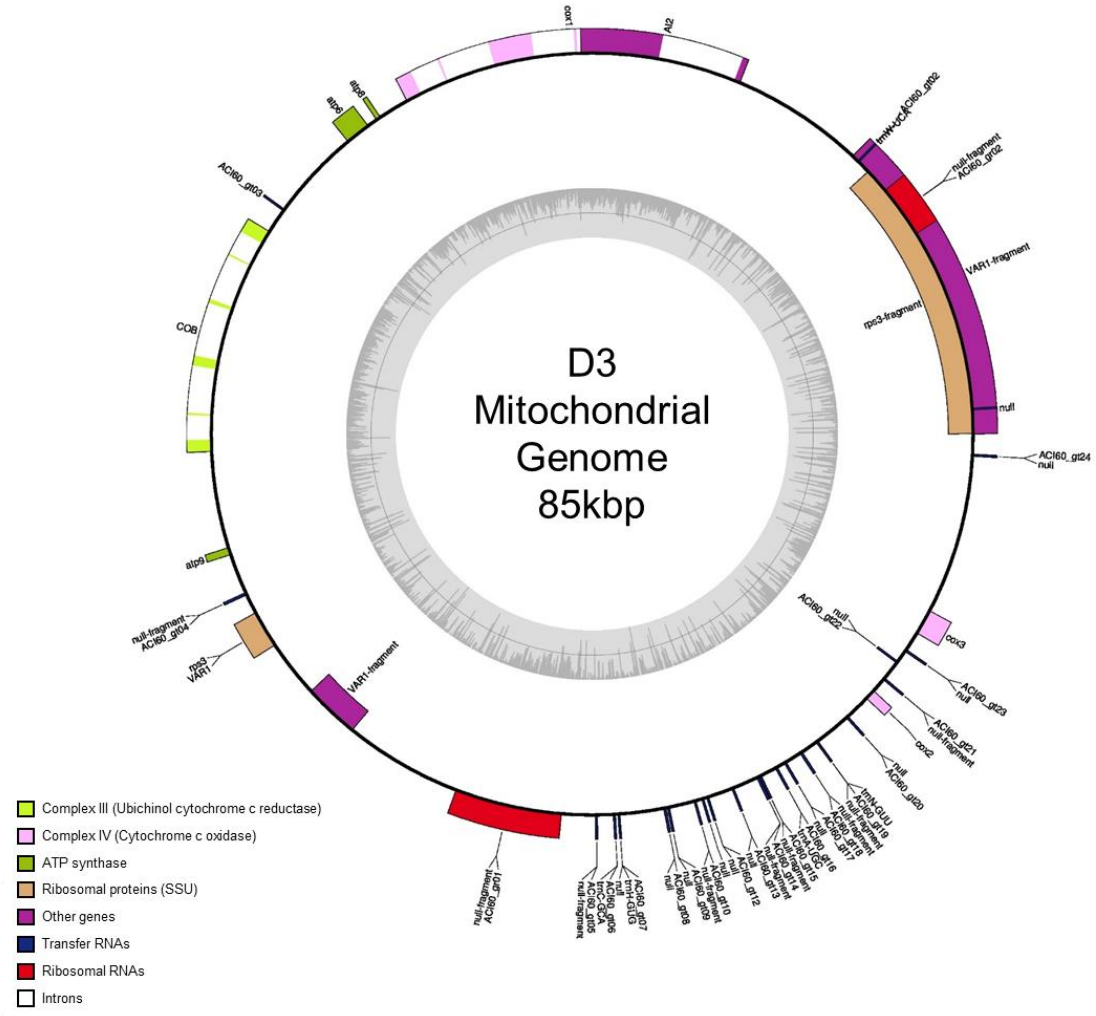


Figure 3.17 mtDNA genome map of the ale yeast strain D3. Relative locations of key genes, coding regions, and introns within the mitochondrial genome are indicated.

In addition to examining and mapping key gene locations, it was also possible to produce a phylogenetic tree to demonstrate the relationship between the different mitochondrial genomes. This was achieved by analysing the data using MEGA 7 software and comparing to other fully sequenced mitochondrial genomes available on the NCBI website, as described in Section 2.6.6. The resulting phylogenetic relationship between the different *Saccharomyces* strains can be seen in Figure 3.18, along with the two additional *S. cerevisiae* strains (the haploid strain S288c and the biofuel yeast Glbbrcy22-3), as well as the type *S. eubayanus* strain (FM1318) and the lager strain W34/70, taken from NCBI and included for reference purposes. Based on this analysis, it can be seen that strain SMCC90 formed a distinct branch when compared to strains SMCC99 and 100, which were more similar to the *S. cerevisiae* strains D3, D23 and S288c (Figure 3.18). As mentioned above, the mtDNA genome obtained from *S. eubayanus* was, rather surprisingly, significantly different to any of the other strains examined, including the two lager production strains. Furthermore, the lager strain cluster was closer to the ale strains examined than to the *S. eubayanus* type strain (Figure 3.18). Given that lager strains are genetically similar in terms of nuDNA content, it might be expected that the same would apply to mtDNA. The reasons why this was not the case are currently unclear, especially since current consensus is that *S. pastorianus* yeast mitochondria are derived from the *S. eubayanus* parent (Rainieri *et al*., 2008; Dunn and Sherlock, 2008). Interestingly, it has recently been demonstrated that mitochondrial recombination events occur when

mating diploid *S. cerevisiae* cells; mapping of potential recombination sites revealed a large number of hotspots at which this could potentially occur (Fritsch *et al*., 2014). Consequently, the view that lager strain mitochondria are derived directly from *S. eubayanus* may be simplistic; it is possible that a series of recombination events with the *S. cerevisiae* parental mitochondria may have occurred before the mitochondria had stabilised within the new hybrid.

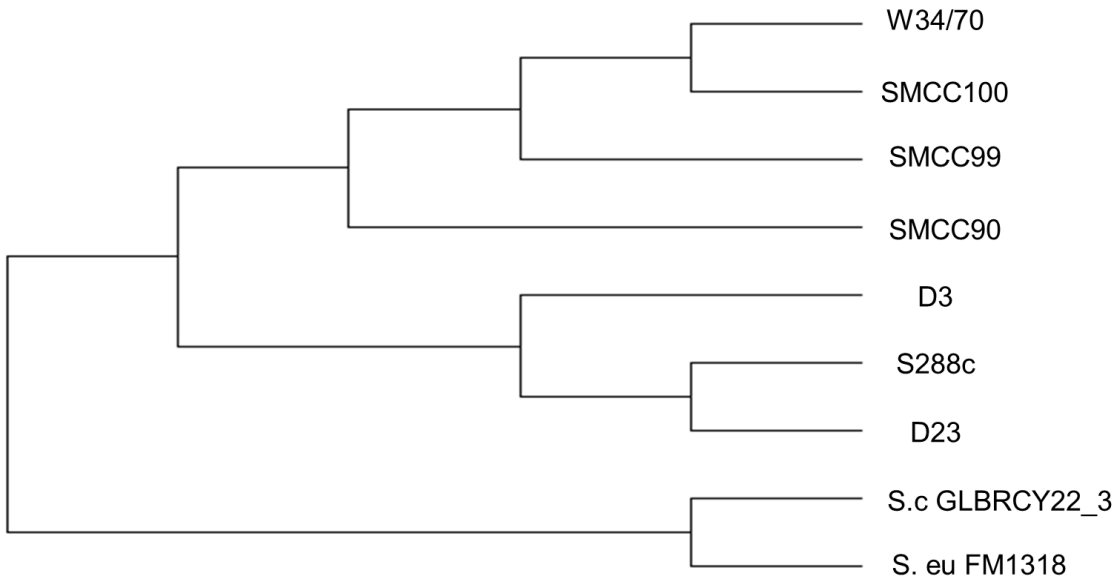


Figure 3.18 Molecular phylogeny of brewing yeast strains, based on mtDNA analysis. The phylogenetic tree was produced using the 'maximum likelihood' based on the Tamura-Nei model (1993), where all sections of the genome with gaps or missing data were excluded. A total of 54440 positions were used to create the final dataset and evolutionary analyses were conducted in MEGA7 (Kumar *et al.,* 2016).

Based on the sequence analysis obtained it was also possible to examine the impact of mitotype on factors impacting functional traits. Figure 3.19 provides an insight into the extent of this variation for different section of the mitochondrial genome. By comparing to the lager strains with reference genome W34/70 and the ale strains with S288c it was possible to determine the % variation in downstream and upstream sections (referring to the location of the DNA downstream positioning towards the 5' end and upstream DNA positioning towards the 3' end), exons (stretches of DNA that represent mature RNAs), introns (stretches of DNA which are non-coding), and intergenic sequences (non-coding regions of DNA). It is evident from this data that all strains showed differences, for example *S. pastorianus* strains were more likely to see intergenic variation with variation occurring at 57.9%, 64.10% and 46% for strains SMCC100, SMCC99 and SMCC90 respectively. While intergenic sequences are non-coding they may influence the transmission of mtDNA to daughter cells (Piskur, 1997). When examining the *S. cerevisiae* strains D23 and D3 the greatest changes could be seen, occurring in the intron regions with indices of 9.40% and 8.91% respectively. It can also be seen that ale strains D23 and D3, and the lager strain SMCC90 experienced a much higher rate of variation downstream. It should be noted that even small changes in downstream sequences can have implications on the transcription of the mtDNA (Foury *et al.,* 1998, Holmes, 2003), having major effects on kinetics of nucleotide addition.

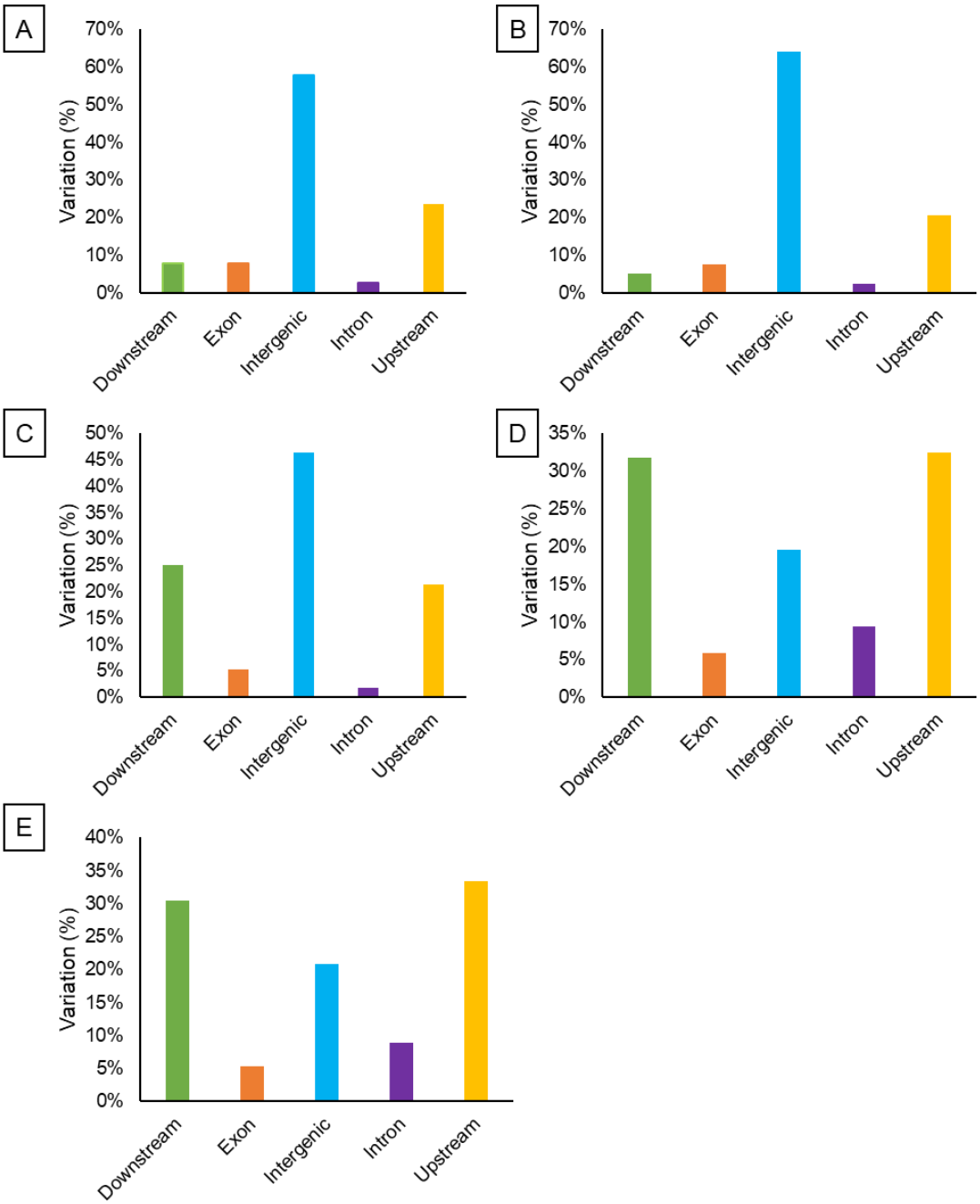


Figure 3.19 Percentage variation which refers to variation compared to the reference genomes of W34/70 for lager strains and S288c for ale strains. Focusing on specific mtDNA regions within brewing yeast strains. A: SMCC100; B: SMCC99; C: SMCC90; D: D23; E: D3.

3.2.4.3 Sequence analysis of brewing yeast mtDNA

Next generation sequencing can function as a powerful tool for examining variations in mtDNA genome between species. As shown above, broad differences could be found in all of the mtDNA sequences examined. In order to achieve more precise insight into the extent of this variation, mtDNA was sequenced and each sequence was examined for single nucleotide polymorphisms (SNPs) using SnpEff, as described in Section 2.6.6. Lager strains were compared to the reference strain W34/70 and ale strains were compared to the haploid strain S288c.

It can be seen from Figure 3.20 that a number of SNPs were found in each genome; the type and frequency of each SNP is also shown. In total, strain D23 yielded the most SNP's (579) and strain SMCC100 the least (17). However, in general there were fewer variations within *S. pastorianus* yeast when compared to *S. cerevisiae* strains. It should be noted that this analysis was based on a comparison of the lager strains against the reference mitochondrial genome from W34/70, and the ale strains compared to the reference mitochondrial genome S288c. Consequently, caution should be taken against reading too much into the extent of variation since it is reasonable to assume that the lager strains would be more similar as a group than the two ale strains to the haploid laboratory reference, S288c. Irrespective, the lager strains examined were particularly similar. The number of SNP’s were typically small (2 – 98 bps), and as such are unlikely have a severe impact on the overall functionality of the yeast mitochondria, depending on their precise location. This is partially indicated by the red colouration (Figure 3.20),

which indicates that only limited SNP's were found in coding sequences. Variation between the ale strains D23 and D3 was greater, with up to 96 bps changes at a single nucleotide location, potentially having a greater impact on functionality. A caveat is that further analysis would be required to determine if these events were contained within essential mitochondrial genes, or that gene functionality was impacted.

SMCC100 Base Changes (SNPs)

	A	C	G	T
A	0	1	1	0
C	3	0	0	1
G	4	0	0	6
T	1	0	0	0

SMCC99 Base Changes (SNPs)

	A	C	G	T
A	0	1	1	0
C	2	0	0	1
G	4	0	0	8
T	2	0	0	0

SMCC90 Base Changes (SNPs)

	A	C	G	T
A	0	1	2	1
C	1	0	0	1
G	5	0	0	7
T	1	0	1	0

D23 Base Changes (SNPs)

	A	C	G	T
A	0	17	64	96
C	36	0	7	82
G	79	4	0	36
T	91	51	16	0

D3 Base Changes (SNPs)

	A	C	G	T
A	0	17	55	88
C	27	0	5	69
G	61	2	0	28
T	83	48	17	0

Figure 3.20 Single nucleotide polymorphisms (SNP's) in mtDNA, compared to the reference genomes derived from strains W34/70 for the *S. pastorianus* strains and S288c for *S. cerevisiae*. The x axis refers to the original base identity, while the y axis indicates the changed base. The colours represent the overall impact of these variations with green being of lesser importance (generally non-coding regions) and red indicating SNP's occurring within coding regions.

Using the sequence data in conjunction with UGENE software (Section 2.6.6.), it was also possible to produce virtual restriction maps for each strain. Essentially, 'cutting sites' present within the different genomes could be identified, allowing more subtle differences to be identified than would be able to be visualised using electrophoresis-based methods. Based on the sequence data, restriction maps were created for each brewing yeast strain correlating to the cutting sites associated with HaeIII (Figure 3.21), HinfI (Figure 3.22) and DdeI (Figure 3.23). From this analysis it was evident that the two ales strains were readily distinguished, as expected based on the mtDNA fingerprinting data displayed previously (Section 3.2.4.1). In contrast, all of the lager strains produced very similar restriction maps, as anticipated; in each instance, where polymorphisms were present they are indicated by red circles (Figures 3.21-3.23). It can be seen that, for lager strains, the restriction enzymes HaeIII and HinfI exposed fewer alterations to the mtDNA genome sequences than DdeI, as indicated by the greater number of red circles in Figure 3.23, compared to Figures 3.22 and 3.23. It is interesting to note that the variation revealed here was not evident when using this enzyme as part of the RFLP digest previously shown above (Section 3.2.4.1). It is possible that this is due to the nature of gel electrophoresis and the size of the expected fragments. If running conditions are such that the particular DNA fragment is not resolved adequately, then this will remain undetected. To support this, analysis of the number of restriction sites and their close proximity indicates that many of the polymorphisms

that do exist would be too small to visualise using gel electrophoresis separation.

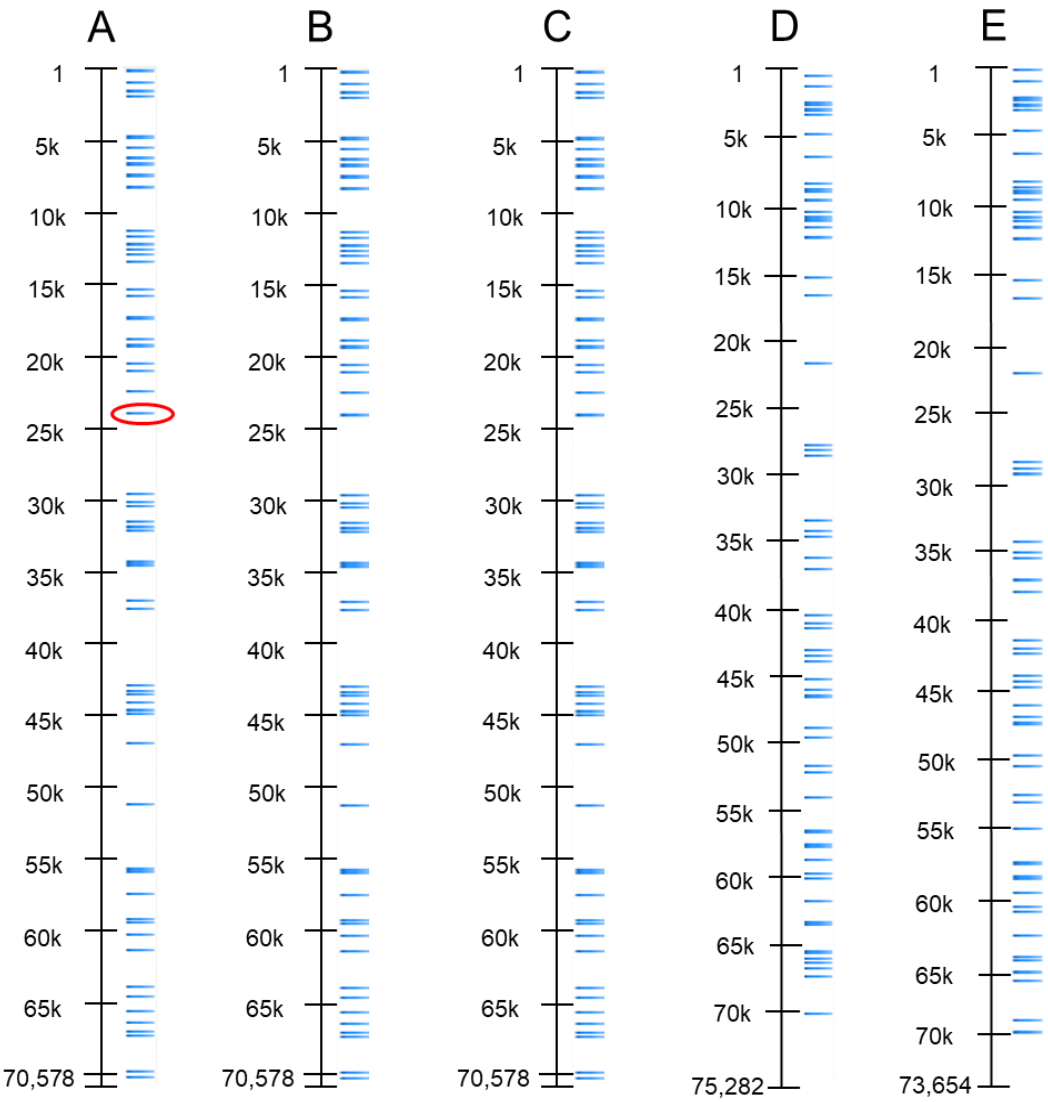


Figure 3.21 Analysis of restriction fragment length polymorphisms using mtDNA genome data. HaeIII cutting sites profiles for A: SMCC100; B: SMCC99; C: SMCC90; D: D23; E: D3. Multiple polymorphisms can be seen in the ale strains, based on broad differences in location and frequency of cutting sites. The red circle indicates polymorphism between lager strains only. Although it may not appear visually obvious from the figure, there are in fact two bands present within this region.

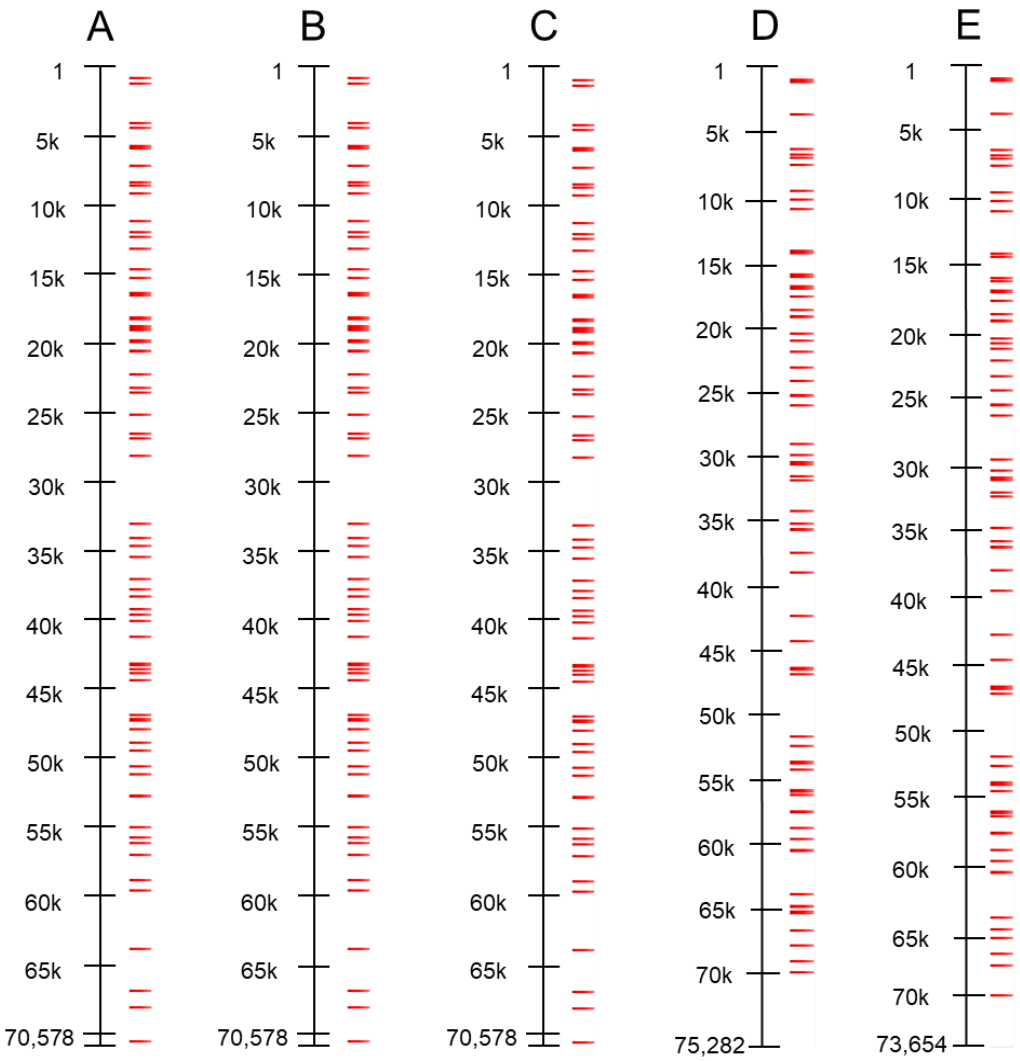


Figure 3.22 Analysis of restriction fragment length polymorphisms using mtDNA genome data. HinfI cutting sites profiles for A: SMCC100; B: SMCC99; C: SMCC90; D: D23; E: D3. No polymorphisms could be identified in lager brewing yeast. Multiple polymorphisms can be seen in the ale strains, based on broad differences is location and frequency of cutting sites.

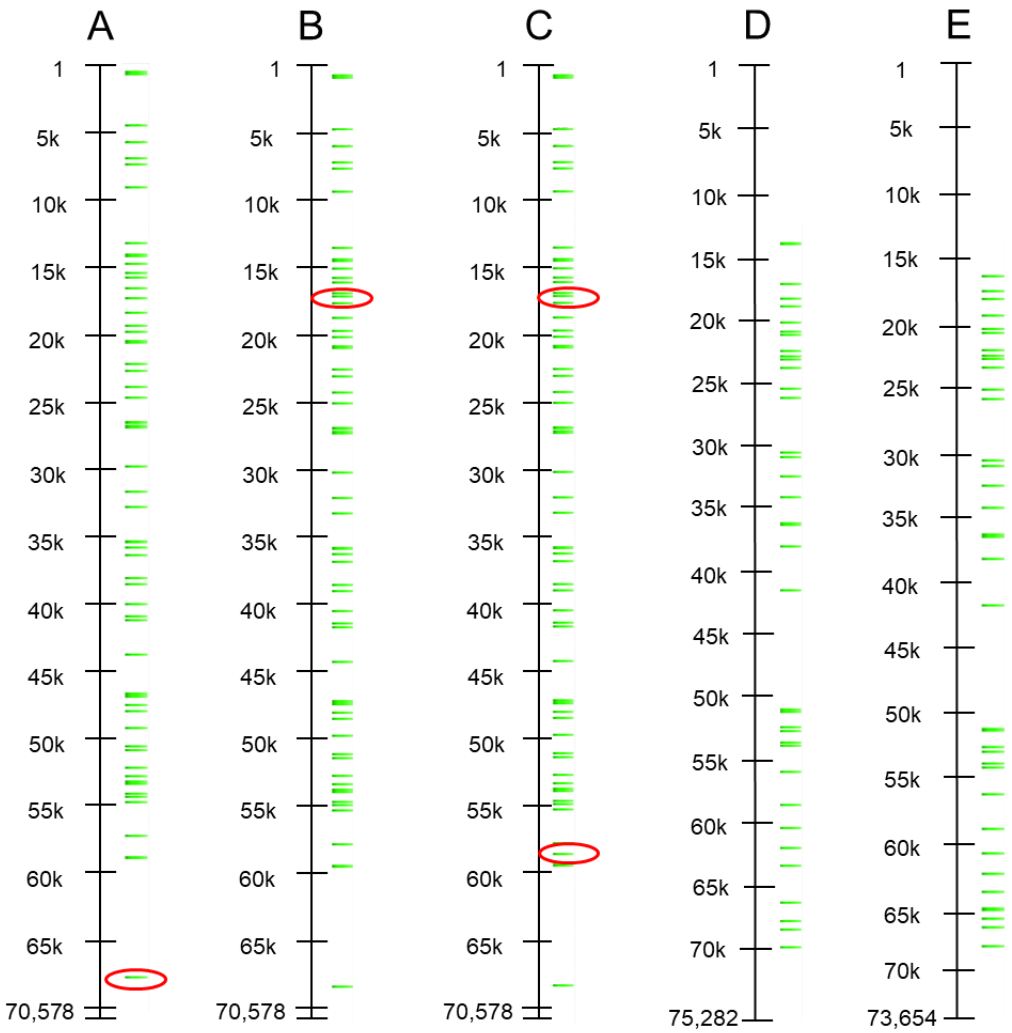


Figure 3.23 Analysis of restriction fragment length polymorphisms using mtDNA genome data. DdeI cutting sites profiles for A: SMCC100; B: SMCC99; C: SMCC90; D: D23; E: D3. Multiple polymorphisms can be seen in the ale strains, based on broad differences is location and frequency of cutting sites. The red circles indicate polymorphisms between lager strains only; in total 4 variations in restriction sites were identified.

3.3 Conclusions

Within this chapter, we investigated a range of physiological and genetic characteristics associated with ale and lager brewing yeast strains, as well as delving deeper into the genetic make-up of the yeast strains under investigation. Brewing yeast type was established using a combination of ITS-PCR and the x-alpha gal protocol to confirm the classification of lager and ale strains. In addition, giant colony morphology analysis and PCR-fingerprinting based on interdelta sequences confirmed that strains were discrete; each strain exhibited its own unique fingerprint.

Once this background information had been obtained, the genetic landscape of the mitochondrial genome in each strain was evaluated. Initial analysis indicated that although ale strains could be readily differentiated based on RFLP analysis of mtDNA, lager strains could not. However, using sequencing it was possible to gain a greater insight into the genetic makeup of brewing yeast mitochondria. Production and examination of mtDNA genome maps reinforced the previous supposition that there was little difference between lager strains in terms of gene location and number of ribosomal units, The greatest diversity observed was within the ale strain grouping. However, variations in the size and location of complex III and IV, involved in the electron transport chain may impact overall functionality and result in less efficient respiration pathways. Whether this has the potential to have major impacts on performance under strictly anaerobic fermentation is unknown, although based on their suitability for commercial use, it would appear not.

Sequence data was also used to develop a phylogenetic tree of yeast strains based on the mitochondrial genome. Analysis of the 5 production strains, along with *S. pastorianus* W34/70, *S. cerevisiae* strains (S288c and GLYBRCY22-3) and the *S. eubayanus* type strain (FM1318) indicated that the lager and ale strains could be clearly differentiated. Of the lager yeast, strain SMCC90 formed its own unique branch indicating that this individual was distinct to the other strains analysed. Interestingly, the type *S. eubayanus* strain was identified as being phylogenetically distinct to all of the strains analysed but was more similar to the *S. cerevisiae* strains than the lager yeasts. This was unexpected since the current consensus is that lager yeast mitochondria are derived from the *S. eubayanus* parent (Rainieri *et al*., 2008; Dunn and Sherlock, 2008). It was interesting to note also that the strain GLYBRCY22-3 which is a *S. cerevisiae* biofuel yeast, appeared to be more similar to the *S. eubayanus* strain than the other *S. cerevisiae* strains.

Analysis of SNP within each genome again revealed that there was little variation between the *S. pastorianus* strains. The greatest degree of variation was seen within the *S. cerevisiae* strains. This observation was supported by subsequent analysis of restriction maps. Using software, it was possible to identify cutting sites associated with common restriction enzymes. With this method we were able to identify subtle differences between *S. pastorianus* strains which couldn't be identified using the conventional mtDNA fingerprinting method (Chapter 3). This analysis offers potential for a more targeted approach to strain identification in the future.

Based on the data shown here, it can be seen that mitochondria from ale strains were distinct. However, there were major similarities within the lager yeast investigated. While this was broadly expected, it does raise questions with regard to the practicalities of fermentation and yeast handling, since it is known that the frequency and occurrence of mitochondrial (petite) mutations varies between strains. One rationale for this is that mitochondria are impacted by nuclear gene expression and affected by other events occurring within the cytoplasm. Hence, it is perhaps inevitable that the scenario is more complex than the data shown here suggests. Furthermore, analysis of mtDNA alone does not provide any indication of the role of mitochondria in fermentation under strictly anaerobic conditions, or the life cycle of mitochondria in brewing yeast. These concepts will be investigated further in Chapters 4 and 5 respectively.

CHAPTER 4 THE IMPACT OF PETITE MUTATION ON FERMENTATION CHARACTERISTICS

4.1 Introduction

Brewing yeast form a robust group of organisms that can be used to ferment a wide range of worts to good effect. This is primarily because strains are able to tolerate stress factors associated with yeast handling, meaning that they can theoretically be used many times during serial repitching without any adverse effects on product quality (Section 1.3.4). However, on occasion, mutations can arise within the population leading to a range of deficiencies, including inconsistent or delayed fermentations, poor ethanol tolerance, and inconsistent flavour development (Ernandes *et al*., 1993; Lodolo *et al*., 2008; Lawrence *et al*., 2012) (Section 1.11.1). When this occurs, it can necessitate changes to the process, including extended maturation times, blending of the final product, or supplementation with processing aids. In addition, the yeast culture is typically discarded and replaced with fresh yeast from propagation or alternative sources (Boulton and Quain, 2001; Smart, 2008) (Section 1.3). Consequently, it can be seen that preventing or minimising mutations is preferable from both a quality and logistical perspective.

The two most widely reported types of mutation encountered in brewing cultures are those associated with flocculation variation and with the accumulation of respiratory deficient cells. Respiratory deficient cells display reduced mitochondrial function and are often termed ‘petite’ due to the small size of colony produced on solid media (Ephrussi *et al*., 1949; Gibson *et al*., 2008; Jenkins *et al*., 2009; Lawrence *et al*., 2012) (Section

1.11). In yeast, petite variants can be divided into two groups: Rho^- mutants contain compromised mtDNA, preventing the mitochondria from functioning correctly; and Rho^0 mutants have a complete absence of mtDNA. While the phenotypes of both Rho^- and Rho^0 petites are not dissimilar, this distinction is important since it implies a different level of damage to the mitochondria, and to the cell. Indeed, it is likely that Rho^0 cells develop out of Rho^- individuals, due to poor inheritance of mitochondria (Piskur, 1997; Osman *et al*., 2015) (Section 1.11). Irrespective, of their form, most brewing yeast slurries contain respiratory deficient cells at low concentrations, often between 1-3% of the total population. At these concentrations they can persist within the population with little noticeable impact on fermentation. However when the number of respiratory deficient cells reaches a certain percentage often over 15% of the yeast slurry population, their presence has a negative impact on yeast growth, fermentation time, the production of desirable flavour compounds, and diacetyl reduction (Lodolo *et al*., 2008; Pires *et al.*, 2014) (Section 1.11.1).

Although it is known that a variety of stress factors can give rise to petite mutations, the development of the petite phenotype has not been fully characterised from the perspective of the individual cell. Interestingly, anecdotal reports have indicated that the rate of mutation may be strain specific, with some yeast showing a greater propensity to form mutations than others. In this chapter the incidence of petites occurring naturally in brewing strains and under artificial conditions designed to promote mutation was determined. In addition, the petites obtained were fully

characterised for growth characteristics, fermentation performance, sugar utilisation and flavour generation. It is anticipated that this information will lead to a greater understanding of the potential impact of petites on fermentation, as well as an indication of how petites are able to survive and proliferate through successive fermentations.

Experimental approach

In order to determine the relative propensity of different yeast strains to form petites, respiratory deficient mutants were isolated from standard media, and from media supplemented with ethidium bromide (Section 2.5.3.) and with ethanol (Section 2.5.4). These were then characterised by mtDNA fingerprinting using RFLP analysis (Section 2.6.5) and subsequently analysed for fermentation characteristics in mini (100mL) fermentations (Section 2.4.5.) and for sugar assimilation in 96 well plates (Section 2.4.3). This was performed primarily to characterise the impact of the petite mutation on fermentation, but also to gain an understanding of the physiological characteristics of the petite cell. The latter was assessed by determining intracellular carbohydrate reserves post fermentation, and by examining stress tolerance in each of the respiratory deficient strains when growing in media supplemented with ethanol as an additional ‘stressor’.

4.2 Results

4.2.1 Relative propensity of brewing yeast strains to petite formation

4.2.1.1 Incidence of petites under non-stress conditions

In order to investigate the natural propensity of brewing yeast to form petite mutations, the lager strains SMCC100, SMCC99, SMCC90, SMCC57, and the ale strains D3 and D23 were analysed for the incidence of mutations under 'optimum' growth conditions. Each strain was grown in liquid YPD media for 72hours (Section 2.5.1) and subsequently cultivated on solid agar (Section 2.5.3) to assess the 'natural' rate of petite production. The latter was facilitated using the TTC overlay method to analyse a minimum of 2000 colonies per strain (Section 2.3.4).

It can be seen from Figure 4.1 that, overall, petite production was low across all of the strains examined. The lowest rate of petite production was for the ale strain D3 (0.26 %), while the highest was for the lager strain SMCC57 (3.25%). It should be noted that even though these values may appear minor, all of the strains produced petites under conditions which were not at all severe. However, strain SMCC57 proved to be of particular interest since this yeast naturally produced significantly more respiratory deficient cells than the other strains. At the time of analysis, there was little information available from SABMiller with regard to any issues associated with this yeast within the brewery. However, it is reasonable to assume that this capacity to form petites may be problematic, especially since it is likely that the quantity of mutant cells

within a yeast slurry would only increase through successive fermentations due to associated stress factors (Powell *et al*., 2007; Gibson *et al*., 2008; Lawrence *et al*., 2012).

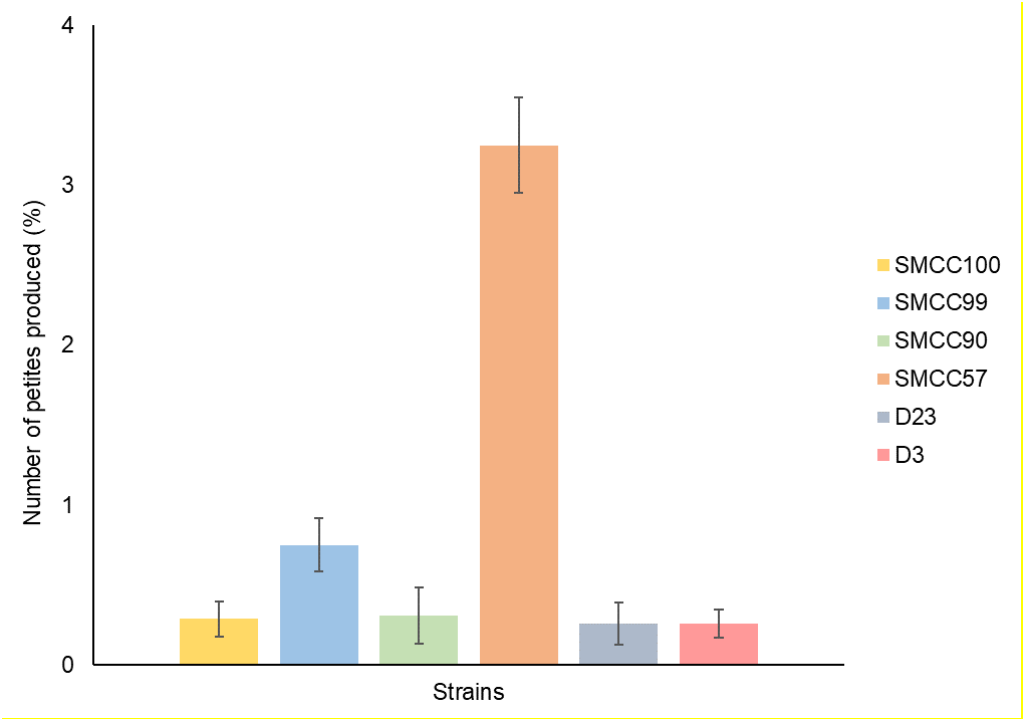


Figure 4.1 Generation of petites by yeast strains cultivated on YPD agar plates under standard laboratory conditions.

4.2.1.2 Incidence of petites under stressful conditions

Once the initial rate of petite production under relatively positive conditions had been established, strains were challenged by a series of stresses to assess their resilience to petite production (Section 2.5.3). In this instance, the ale strains were eliminated since the original analysis described above showed they had the lowest capacity to form petites. In contrast, the lager strains appeared to be more susceptible and, given their commercial significance, these strains became the main subject for further experimentation. In an attempt to induce the petite mutation,

ethidium bromide (100 µg/mL) (Section 2.5.3) and ethanol (5 and 10%) (Section 2.5.4) were applied to cells in separate reactions. Ethidium bromide was employed since it is a well-known mutagen which acts by inhibiting the replication and translation of mitochondrial DNA (Warren *et al*., 2017). This agent has also been used previously to examine resilience to petite production under different conditions, for example when comparing the propensity of mother cells verses daughter cells to produce petites (Nagley and Mattick, 1977). Ethanol was also applied as a stress factor, since this is known to cause damage to DNA structures in yeast cells (Ibeas and Jimenez, 1997) and is more relevant from a brewing perspective; in this instance the concentrations of ethanol were selected based on typical concentrations found during fermentation and in the final product. Once cells had been stressed, aliquots were plated onto YPD media and tested for petite generation using the TTC protocol (Section 2.3.4.). In total approximately 2,000 colony forming units were assessed for each strain under each set of conditions.

When yeast cells were challenged with ethidium bromide, a similar pattern of results could be seen to those reported previously (Section 4.2.1). However, in contrast to the previous data, the incidence of petites was enhanced significantly. Strain SMCC57 produced the greatest percentage of petites (30.97%), representing a 10-fold increase in production. While this data represented the greatest total quantity of petites generated, the lager strains SMCC100, SMCC99 showed the greatest *increase* in petite production, from 0.29% to 10.29%, and from 0.75% to 17.05%, respectively. Strain SMCC90 was particularly resilient

and when subjected to ethidium bromide stress, only 1.87% of the population comprised petite mutants.

Subsequently, a similar analysis was conducted using both 5% and 10% ethanol as the stressor. From Figure 4.3, it can be seen that, as before, all of the strains produced cells exhibiting the petite mutation. This was within the range of 0.5% to 6%, depending on the strain and the concentration of ethanol applied. A similar pattern of results was seen, with strain SMCC57 continuing to yield the greatest percentage of petites under both ethanol conditions: 4.47% and 5.62% for 5% and 10% ethanol respectively, while SMCC90 remained the lowest at 0.77% and 0.93%. The other two strains analysed displayed characteristics in-between these extremes: SMCC100 produced petites at 0.64% at 5% ethanol, increasing to 2.92% in 10% ethanol. Under 5% ethanol stress, SMCC99 produced 0.56% petites, and at 10% this increased to 1.7%. This data, in conjunction with those presented in Figures 4.1 and 4.2, suggests that strain SMCC90 is robust and less likely to produce the petite mutation, while strain SMCC57 is particularly susceptible to developing respiratory deficient cells. Furthermore, the data also illustrates the impact that different stress factors may play on the rate of petite production. Although ethidium bromide is a known mutagen and not associated with brewing processes, ethanol at low concentration is a readily encountered stress during fermentation. Given that the brewing process is complex, with a myriad of interwoven stress factors being applied consecutively across yeast handling, it is important to recognise that in reality the pressures on each cell in ‘real life’ are likely to be elevated. Consequently, although the

primary goals of this work were to generate petite mutants for analysis, and to determine their rates of production between strains, this data does indicate that a more holistic approach to the analysis of stress factors associated with yeast handling on petite production would be insightful.

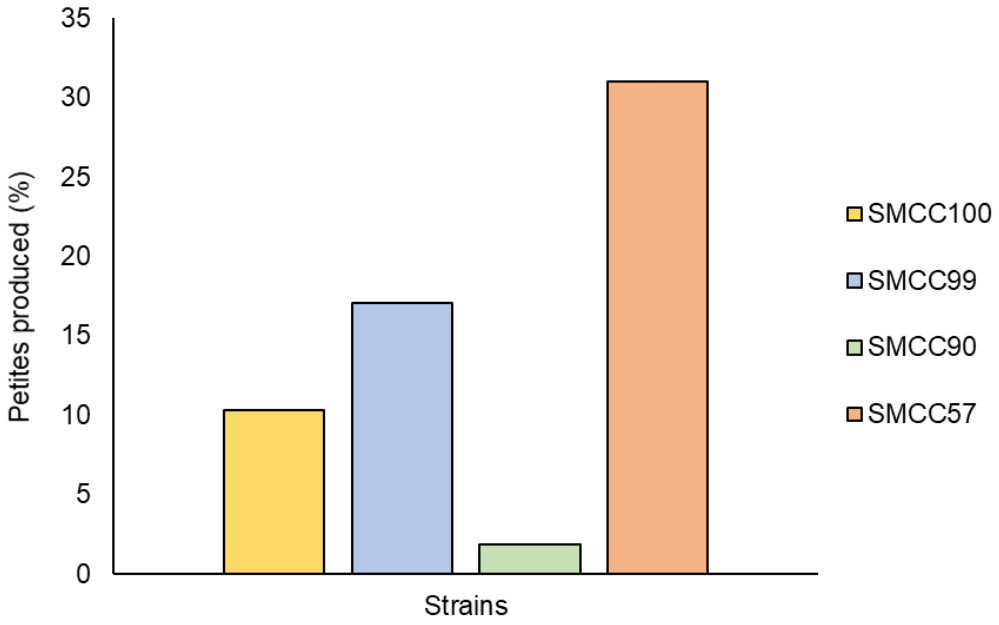


Figure 4.2 The rate of petite production in *S. pastorianus* yeast strains when exposed to the mutagen ethidium bromide.

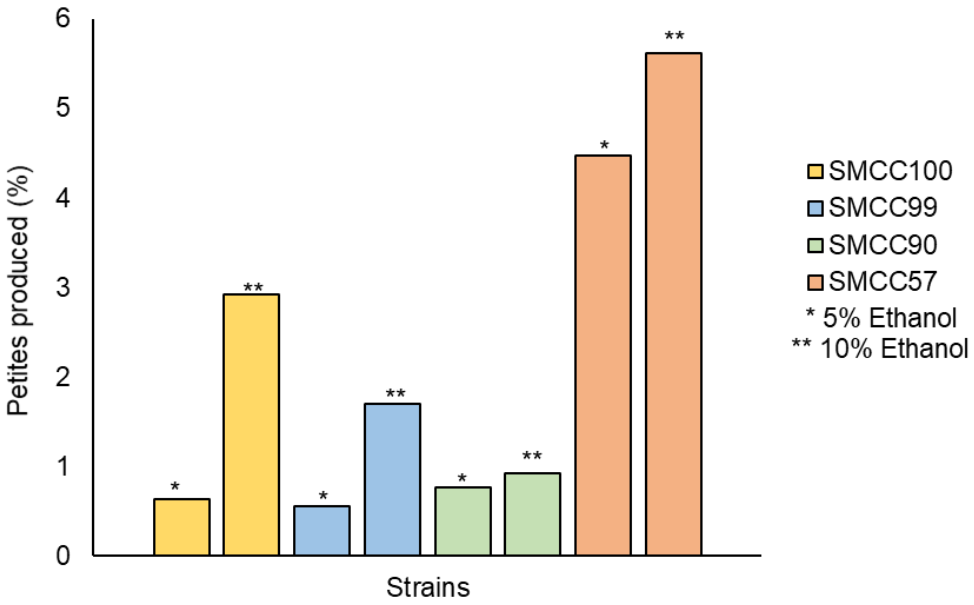


Figure 4.3 The rate of petite production in *S. pastorianus* yeast strains when exposed to 5% (*) and 10% (**) ethanol stress.

4.2.2 Confirmation of the petite mutation

While petites were produced more readily when strains were exposed to ethanol or ethidium bromide, those produced naturally were chosen for further study since it was reasoned that they would be more representative of petites present in the brewery than those produced 'artificially'. It should be noted that for each strain, a single petite colony was selected at random for further analysis. This was performed since the primary goal of subsequent experiments was to analyse the performance of petites against a range of brewing strains, rather than to compare the performance of different petite strains *per se*. These naturally occurring petite colonies (from this point referred to using the suffix 'p') were collected and re-cultivated on both YPD and YPGly agar plates. Since petite mutants cannot grow on non-fermentable carbon sources, this acted to provide additional reassurance that they were

indeed respiratory deficient (Section 2.2.1). An example of the comparative growth of petites on YPD and YPGly can be found in Figure 4.4 .

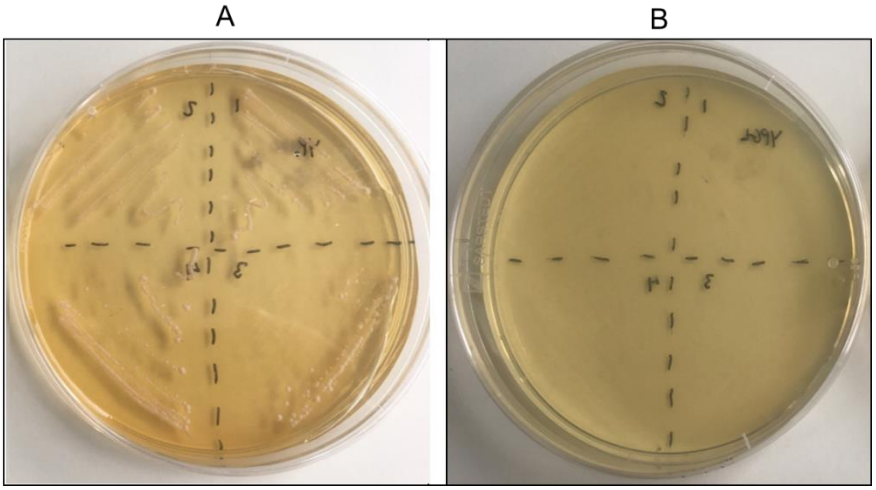


Figure 4.4 Confirmation of the petite mutation by comparing the capacity to grow on the fermentable carbon source, YPD (A) with the inability to be cultivated on the non-fermentable carbon source, YPGly (B).

Once the petite colonies had been confirmed to be respiratory deficient based on growth requirements, each isolate was further examined by RFLP of the mtDNA (Section 2.6.5). This was performed to provide information on the mtDNA genome, including analysis of the broad differences between petites and parental strains, and also to identify whether the mutations were in the form of Rho^- or Rho^0. Examination of each RFLP profile indicated the extent of damage to the mtDNA, as shown in Figure 4.5. In each instance it can be seen that, compared to the respiratory competent parent strains (Figure 4.5A), the petite profiles were considerably different (Figure 4.5B). In each instance the mtDNA profiles exhibited less fragments than the parent strains and the majority

of fragments that were produced were much smaller in size. This was not unexpected, since a previous study into brewing yeast mitochondrial DNA indicated a similar pattern of results (Lawrence *et al*., 2012). Closer analysis of the size of fragments indicated that in each case the largest fragment was approximately 1000 bp in size, where respiratory competent cells contained a number of fragments greater than this. One notable exception was SMCC99p which exhibited a very large band of over 10,000 bp. The nature of this fragment is unknown, although it could have arisen due to the incorporation of 'nonsense' DNA, leading to errors during duplication causing a large mass of DNA to be generated. Interestingly, this strain did continue to show some smaller bands, in line with the other petite mutants. Furthermore, although each petite mutant evidently contained less mtDNA (based on the low intensity of the digested fragments), they seemed to show similarities in terms of the final profile, with bands at 900, 750 and 500 bp. The exception to this was for strain SMCC90p which showed virtually no mtDNA restriction fragments at all. Previous studies have suggested that the presence of smaller mtDNA fragments is not unusual in petite mutants (Piskur *et al*., 1998; Lawrence *et al*., 2012), and the uniform sizes observed are likely to be caused by hotspots within the mtDNA. Hotspots represent sequences that are more susceptible to damage and these have been reported in nuDNA and mtDNA (Zhou *et al*., 2013). Their presence may go towards explaining the similarity in banding patterns observed here since hotspots are more likely to reduce the size of large restriction fragments, rather than smaller pieces of mtDNA.

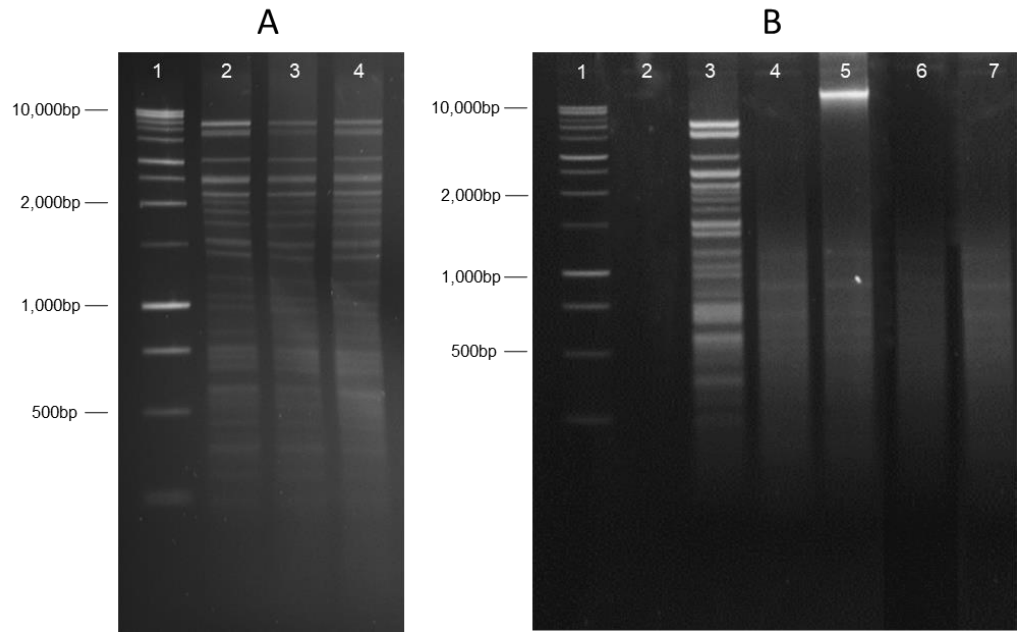


Figure 4.5 RFLP profiles of mtDNA *S. pastorianus* strains. (A) Wild-type profiles; lane 1: 1kb ladder; lane 2: SMCC100; lane 3: SMCC99; lane 4: SMCC90. (B) Naturally produced petite strains profiles; lane 1: 1kb ladder; lane 2: Blank; lane 3: SMCC100; lane 4: SMCC100p; lane 5: SMCC99p; lane 6: SMCC90p; lane 7: SMCC57p.

4.2.3 Physiological properties of petites

It might be expected that petite cell populations should be less viable than their respiratory competent counterparts, since the mitochondria is a key cellular organelle. However, it is accepted that petites can persist through fermentations and in fact can occur with increasing frequency during yeast handling (Jenkins *et al*., 2009). In order to provide an insight into the relationship between petite generation and viability in the strains generated, all strains were grown in YPD media under standard

laboratory conditions for 3 days and viability was assessed according to Section 2.3.2. Although this was a rather simplistic experiment, the data indicated that generation of the petite phenotype did not necessarily lead to a significant reduction in population health. It can be seen from Figure 4.6 that there was no difference in viability between the strains and their petite counterparts. The only exception to this was for the petite strain SMCC100p, which displayed significantly reduced viability (94% compared to 97.5% in the grande strain) according to ANOVA analysis ($P<0.001$). However, even this difference was relatively small and certainly within the acceptable limits imposed by many breweries.

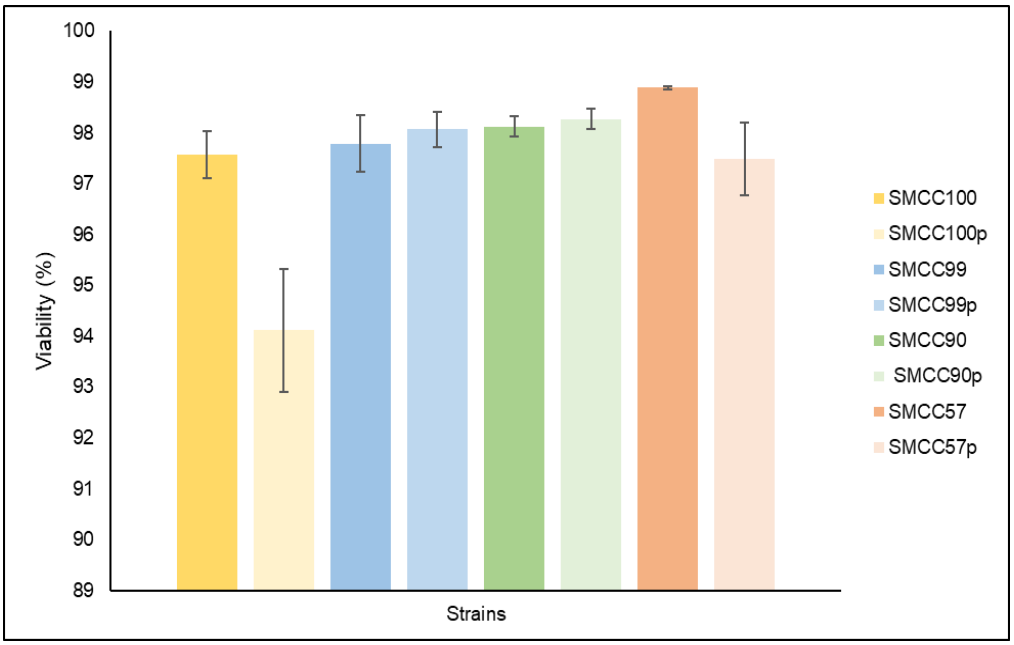


Figure 4.6 Comparison of cell viability between grande strains (SMCC100, SMCC99, SMCC90, SMCC57) and petites (SMCC100p, SMCC99p, SMCC90p, SMCC57p) following growth in standard YPD media, and in the absence of any stress factors. Error bars indicate the standard deviation of the mean of triplicate samples.

In addition to examining strain viability, the membrane potential of the cells was investigated as an alternative measure of yeast quality. Cell membrane potential has been shown to be a good indicator of cell overall functionality and health (Hewitt and Nebe-Von-Caron, 2001), as maintaining membrane potential prevents environmental toxicants from entering the cell and causing damage. Membrane potential can be analysed using the fluorescent stain DisBAC2 (3). This stain enters cells where it binds to intracellular proteins and membrane structures, creating fluorescence in the red spectrum. When cells have a reduced or compromised membrane polarity an influx of the dye occurs leading to an increase in fluorescence (Hewitt and Nebe-Von-Caron, 2001). Each petite strain and its respiratory competent parent were analysed using DisBAC2(3) in conjunction with propidium iodide as a viability stain, and populations were examined using flow cytometry to analyse 20,000 cells per sample in triplicate as outlined in Section 2.3.7. In addition to the experimental samples, control samples were prepared in order to provide 'gates'; these additional samples contained 100% dead and 50/50 live/dead cells ensuring that only data from living (viable) cells was collected.

It can be seen from Figure 4.7 that in each instance petite cell populations produced a greater fluorescent value than the parent strain, indicating that the membrane may have been compromised to a certain extent, despite cells remaining viable. It is possible that the ATP supplied by functional mitochondria is involved in the polarisation of the cell membrane and that respiratory deficient yeast are unable to supply the

required ATP to maintain the cell membrane potential (Kováĉ and Varečka, 1981; Maresova *et al*., 2006). However, it should be noted that only the differences between SMCC99 and SMCC99p, and between SMCC90 and SMCC90p were statistically significant (P value 0.005 and P value 0.02). Furthermore, all values were within a similar range, and it is difficult to draw strong conclusions from this data.

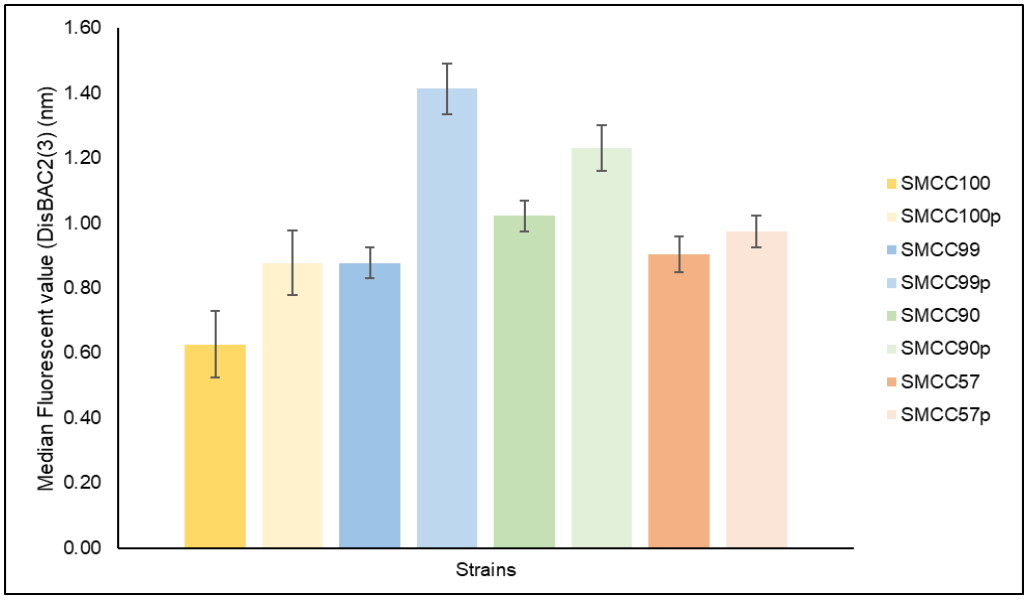


Figure 4.7 Comparison of membrane potential between grande and petite yeast strains. Polarity was determined by $DisBAC_2(3)$ in conjunction with flow cytometry. Data is expressed according to the median fluorescent values generated, using gated results showing fluorescence derived only from viable cells. Error bars indicate the standard deviation of the mean of triplicate samples. Petite strains are indicated by the suffix ‘p’ in each instance.

4.2.4 Growth characteristics of petite mutants under ethanol stress

Following the initial examination of petites and their physiological properties, analysis of growth characteristics was conducted in 96 – well plates containing 15 °P wort as described in Section 2.4.3. It is evident from Figure 4.8 that, comparatively, petites do not grow as vigorously as their grande counterparts. In each instance differences were noticed almost immediately; petite strains display an increased lag phase and reduced rate of logarithmic growth. Furthermore, the time required to reach stationary phase was extended: 45h compared to 28h, for strains SMCC100 and SMCC99, and even longer for strain SMCC57. This pattern was not unexpected since slow growth is one of the primary attributes which was initially used to define the petite mutation (Ephrussi *et al*., 1949).

Once typical growth dynamics had been confirmed, each strain was analysed for growth under stress conditions, created by supplementing media with 5 and 10% ethanol. This was performed to determine the relative tolerance of petite strains to their grande counterparts. It can be seen from Figure 4.9 that, when supplemented with 5% ethanol, the growth of all strains was affected. Although the length of lag phase in the respiratory competent parental strains remained similar to that observed in the absence of stress, log phase was disrupted, perhaps as a result of diauxy, and stationary phase was delayed. However, when analysing the petite mutants, it can be seen that these strains were more severely impacted (Figure 4.9). While the grande strains were able to enter log

phase after 1h growth, the petites exhibited an extended lag phase ranging from of 8-10h. Furthermore, after 45h growth stationary phase had yet to be reached.

When the concentration of ethanol was increased to 10%, the effects were exacerbated further. All strains suffered restrictions to growth, with extended lag phase and poor overall biomass production after 45h analysis. In each instance the petite strains were more affected than the parental strains, demonstrating the same trend reported previously for 5% ethanol stress. This data reveals that under aerobic growth conditions the respiratory deficient petites were unable to grow as effectively as the grande parental strains. Even in the absence of any stress factor, this trend is clear; they were far less effective than the respiratory competent parental strains.

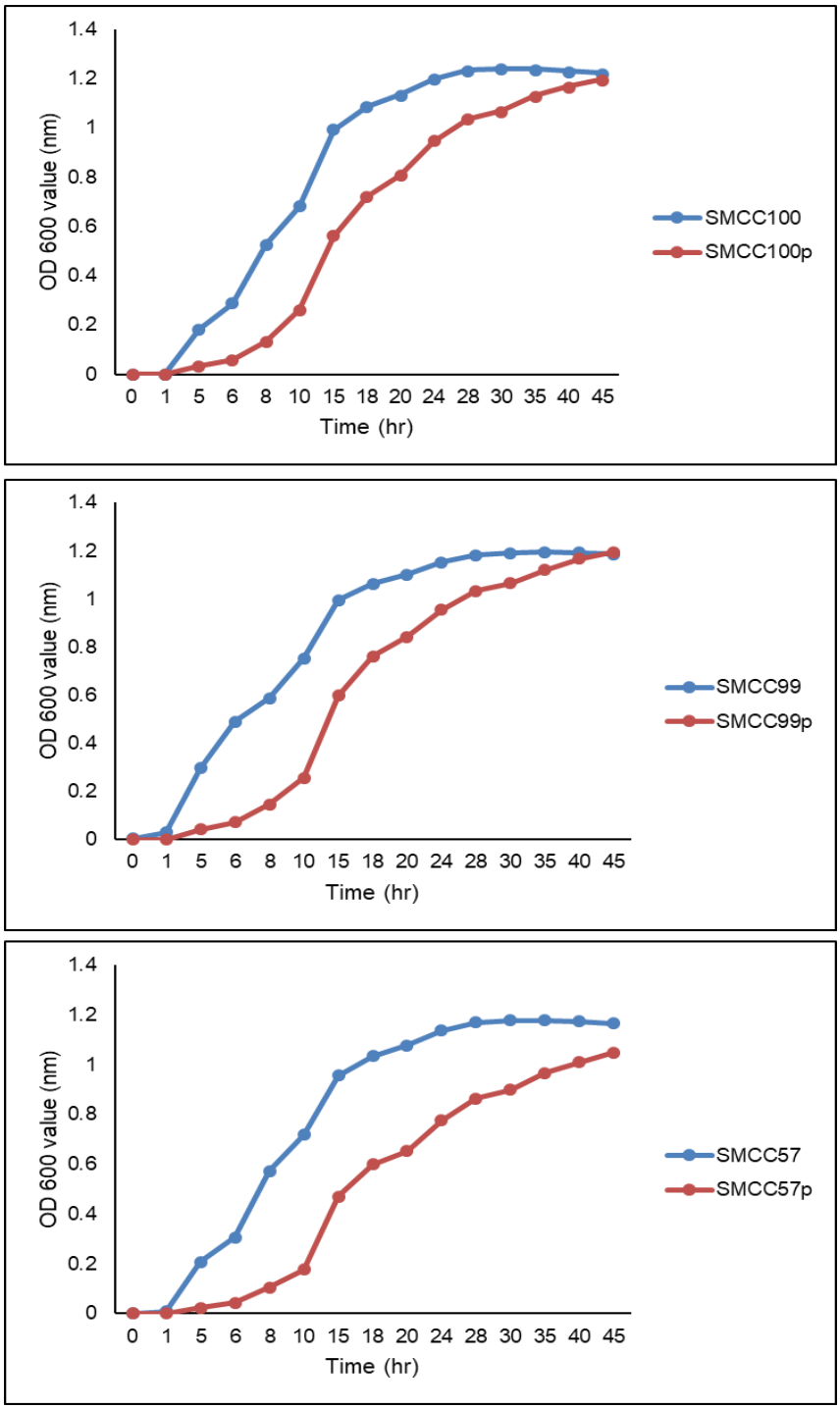


Figure 4.8 Analysis of the growth characteristics of lager yeast strains SMCC100, SMCC99, SMCC57 and their petite counterparts, denoted by the suffix ‘p’. Cell growth was examined by cultivating cells aerobically in 96-well plates containing 15 °P wort. Growth was measured by monitoring absorbance at 600nm over 45 hours.

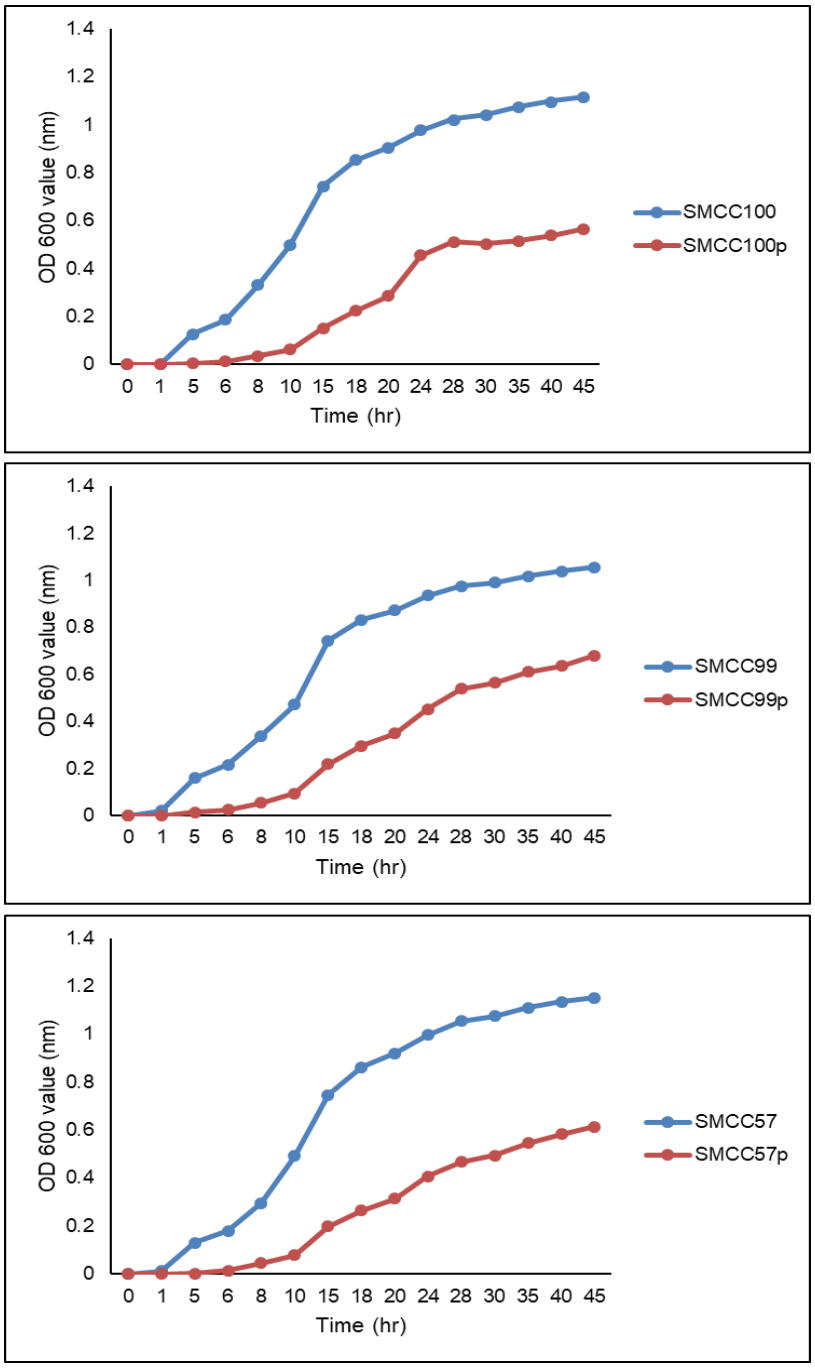


Figure 4.9 Analysis of the growth characteristics of lager yeast strains SMCC100, SMCC99, SMCC57 and their petite counterparts, denoted by the suffix ‘p’. Cell growth was examined by cultivating cells aerobically in 96-well plates containing 15 °P wort and 5% ethanol. Growth was measured by monitoring absorbance at 600nm over 45 hours.

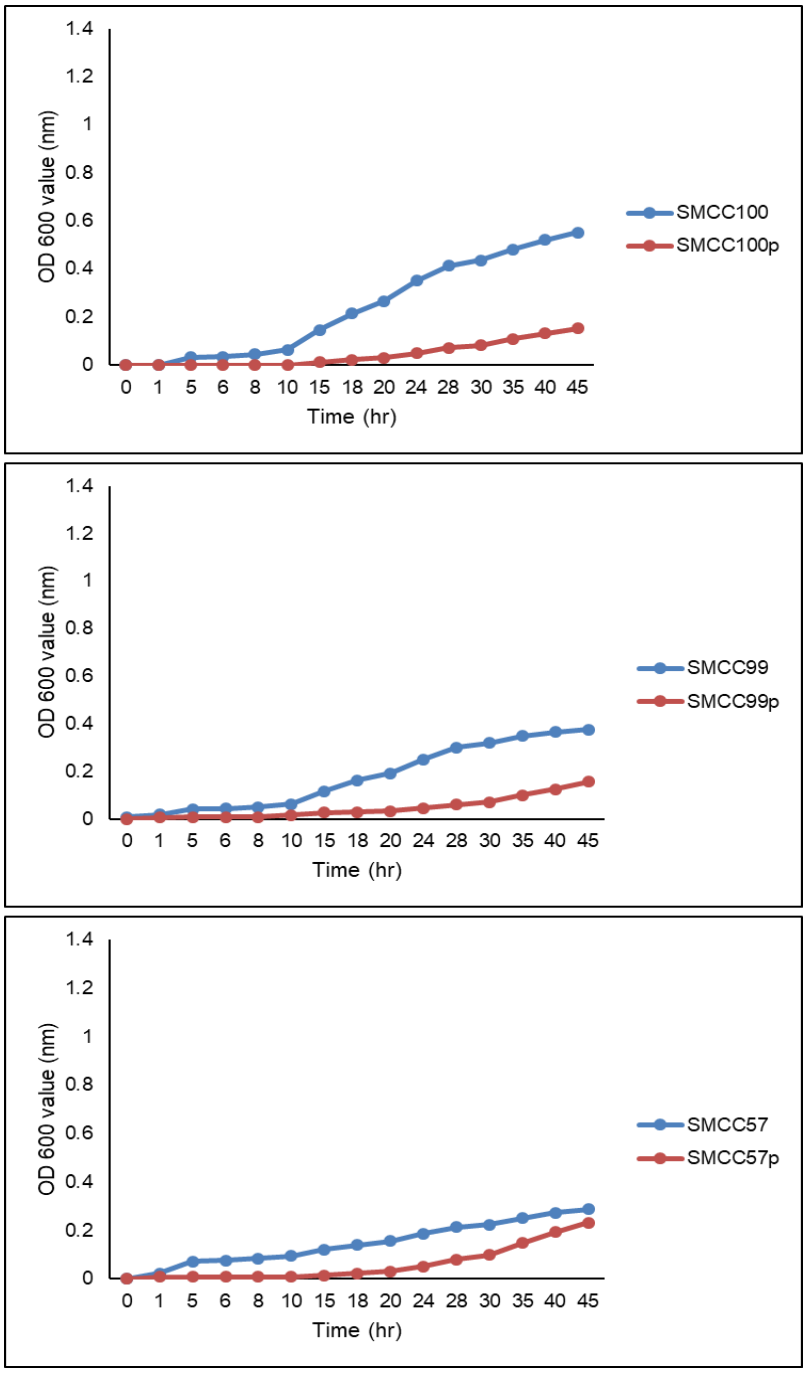


Figure 4.10 Analysis of the growth characteristics of lager yeast strains SMCC100, SMCC99, SMCC57 and their petite counterparts, denoted by the suffix ‘p’. Cell growth was examined by cultivating cells aerobically in 96-well plates containing 15 °P wort and 10% ethanol. Growth was measured by monitoring absorbance at 600nm over 45 hours.

4.2.5 Fermentation characteristics of petite mutants

While the data presented above provides some insight into the growth potential and stress tolerance of petite strains, this has little bearing on fermentation performance. This is because cellular division in fermentation is restricted to 2-3 divisions on average (Boulton and Quain, 2001), and because conditions are strictly anaerobic. Consequently, small scale (100mL) fermentations were conducted in order to gain greater insight into the impact of petite mutation on fermentation characteristics. Propagated yeast cells (1.5×10^7 cells/mL) (Section 2.4) were pitched into 15 °P wort, pre oxygenated at 15 °C for 24 hours as described in Section 2.4.4. Fermentation progression was monitored by tracking weight loss over time as described in Section 2.4.5.

From Figure 4.11 it can be seen that initially there was little difference in fermentation profile between the petite and grande strains. A similar weight loss percentage was observed in each instance over the first 33 hours. However, after this timepoint differences started to occur between the grande and petite strain. All of the petite strains were unable to attenuate to the same level as the grande strains, which may suggest a reduced ability to consume the more complex carbohydrates such as maltose and maltotriose which predominate towards the end of fermentation. This data confirms previous reports that petites are less effective at fermentation than grande strains (Ernandes *et al*., 1993; Lodolo *et al*., 2008; Lawrence *et al*., 2012), however it also provides

assurance that the petite strains developed in the current study are typical to those encountered within the brewery.

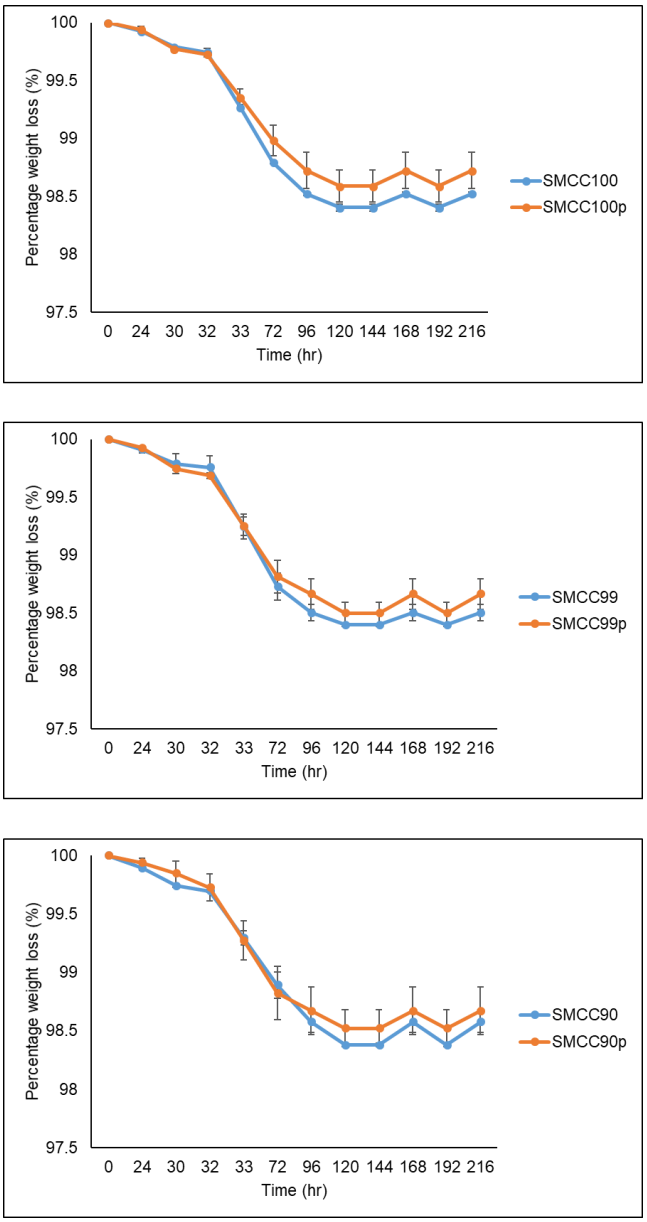
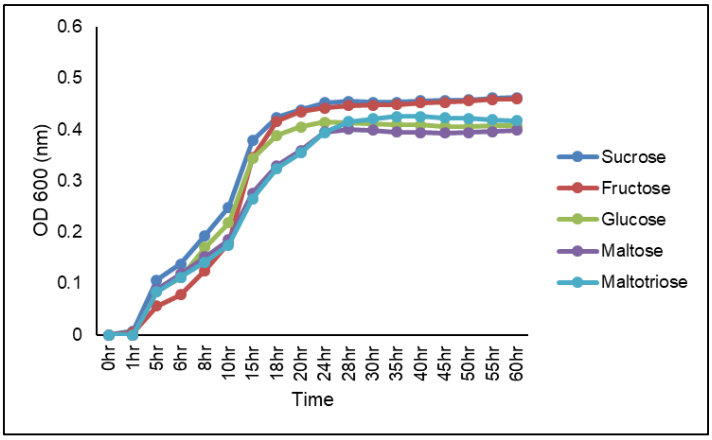


Figure 4.11 Analysis of the fermentation profiles of lager yeast strains SMCC100, SMCC99, SMCC57 and their petite counterparts, denoted by the suffix ‘p’. Mini (100mL) fermentations were conducted in 15°P wort at 15 °C and progression was monitored by measuring weight loss over time. Error bars indicate the standard deviation of the mean of triplicate samples.

Given that the petite mutants investigated here were unable to attenuate to the same level as the parental strains, the lager strain SMCC100 was selected (primarily due to its commercial relevance) and investigated for the capacity to utilise specific wort sugars. Cultures of SMCC100 and its derived petite were cultivated in 96 well plates in the presence of sucrose, fructose, glucose, maltose and maltotriose, and growth was determined by monitoring absorbance at OD600 nm as described in Section 2.4.3. It can be seen from Figure 4.12 that the grande strain was able to assimilate all sugars with little problem. However, the petite isolate showed significant variation. While sucrose could be metabolised without any issues, surprisingly, glucose and fructose uptake was impaired. This was unexpected since sucrose is believed to be largely broken down to glucose and fructose in the periplasmic space, hence this data is difficult to explain. However, it has been shown that wine yeast with disrupted hexokinase genes can show altered affiliation for certain sugars (Guillaume *et al*., 2007). Furthermore, even though fructose can be broken down in the periplasm, it is known that yeast also contain cytosolic forms of invertase (Marques *et al*., 2015). Consequently, it is possible that variation in sugar assimilation may be largely determined by transport mechanisms through the cell membrane. This is supported by data from analysis of the uptake of the complex sugars, maltose and maltotriose whose primary route into the cell is via active transport. For these sugars, growth was significantly impaired compared to the parental strain (Figure 4.12). Given that ATP is required to assimilate long chain carbohydrates (as well as simple sugars when present at low

concentrations), it is possible that this data implies that there may be an energetic requirement which cannot be met by the yeast. This theory is supported by previous analysis showing that the respiration pathway in yeast is blocked using antimycin A, causing reduced utilisation of maltotriose due to a lack of available energy (Londesborough, 2001; Zastrow *et al.,* 2001; Vidgren *et al*., 2009). Additionally it may be possible that reduced transport of maltose and maltotriose is a consequence of the reduced cell membrane potential present in petites, which could also disrupt transport systems (Van Leeuwen *et al*., 1992; Vidgren *et al*., 2010). Despite this, it is evident that more research is required to better understand why respiratory deficient mutants are unable to utilise maltotriose and maltose as readily as the respective grande strain.

A



B

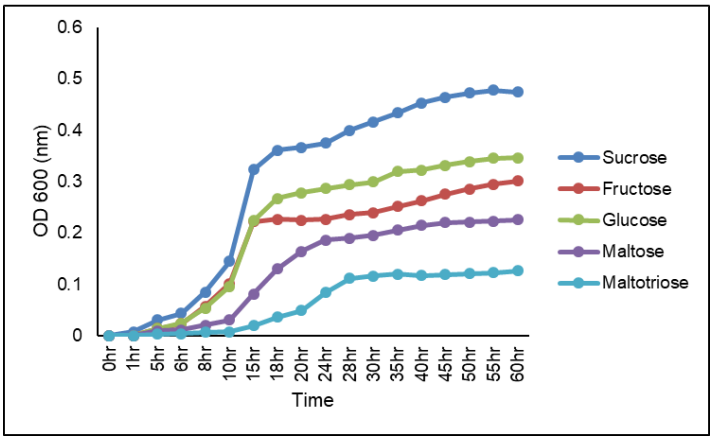


Figure 4.12 Analysis of the growth characteristics of lager yeast strains SMCC100, and the petite counterpart, denoted by the suffix ‘p’. Cell growth was examined by cultivating cells aerobically in 96- well plate containing 197 µL laboratory growth media comprising 1% yeast extract, 2% peptone and 1% of the respective sugar (sucrose, fructose, glucose, maltose, maltotriose) and 3 µL yeast sample and the absorbance was measured at 600nm over 45 hours conducted aerobically.

4.2.6 Yeast quality pre and post fermentation

Once fermentations had been conducted, the quality of the yeast was assessed to determine the capacity of petites to withstand the process. Consequently, viability was analysed using methylene blue staining (Section 2.3.2) and concentrations of the intracellular carbohydrate reserves glycogen and trehalose were quantified. These sugars are known to be key factors in determining yeast quality since glycogen forms a readily available source of sugar, while trehalose is known to be formed in response to stress and acts to protect membrane structures within the cell (Hounsa *et al.,* 1998; François and Parrou, 2006; Bandara *et al*., 2009). Hence high levels of glycogen in respiratory competent cells are often used as a measure of cell health, while the presence of trehalose can indicate that cells are stressed. Each sugar was enzymatically digested to glucose using α-amyloglucosidase and trehalase (for glycogen and trehalose respectively) and glucose was then quantified spectrophotometrically according to Section 2.3.6.

Analysis of viability indicated that, post fermentation, the respiratory competent populations comprised 95%, 97% and 95% live cells for strains SMCC100, SMCC99 and SMCC90 respectively. A similar analysis of petite strains demonstrated that viability post fermentation was reduced in each instance to 94%, 93% and 92% live cells for strains SMCC100p, SMCC99p and SMCC90p respectively. This decrease was not surprising based on the data reported above, which indicated that petite cells were generally less resistant to stress. Consequently, as an

additional measure of quality, intracellular glycogen and trehalose were quantified. It was anticipated that this would perhaps indicate the reasons behind poor stress tolerance, but also indicate the capacity of petites to employ carbohydrate reserves. It can be seen from Figure 4.13 that in each instance there were greater quantities of trehalose before fermentation than post-fermentation. This data was a little confusing since it has previously been demonstrated that trehalose levels are higher at the end of fermentation than the start (Powell *et al*., 2000; Smart, 2013). However, this difference could be attributed to the fact that previous work was conducted with industrial samples, where yeast had been maintained for a period of time within the cone of the fermentation vessel. Consequently, the industrial samples would have been subjected to an extended period of stress associated with starvation and ethanol toxicity, whereas in this instance samples were removed immediately and analysed. Irrespective, it can also be seen that all of the petite strains showed lower levels of trehalose than their grande counterpart. Although this could indicate that these cells were less *stressed* it may also suggest that they were less efficient at producing trehalose and hence less *tolerant* to stress (Bandara *et al*., 2009; François and Parrou, 2001; Trevisol *et al*., 2011). The latter does partially support the observations above in terms of stress resistance, but it is difficult to be confident in this conclusion, especially since the SMCC90p strain contained a high proportion of trehalose pre-fermentation. It is suggested that a more detailed study in the future focusing on expression of TPS1 and TPS2, involved in the production of trehalose from glucose-6-phosphate

(Noubhani *et al*., 2009) would be useful. Indeed, there is some evidence to suggest that some mitochondrial respiratory mutants may be deficient in trehalose synthesis (Filipak *et al*., 1992).

Analysis of glycogen revealed a less consistent pattern of results. Of the grande strains, all except for SMCC100 showed lower levels of glycogen post fermentation, as would normally be expected. A similar trend was seen for the petite strains, except for SMCC99p, where there was no significant difference between pre- and post-fermentation. Interestingly, it has been reported that petite mutants very rapidly mobilize glycogen reserves when other sugars are no longer available Enjalbert *et al.* (2000). Consequently, the data obtained here could simply be an artefact of the stage of fermentation cells were isolated at. For example, petite populations which had attenuated slightly earlier may have been required to utilise glycogen reserves immediately, and hence would appear to have lower concentrations. This theory is partially supported by the fact the grande strains contained greater concentrations of glycogen than the petite variants in each instance; in the same study, Enjalbert *et al.* (2000) report that degradation of glycogen in grande cells is considerably more transient in nature.

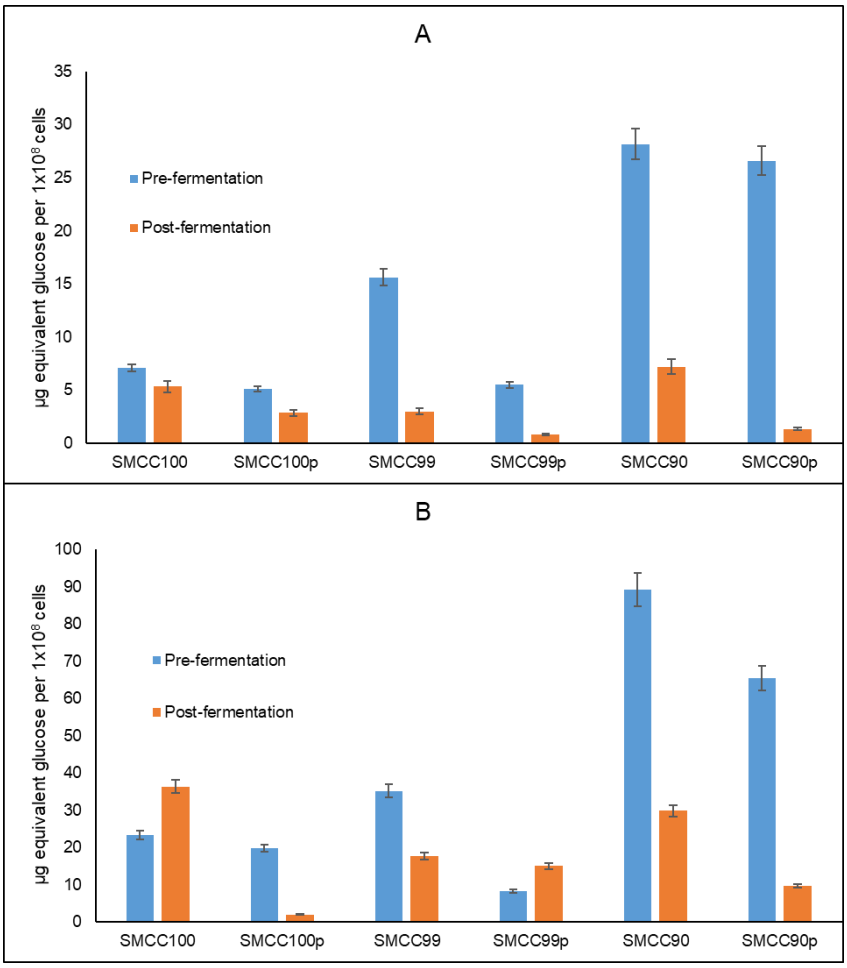


Figure 4.13 The impact of petite mutation on storage carbohydrate reserves. The lager yeast strains SMCC100, SMCC99 and SMCC90 were compared to their corresponding petite mutants, designated by the suffix ‘p’. Each sample was analysed for trehalose (A) and glycogen (B) both pre- and post-fermentation. Error bars indicate the standard deviation of the mean of triplicate samples.

4.2.7 Examination of volatile compounds and VDKs using GC analysis

Once fermentations were complete, final samples were collected and analysed for volatile compounds and vicinal diketones (VDKs) in order to profile beers produced using respiratory competent cells and respiratory deficient mutants. Specifically, acetaldehyde, ethyl acetate, isobutanol, 3-methyl 1-butanol and diacetyl were quantified from each sample were quantiied in beers produced using the strains SMCC100, SMCC99 and SMCC90 and their petite counterparts. These flavour compounds were selected since the other volatiles namely propanol, isoamyl acetate, ethyl hexanoate, and ethyl octanoate were below the detectable range of the GC.

It can be seen from Figure 4.14, that beer produced using SMCC100 and SMCC100p, yielded different quantities of volatile compounds. There was a higher incidence of acetaldehyde present in beer produced using the petite strain (8.3 mg/ L) compared to the grande (1 mg/ L) (P value < 0.05), this increased quantity of acetaldehyde may be due simply to incomplete fermentation. Alternatively, it may be due to ethanol oxidation by mitochondrial acetaldehyde dehydrogenase (Brányik *et al*., 2008; Datta *et al*., 2017), which may be required to generate carbon to be used in the glyoxylate system in order to produce functional metabolites (Wang *et al*., 1998). Despite this theory, it is worth noting that acetaldehyde was not detectable in any of the other samples analysed (Figures 4.15 and 4.16).

When examining the quantity of ethyl acetate produced by the yeast strains, it can be seen that the values were higher in all grande strains compared to the petite mutants (Figures 4.14 – 4.16). This may be due to the grande strains elevated growth characteristics, which require acetaldehyde to be diverted to acetyl-co-A, where it can react with ethanol to produce ethyl acetate (Nordström, 1963; Saerens *et al*., 2010). When examining higher alcohol production (isobutanol and 3-methyl-1-butanol), it can be seen that the petite strain SMCC100p produced higher concentrations of both compounds than the parent strain While this difference is highly significant (P value < 0.0005) (Figure 4.14). However, strains SMCC99p and SMCC90p only produced higher concentrations of iso-butanol; 3-methyl-1-butanol remained similar to beer produced by the grande strain in each case. Isobutanol biosynthesis occurs via the Ehrlich pathway and is linked in part to the mitochondria (Generoso *et al*., 2015; Pires and Brányik, 2015) (Section 1.10). A series of studies conducted by Brat *et al,* (2012; 2013) demonstrate that a fully cytosolic Ehrlich pathway can produce greater quantities of isobutanol, which may be a cause of the elevated concentrations observed.

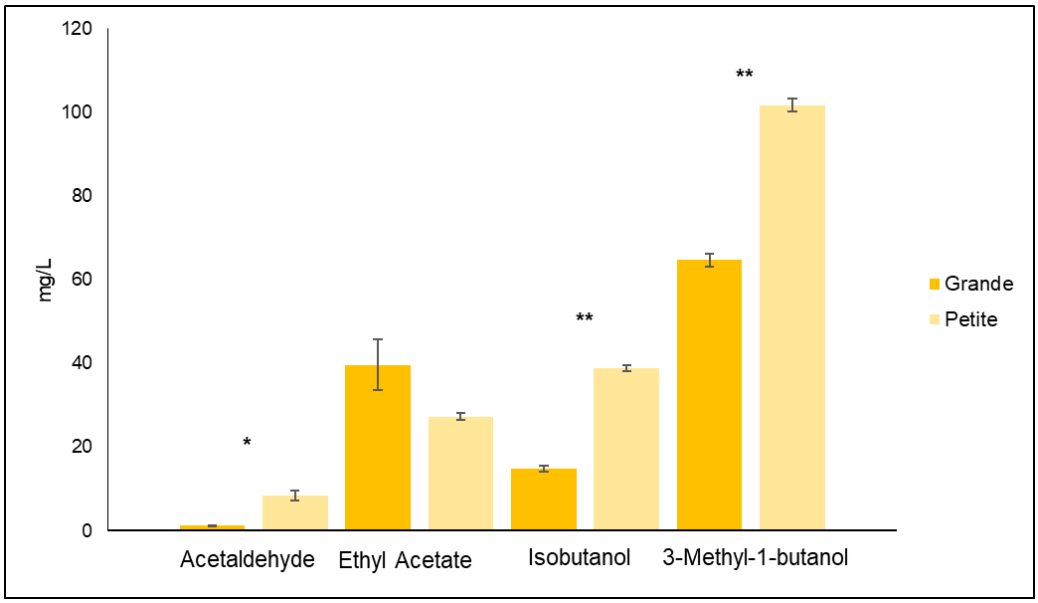


Figure 4.14 Volatile flavour compounds produced by *S. pastorianus* strain SMCC100 and the derived petite mutant SMCC100p. Flavour compounds were determined on completion of 100 mL fermentations conducted in 15°P wort. The degree of significance is indicated where * denotes P<0.005, and ** indicates P<0.0005. Error bars show the standard deviation of the mean of triplicate samples.

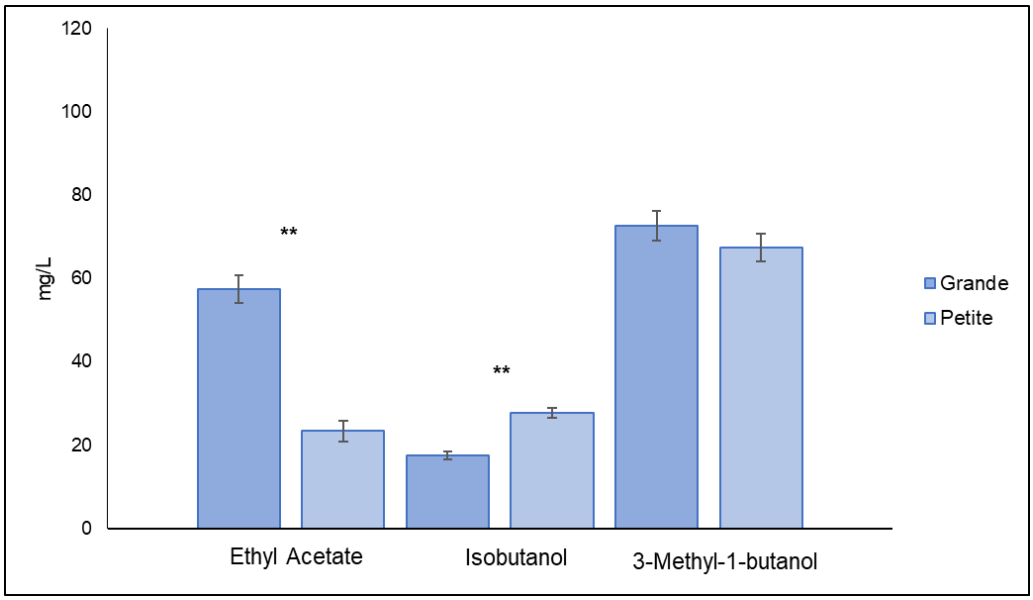


Figure 4.15 Volatile flavour compounds produced by *S. pastorianus* strain wild type (SMCC99) and petite mutant (SMCC99p) measured at the end of 100 mL fermentations conducted in 15°P wort. The degree of significance is indicated where ** indicates P<0.0005. Error bars show the standard deviation of the mean of triplicate samples. Note acetaldehyde is not shown here since concentrations were below detectable levels.

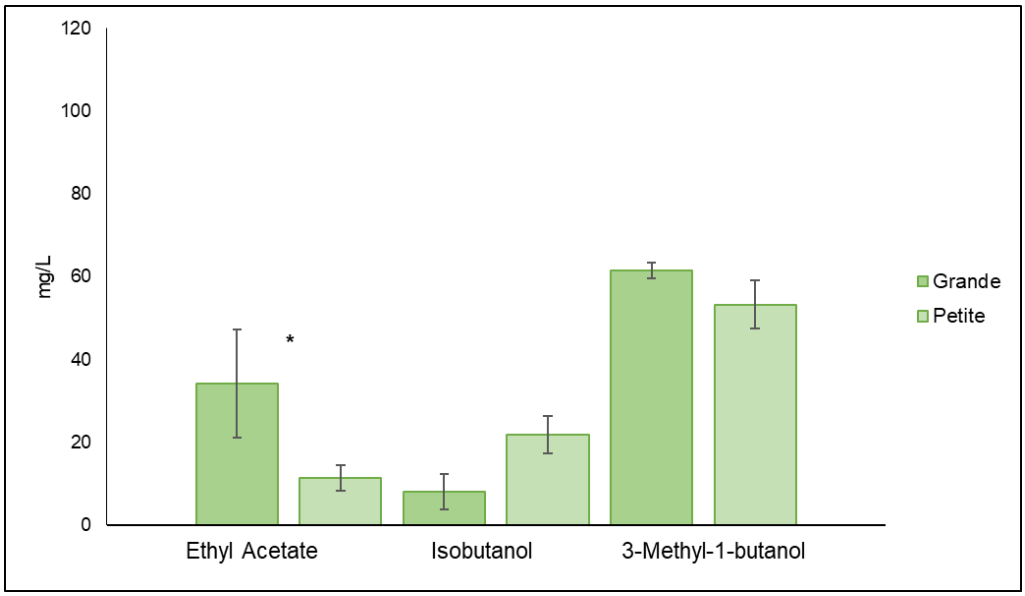


Figure 4.16 Volatile flavour compounds produced by *S. pastorianus* strain wild type (SMCC90) and petite mutant (SMCC90p) measured at the end of 100 mL fermentations conducted in 15°P wort. The degree is significance is indicated where * denotes P<0.005. Error bars showing standard deviation of the mean of triplicate samples. Note acetaldehyde is not shown here since concentrations were below detectable levels.

Following the assessment of volatile compounds the grandes and petites, beer was also examined to determine the impact of petite mutation on the production of VDKs, particularly focusing on diacetyl. Diacetyl is a particularly important compound, since its reduction at the end of fermentation is often considered to be a rate limiting step in the process (Section 1.10 and 1.11.1). It is evident from Figure 4.17 that the quantities of diacetyl produced by the respiratory deficient mutants are considerably higher than those of the grande strains. In addition, their concentrations are significantly above the detectable range of 0.1 - 0.15 mg/L (Pires *et*

al., 2015). Diacetyl concentrations were particularly elevated in beer produced using the strain SMCC100p which was observed to produce greater than 1.2 mg/L diacetyl, compared to the respiratory competent SMCC100 which produced just 0.03 mg/L. However, it is interesting to note again that not all petites were the same; even though each petite produced greater than the detectable threshold for diacetyl, SMCC99p and SMCC90p produced significantly less than SMCC100p. The method by which petites produce more diacetyl is well documented (Duggleby *et al*., 2000; Dasari and Kölling, 2011; Pires *et al*., 2014) but essentially this is because petite cells excrete alpha acetolactate, due to the inability to internalise acetohydroxy acid synthase inside the mitochondria (Pires *et al*., 2014) (Section 1.11.1)

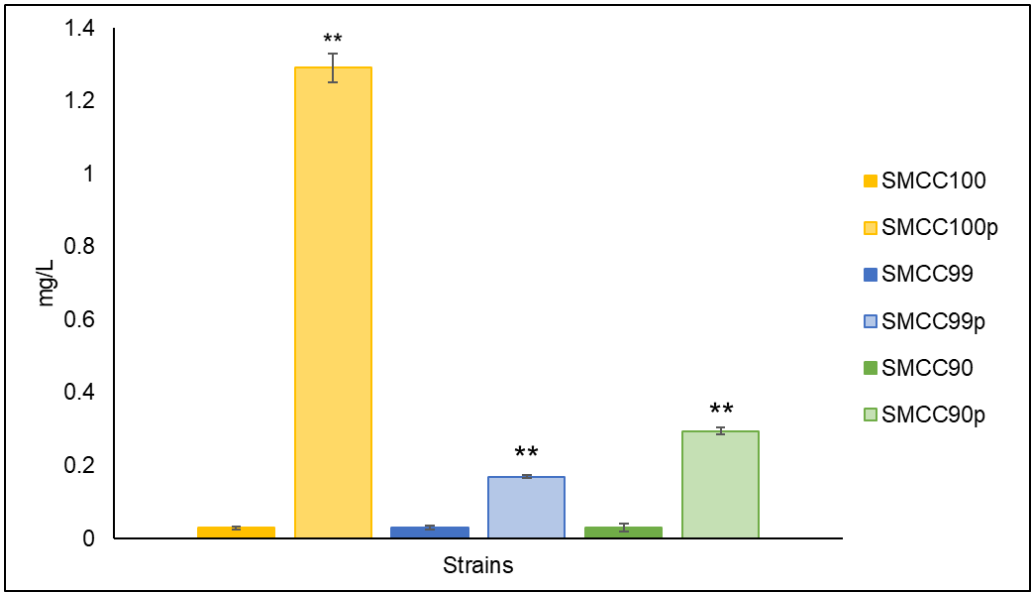


Figure 4.17 Quantity of diacetyl post fermentation produced by the lager yeast strains SMCC100, SMC99, SMCC90 compared to their petite mutants (SMCC100p, SMC99p, SMCC90p respectively). The degrees of significance are indicated where * denotes P<0.005, and ** indicates

P<0.0005. Error bars showing standard deviation of the mean of triplicate samples.

Based on the flavour analysis conducted, it can be seen that, aside from diacetyl, there were few trends observed that could be attributed to the petite mutation. Each petite produced its own specific profile that was occasionally, but not exclusively, similar to the parental strain. However, it is important to note that there are a huge number of elements that can impact on flavour development, not least yeast growth. Consequently, from the analysis here, it is difficult to accurately predict if certain biochemical pathways are preferentially utilised due to respiratory deficiency, irregular gene activity, or simply due to the fact that grande strains are able to grow quicker. Whatever the reason, it is important to appreciate that petite yeast cells produce different volatile profiles to their grande counterparts.

4.3 Conclusions

In this chapter, brewing lager yeast strains were examined for their propensity to develop the respiratory deficient phenotype. Initially, analysis of standard laboratory grown cells indicated that all strains naturally generated petites. However, by incorporating stress agents (ethidium bromide or ethanol) into the media, the rate of petite production was increased significantly. Analysis of each strain under these stress conditions revealed that strain SMCC90 was particularly resistant to petite mutation, while SMCC57 was highly susceptible.

Following the production of the petites, examination of their physiological characteristics was performed. Specifically, each strain was analysed for viability, response to ethanol stress (via growth curve analysis) and cellular membrane potential. Although viability was generally not impaired under standard conditions, petite strains were much more sensitive to concentrations of ethanol, showing much reduced growth rates and poorer viability overall. Cell membrane functionality analysis also indicated that there were significant differences between the grande and petite strains (with the exception of SMCC57 and SMCC57p). In each instance, petite cells showed membranes which were compromised, indicating that these cells may have issues with homeostasis and in regulating transport mechanisms (Vidgren et al., 2010). Indeed, it was also shown that petite cells fermented more slowly than grande strains, as expected. Furthermore, they were unable to quickly assimilate more complex sugars (maltose and maltotriose), while transport of simple sugars appeared to be disrupted.

Analysis of yeast quality post-fermentation revealed that viability had been lost to a greater extent in petite populations. This was partially rationalised by a comparative analysis of the storage carbohydrates glycogen and trehalose pre- and post-fermentation. It was revealed that petite strains were unable to accumulate trehalose or glycogen to the level seen in grande strains. This data was contradictory in nature since it would be expected that failure to accumulate one sugar might also lead to an increase in the other. However, this pattern of results was rationalised by the fact that petite mutants are known to rapidly utilise

glycogen reserves once assimilable glucose is gone from the growth media (Enjalbert *et al.,* 2000), and that trehalose synthesis may be deficient in petite cells (Filipak *et al*., 1992).

Finally, the impact of the petite mutation on volatile flavour production and diacetyl were also analysed. It was evident from the results that the petite yeast strains produced greater quantities of less desirable compounds such as diacetyl, as well as isoamyl alcohol and isobutanol. While the causes behind this are likely to be complex, this data does support previous reports of the nature of petite mutants, and lends further support to demonstrate the potentially negative impacts on fermentation consistency and final product quality.

CHAPTER 5 THE IMPACT OF FERMENTATION AND YEAST HANDLING ON MITOCHONDRIAL STRUCTURE AND CONTENT

5.1 Introduction

One compelling and broadly accepted theory on the origins of mitochondria is that they may be descendants of specialised bacterial cells that survived an endocytotic process, culminating in them being embedded into the cytoplasm of a host cell (Gray *et al*., 2001) (Section 1.7). A key factor in support of this theory is that mitochondria cannot be produced *de novo*, but instead require division of pre-existing mitochondria within the cell (Shiota *et al*., 2014). Although nuclear DNA replication can be linked to the cell cycle, production of new mitochondria also occurs independently, leading to multiple mitochondria per cell (Shaw and Nunnari, 2002). In addition, it is known that mtDNA copy number is not always related to the number of mitochondria, indicating that some mitochondria will have multiple copies of the mtDNA genome (Shadel, 1999). It is evident therefore that mitochondrial reproduction and genomics is a complex scenario, which is still poorly understood.

Further to the above, it is evident that there are a number of cytoplasmic and genetic factors which control the process of mitochondrial division (Section 1.8), hence mitochondria are not completely autonomous in this regard; factors which impact the yeast cell will also affect mitochondrial dynamics. One peculiarity of the mitochondria is their plasticity; they are able to rapidly undergo fission and fusion events in response to a host of environmental conditions (Youle and Bliek, 2012; Westermann, 2012; Bliek *et al*., 2013) (Section 1.6.). Some of these (for example, anaerobiosis) can also affect the conformation of the mitochondrial membrane both externally and internally (Visser *et al*., 1995; Rosenfeld

et al., 2004; Okamoto and Shaw, 2005); (Section 1.5). These membrane structures are important for functionality; it is known that mitochondrial membrane potential (MMP) plays a major role in mitochondrial homeostasis (O'Rourke *et al*., 2005). Indeed, the MMP has been used as a tool indicating both mitochondrial functionality and the overall health and condition of mitochondria (Zorova *et al.,* 2017). Previous analysis of the relationship between MMP and fermentation performance during sake production has indicated that ensuring MMP is required to produce specific aroma compounds, and that respiratory deficient yeast fail to produce these compounds (Oba *et al.,* 2014). However, no studies have previously been performed to analyse this in brewing yeast systems. Furthermore, although much is known about mitochondrial dynamics during growth and division, the majority of this work has been conducted in haploid strains under laboratory conditions. With regard to industrial yeast strains, reports have typically focused on the petite mutation and its impact on processes, but few studies have examined the physiology of mitochondria within actively fermenting cells. In this chapter, we examine mitochondrial development within brewing yeast cells, focusing on their morphology, quantity (biomass) and functionality. In order to achieve this, we applied a combination of fluorescent stains specific to mitochondria, mitochondria membrane structures, and mtDNA.

Experimental approach

In order to assess mitochondrial characteristics through propagation, fermentation and storage, a series of fluorescent stains were developed and used to evaluate cells via confocal microscopy, structured illumination microscopy (SIM) and flow cytometry. Specifically, stains were developed to analyse mitochondrial morphology, to quantify mitochondrial DNA and the 'biomass' of the mitochondrial membrane, and finally to assess mitochondrial membrane potential.

5.2 Results

5.2.1 Structural visualisation of mitochondria

Initially a number of fluorescent stains were tested in order to analyse mitochondria in brewing yeast populations. MtDNA was analysed using the dyes fluorescent stain Syto18, which actively binds to mtDNA (Ernst and Downs, 2018). The fluorescent stains Rhodamine b hexyl ester and MitoTracker Green were used as a means of visualising and quantifying membrane structures. In order to investigate yeast mitochondrial membrane potential (MMP), the dyes $DiOC_2(3)$ and Rhodamine123 were employed. Both $DiOC_2(3)$ and Rhodamine123 are lipophilic cationic compounds which can cross the membrane barrier and accumulate inversely to the proportion of MMP, i.e. the more negative the membrane potential the higher the amount of fluorescence that is emitted (Perry *et al.,* 2011).

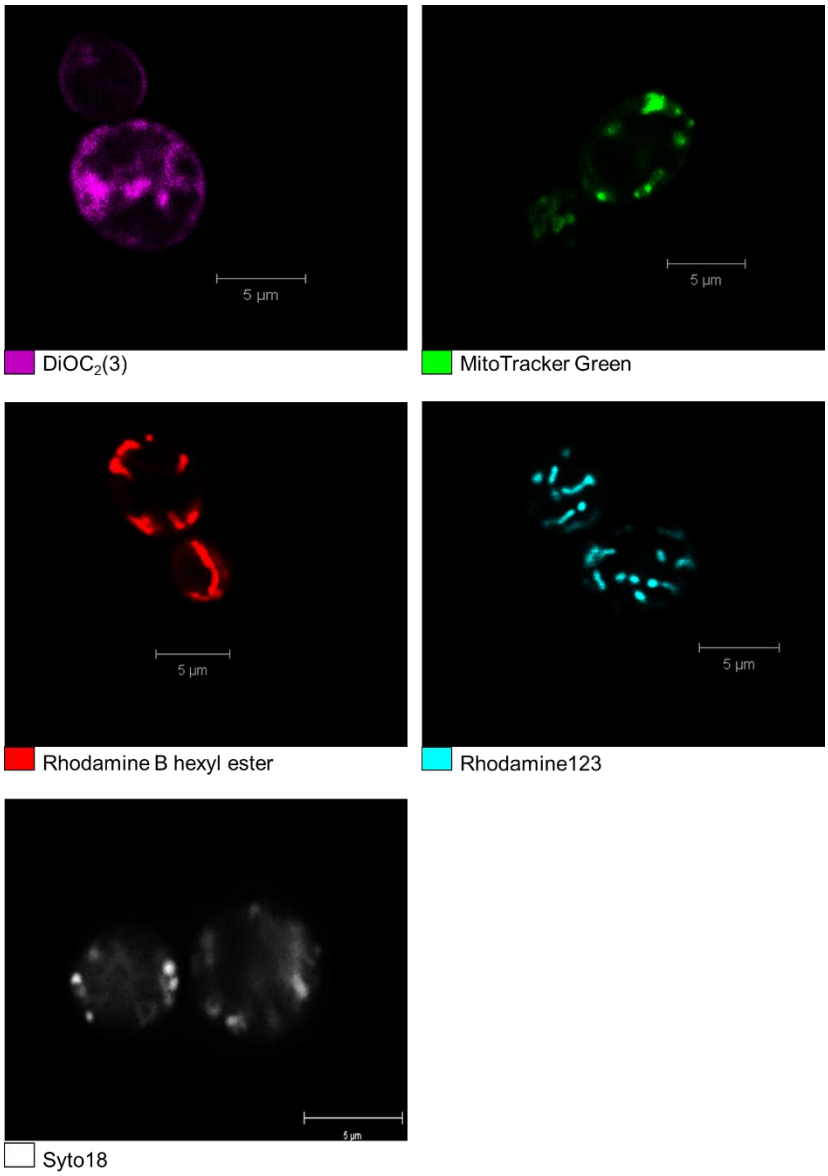


Figure 5.1 Determination of stain efficacy in the lager yeast strain SMCC100. Cells were grown under standard laboratory conditions in YPD over 24 hours prior to staining.

From Figure 5.1, it can be seen that some dyes were more effective at staining mitochondria than others. Syto18 was able to detect mtDNA to good effect. However, although Rhodamine123 can be seen to be effective for visualising mitochondrial membrane structures, $DiOC_2(3)$ yielded some non-specific staining of the cell wall, apparent in the mother

and daughter cell. Both MitoTracker green and Rhodamine B hexyl ester allowed visualisation of mitochondria which could be used to determine MMP. However, the emission spectra of MitoTracker was similar to that of Syto18, used to stain mtDNA. Due to this, MitoTracker was excluded since it's use would have precluded duel staining. A similar decision was made for Rhodamine123 and hence the dyes selected for use were: Syto18 for mtDNA, Rhodamine B hexy ester for cell membrane analysis and Rhodamine 123 for determination of MMP. These dyes provided specific staining as shown in Figure 5.1 and could be visualised clearly.

5.2.2 Mitochondrial morphology in brewing yeast

It has previously been established that mitochondria are dynamic organelles undergoing fusion and fission events depending on the cell environment, growth conditions and cell cycle phase (Sesaki *et al*., 1999; Nunnari *et al*., 1997; Shaw *et al*., 2002; Osman *et al*., 2015) (Section 1.5). However, much of this work has been conducted in laboratory strains and little is known about the confirmation of mitochondria in polyploid/aneuploid industrial brewing yeast strains. Furthermore, analysis of mitochondrial structure and changes over time in Rho^- petite cells have not been reported. In order to investigate this, variations in the mitochondrial topologies of ale and lager brewing yeast cells (grande and petite) were examined.

Analysis of mitochondrial morphology in all grande strains grown in YPD for 24 hours indicated that the preferred mitochondrial topology at this point was tubular / networked, evidently caused by fusion events. An

example of this morphology can be seen in Figure 5.2. This result was deemed to be positive since the form has been linked with functioning mitochondria and healthy mtDNA (Shepard and Yaffe, 1999; Shaw and Nunnari, 2002; Aung-Htut et al., 2013). Further analysis was carried out to compare mitochondrial morphology between grande and petite cells. From the images displayed in Figure 5.2 (SMCC100 grande) and Figure 5.3 (SMCC100 petite), it can be seen that the petite morphology was considerably different. Although the grande morphology retained its fused state, the petite strain appeared more globular / aggregated, with some smaller fragments dispersed throughout the cell. Although the mitochondrial membrane analysis showed differences in dispersal through the cell, analysis of mtDNA indicated that the nucleoids visualised did not appear to be different in terms of quantity or shape. However, it should be noted that the images represent a single plane of view, and the subject of mtDNA content in grande and petties is investigated in more detail in Section 5.2.3

Although it is known that mitochondria in tubular form are more likely to be healthy, understanding the rationale for adopting a different confirmation in petites is unknown. However, analysis by Okamoto *et al*. (2016) revealed similar aggregated morphologies to those reported here. These authors suggested that the clustered mitochondrial morphology may occur as a result of mtDNA repair systems, which require mitochondrial fusion (Contamine and Picard, 2000; Hori *et al.,* 2001; Shaw and Nunnari, 2002).

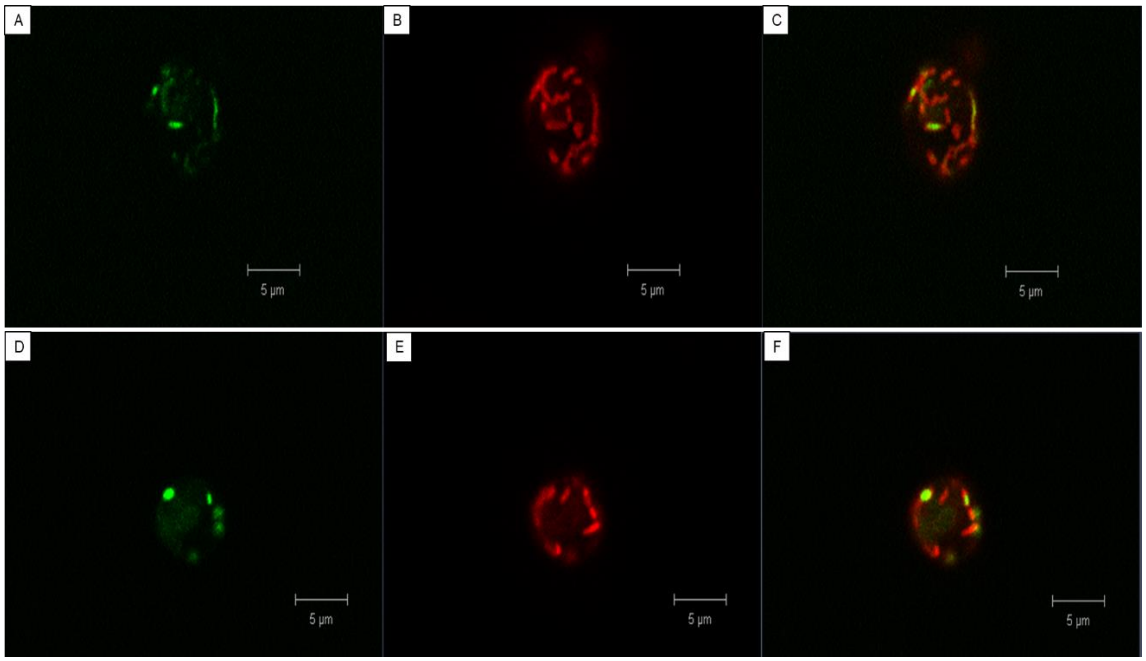


Figure 5.2 Analysis of mtDNA (green), and mitochondrial membrane (red) by staining with Syto18 and rhodamine b hexyl ester respectively. SMCC100 yeast cells were grown under standard laboratory conditions in YPD over 24 hours before staining. A: Green channel; B: Red channel; C: merged overlay; D: Green channel; E: Red channel; F: merged overlay.

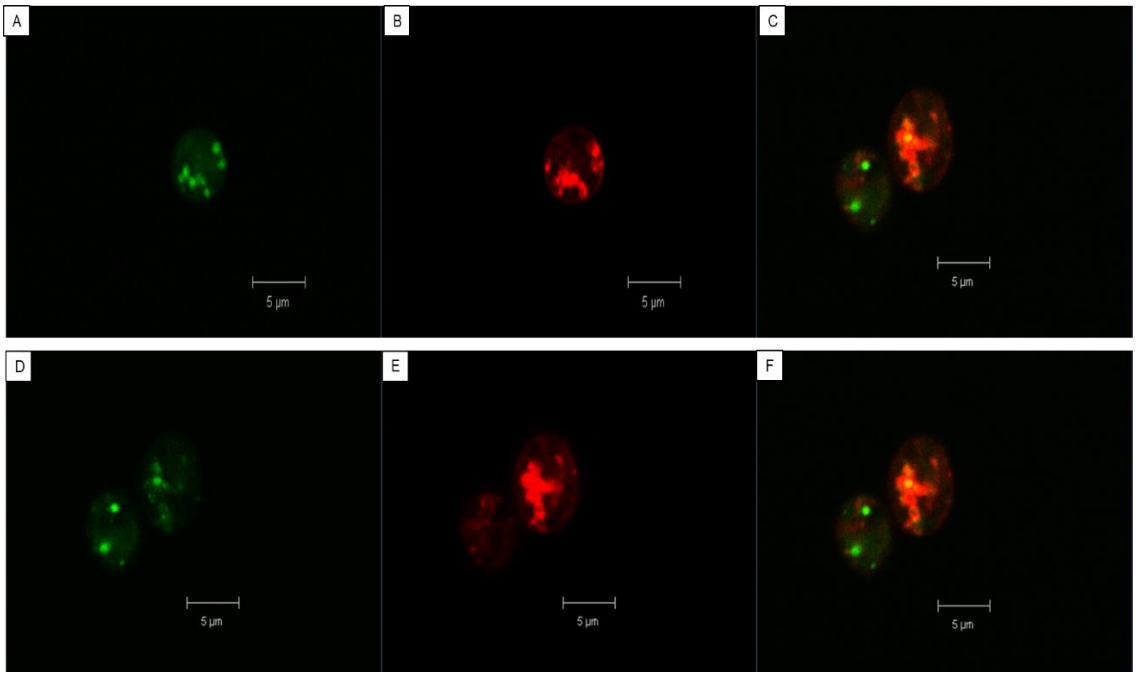


Figure 5.3 Analysis of mtDNA (green), and mitochondrial membrane (red) by staining with Syto18 and rhodamine b hexyl ester respectively. SMCC100p cells were grown under standard laboratory conditions in YPD over 24 hours before staining. A: Green channel; B: Red channel; C: merged overlay; D: Green channel; E: Red channel; F: merged overlay.

5.2.3 Mitochondrial content and mitochondrial membrane potential

Based on the mitochondrial structure data obtained above, other indicators of mitochondrial health were investigated, specifically mitochondrial content (mass), mtDNA content and mitochondrial membrane potential. In order to achieve this flow cytometry was employed in conjunction with the stains optimised previously (Section 5.2.1.) to quantitatively analyse these aspects of the *S. pastorianus* lager yeast strains SMCC100, SMCC99, SMCC90 and SMCC57 and their respective petite mutants isolated previously (Section 4.2.1.1). These

strains were chosen by virtue of the fact that they revealed the greatest similarity in mtDNA profiles (Section 3.2.4.1), yet the greatest diversity in petite production rate (Section 4.2.1.1), raising questions regarding the underpinning causes of respiratory deficiency.

All strains were analysed for mitochondrial membrane mass and mtDNA content after 24 hours growth in 15 °P wort. Each population was analysed using flow cytometry and median values were calculated, in order to exclude potential outliers influencing the overall data. In Figure 5.6, the mitochondrial mass of each strain and its petite counterpart can be seen. The median fluorescent values obtained indicated that there were no clear statistical differences between the grande strains and their petite counterparts, with the exception of SMCC90 and SMCC90p respectively. This result was surprising, given that MMP is known to be an indication of healthy mitochondria and that mtDNA damage can influence mitochondrial inheritance (Westermann, 2014). Furthermore, the reason why SMCC90 was the only strain to show a difference between the petite and grande is also unclear. However, it should be noted that this petite mutant exhibited mtDNA profiles which were extremely degraded (Chapter 4), hence it is possible that this mtDNA was damaged to a greater extent than the other cells examined. This may have led to SMMCC90p being less capable of producing or maintaining mitochondria, due to extensive damage or loss of mtDNA *(*Berger and Yaffe*,* 2000; Parone *et al*., 2008; Westermann, 2014).

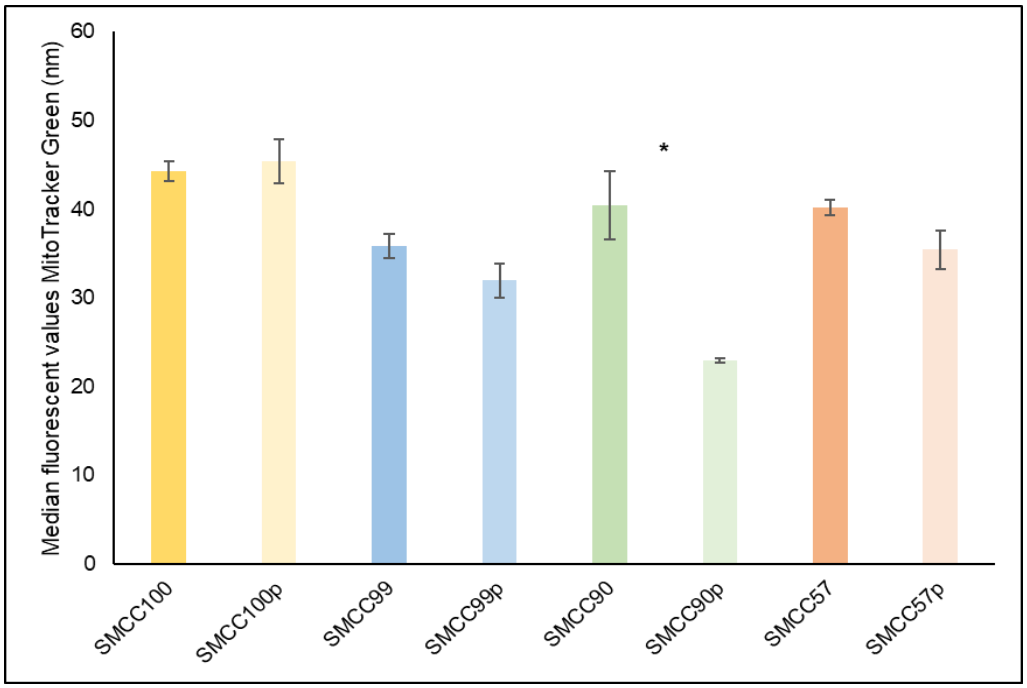


Figure 5.6 Analysis of mitochondrial membrane content using MitoTracker green. Data indicates the median fluorescent values produced by grandes (strain number only) and petite cells (designated by the suffix, p). * indicates a P value of < 0.0131. Error bars showing standard deviation of the mean of triplicate samples.

To test whether mtDNA loss had impacted mitochondrial mass, the mtDNA content of strains SMCC100, SMCC99, SMCC90 and their petite counterparts were analysed. From Figure 5.7 it can be seen that there were significant differences between mtDNA content in grande and petite strains. In each instance the fluorescence produced by grande strains in response to Syto18 staining was higher than in the petites (P value <0.0001). Furthermore, although each parental strain showed a similar median fluorescent intensity, MtDNA content in the petite strains was variable. Strain SMCC100p contained the least amount of mtDNA of the three petites examined, while SMCC99p and SMCC90p were more

similar. Although this contradicts the hypothesis above, with regard to the impact of mtDNA on MMP, it should be noted that the current analysis only determines total mtDNA content and provides no insight into the integrity of the DNA itself. However, using this method it is clear to see that all petite strains suffered a loss of mtDNA overall. It is suggested that the respiratory deficient yeasts may have smaller amounts of mtDNA due simply to the physiochemical instability of the ρ^- genome (Morita *et al.,* 1975; Jones and Fangman 1992; Zhang *et al.,* 2014). Furthermore, it is known that in healthy grande cells, multiple copies of mtDNA can be found in each mitochondria (Solieri, 2010). Since the analysis of membrane mass revealed little difference between grande and petite cells, it is likely that the copy number of mtDNA per mitochondria (and hence per cell) was reduced. This may also explain the globular / aggregated appearance in the images shown previously (Figure 5.3), since it is possible that mitochondrial fission events were no longer able to take place; a phenomenon that has been shown to result in loss of mtDNA (Parone *et al*., 2008). Irrespective, a loss of mtDNA has been linked with a reduction in overall functionality of mitochondria, as would be expected.

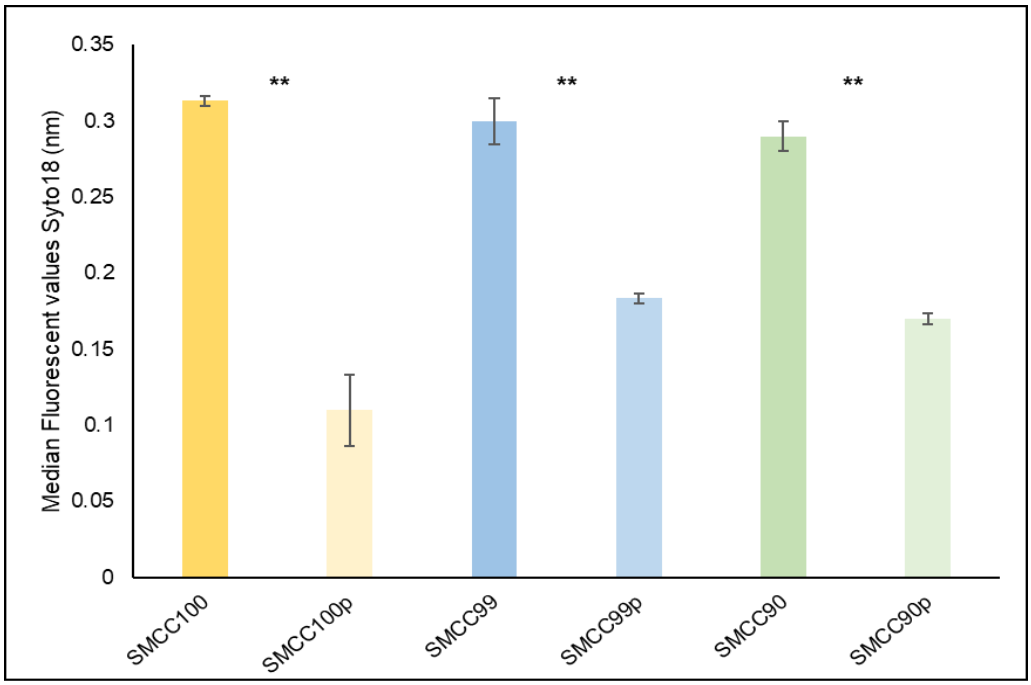


Figure 5.7 Analysis of mtDNA content using Syto18. Data indicates the median fluorescent values produced by grandes (strain number only) and petite cells (designated by the suffix, p). ** indicates a P values of < 0.0001. Error bars showing standard deviation of the mean of triplicate samples.

Interestingly, it has also been suggested that mtDNA loss results in reduced mitochondrial membrane potential (Ehlers et al., 1999; Santos et al., 2003; Hebert et al., 2010). This occurs once key gene sequences (ATP8, 6 and 9) encoding for mtDNA proton ATPase's (which maintain membrane potential) become affected (Ehlers et al., 1999; Santos *et al.,* 2003; Reyes *et al.,* 2016). This is important since although the primary role of MMP is for aerobic respiration, the membrane must be maintained even under hypoxic conditions, since it is vital for mitochondrial transmission (Geissler *et al.*, 2000; Vevea *et al*., 2014; Zorova *et al*., 2018). In order to investigate the relationship between MMP and mtDNA

content in yeast cells, the fluorescent stain Rhodamine 123 was employed.

The data shown in Figure 5.8 indicates that there were differences between all of the parental grande strains. Strain SMCC57 exhibited the highest value (50 nm), with SMCC100 the lowest (25 nm) and SMCC99 lying in-between (40 nm). This was perhaps surprising given that grande strains were similar in other aspects of mitochondrial morphology. However, it is interesting to note that MMP was variable between petites. Indeed, the petite strain SMC99p showed a greater MMP than the grande strain SMCC100. However, all of the petite strains showed reduced MMP compared to their respective parental strain (Figure 5.8). This supports the previous results where the amount of mtDNA was lower in the petite cells, indicating a relationship between DNA damage and MMP. However, it is interesting to note that strain SMCC57p displayed the greatest reduction in MMP of all the strains (Figure 5.8), despite mitochondrial mass being similar to the original grande strain (Figure 5.6), this indicates that membrane mass alone does not correlate to efficiency or functionality. In fact, variations in mtDNA content are known to have a greater influence over MMP of the yeast cell, due in part to the action of proton ATPase's, as described above. However, it is likely that there are a number of other functional parameters that are also impacted by MMP. For example it has been shown that a reduced MMP can trigger a retrograde response pathway which induces a broad array of nuclear target genes including those involved in autophagy and homeostasis (Woo and Poyton, 2009; Miceli *et al*., 2012). While it is unknown to what

extent this occurs in petite cells, it does suggest that general repair mechanisms may be activated, perhaps offering an explanation as to how petites persist through successive fermentations, despite exhibiting reduced viability and vitality (Chapter 4).

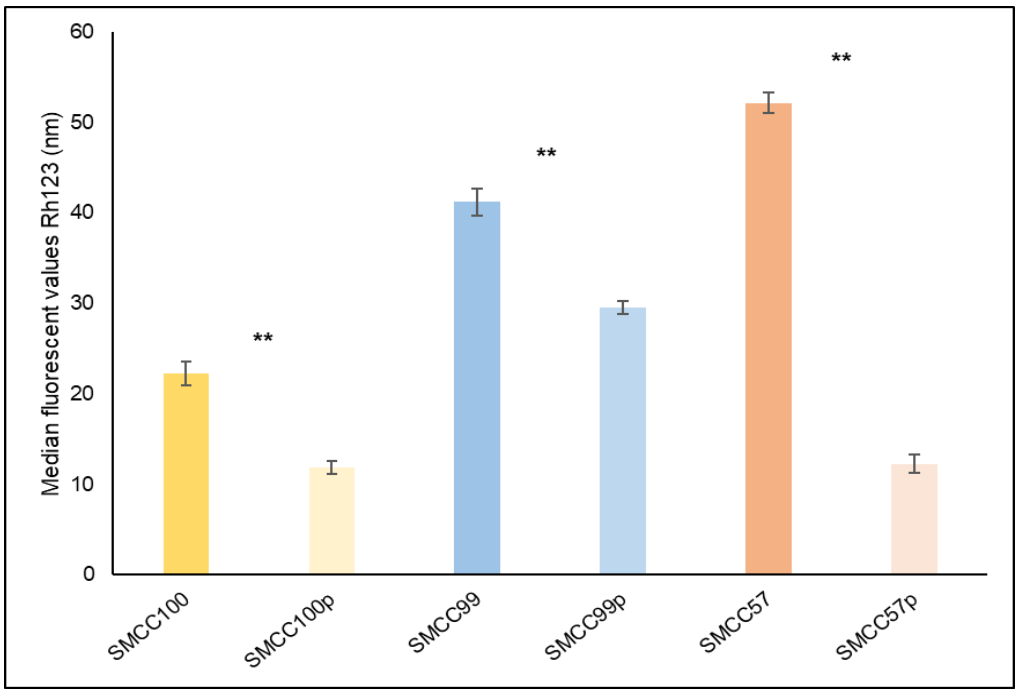


Figure 5.8 Analysis of MMP using Rhodamine 123. Data indicates the median fluorescent values produced by grandes (strain number only) and petite cells (designated by the suffix, p). ** indicates a P values of < 0.0001. Error bars showing standard deviation of the mean of triplicate samples.

5.2.4 Impact of ethanol stress on mitochondrial content and functionality

In Chapter 4 it was established that strains are more likely to produce petites under conditions of stress, and especially in response to ethanol. However, the previous study did not investigate potential causes of the petite mutation and it is unknown whether they arise due to mtDNA

damage, inefficient functionality, or failure to inherit mtDNA correctly. In order to determine the short-term effect of ethanol stress on mitochondrial morphology, the two yeast strains producing the most and least number of petites (SMCC57 and SMCC100 respectively) were evaluated. Strains were grown in 15 °P wort over 24 hours in the presence of ethanol at concentrations of 0 (control), 5 and 10%. Each population was then analysed for mitochondria mass, MMP and mtDNA content as described in Sections 2.73 and 5.23 respectively.

From Figure 5.9 it can be seen that there is a decrease in mitochondrial content as the concentration of ethanol increases. Strain SMCC57 showed an initial value of 28 nm however when exposed to 5% ethanol this dropped to about 14 nm, and strain SMCC100 a started at the highest value with no ethanol present at 37 nm then dropping to 24 nm and 18 nm in 5 % and 10 % respectively. This trend continued when grown in 10% ethanol, it can be seen that there was a further 41% loss in mitochondria for SMCC57 and 19% for SMCC100. This reduction may be due to mitochondrial fragmentation induced by the increased ethanol concentrations (Kitagaki *et al*., 2007; Bonet-Ponce *et al*., 2015) and may provide a reason as to why the mitochondria present within SMCC100 are more capable of resisting damage caused by ethanol than SMCC57 (Aguilera and Benítez, 1985; Bandara *et al*., 2009).

In addition to examining the mitochondrial content, the membrane potential was also determined. Initial values for SMCC100 and SMCC57 were statistically different, supporting the data presented above (Figure

5.8). However, in this instance it can be seen that the difference between strains was reduced as the ethanol concentration increased. This is supported by reports from the literature which suggest that mitochondria in yeast and mammalian cells become hyperpolarised when exposed to increased concentration of ethanol (Mashimo and Ohno, 2006; Bonet-Ponce et al., 2015). Consequently, it is possible that as mitochondrial content decreases the remaining mitochondria had to increase membrane potential in order to compensate for the loss of biomass.

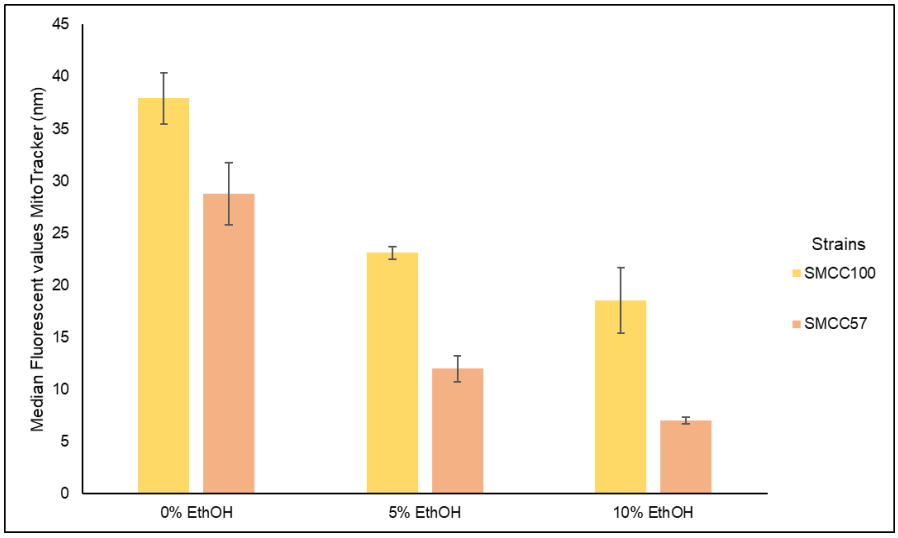


Figure 5.9 Relationship between ethanol stress and mitochondrial content in lager strains using Mitotracker green. Mitochondrial content in SMCC100 and SMCC57 when exposed to ethanol stress 5% and 10% respectively. Error bars showing standard deviation of the mean of triplicate samples.

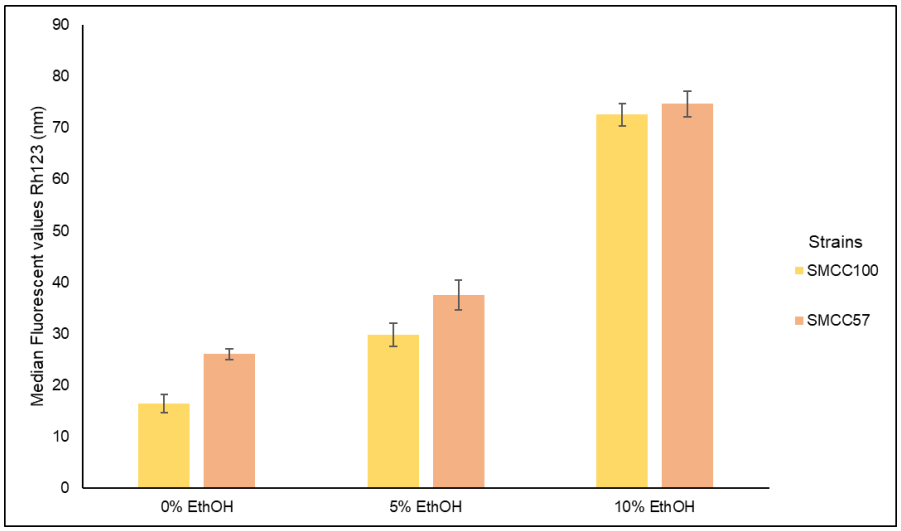


Figure 5.10 Impact of ethanol on mitochondrial membrane potential using Rhodamine 123. Mitochondrial membrane potential in SMCC100 (grande) and SMCC57 (grande) when exposed to ethanol stress 5% and 10% respectively. Error bars showing standard deviation of the mean of triplicate samples.

5.2.5 Mitochondrial content during yeast handling

In Chapter 4 it was established that strains are more likely to produce petites under conditions of stress, and especially in response to ethanol. However, the previous study did not investigate how the petite mutation arose; whether it was due to mtDNA damage, impaired membrane functionality over time, or the failure to inherit mtDNA correctly. Furthermore, the impact of fermentation on these parameters is currently unknown. Consequently, it was anticipated that an investigation into the relationship between fermentation progression and mitochondrial dynamics might assist in understanding the role of mitochondria under anaerobic conditions, as well as the propensity to form petites.

Having established methodology to assess aspects of mitochondrial physiology, strain SMCC100 was sampled over the course of a replica yeast handling process deigned within the laboratory. Specifically, samples were taken during yeast propagation (Section 2.4.1), fermentation (Section 2.4.6) and from storage. During propagation, cells were continuously oxygenated at 15 °C for 24h. Fermentations were conducted in 15 °P wort in closed stirred 2L vessels, maintained at 15 °C until attenuation had been achieved, while cropped yeast was stored statically at 5°C for a maximum of 82h. Samples were obtained throughout each stage and assessed for mitochondrial biomass and mtDNA content using rhodamine B hexyl ester and Syto18 respectively (Section 2.7.3).

It can be seen from Figure 5.11 that the quantity (biomass) of mitochondria present over the entire process of yeast handling does not remain constant. There appear to be key points where the mitochondrial content is higher, and points where mitochondrial mass remains low. This information is not surprising, since mitochondria are dynamic organelles and fluctuate in morphology as the environment requires (Shaw and Nunnari, 2002; Mitra and Lippincott-Schwartz, 2010). The environment in which brewing yeast cells find themselves regularly changes, partly due to the physical process of being moved from one stage of the yeast handling process to the next, but also due to events within each phase. For example, fermentation in particular is characterised by major environmental challenges which change over the duration (Section 1.3.1). Minor fluctuations in conditions may occur during storage at large

scale, for example the development of hot spots within large vessels, but these can be controlled at small scale. Irrespective, it can be seen that the amount of mitochondrial mass tended to increase during propagation. It is suggested that this may be an artefact of the respiro-fermentative nature of brewing yeast. It is possible that under highly oxygenated conditions yeast mitochondrial activity may be promoted since there is a strong requirement to produce amino acids and proteins required for structural and enzymatic purposes, as well as lipids for biosynthesis of cell membranes. Many of these, particularly the early stages of lipid synthesis, require access to the truncated TCA cycle and hence mitochondrial activity (O'Connor-Cox *et al*., 1995). Alternatively, due to the amount of budding occurring mitochondrial generation may be instigated by the nuDNA, promoting generation of mitochondrial biomass.

Once yeast was transferred from propagation to fermentation, a sudden decrease in mitochondrial mass was noticed. The reason for this is unknown, however it is likely that this is due to the condition of the yeast cells immediately prior to inoculation and on introduction to the wort. It is known that once yeast are placed into a new medium, cells undergo a transitional period of stabilisation during which they accumulate to their new environment. This forms the basic principles of the lag phase in yeast (Boulton and Quain, 2001). However, it is also known that yeast at the start of fermentation show synchronicity, and that most cells (>99%) are in G0 of the cell cycle (Miller et al., 2013). It is possible that this shift in environment, coupled to cell cycle arrest may have resulted in an inhibition of mitochondrial replication.

Finally, analysis of membrane biomass during fermentation indicated that the quantity of mitochondria per cell increased after approximately 12h into fermentation until a peak was reached at 26-30h. Initially this result was very surprising; given that mitochondrial bio-energetic function is not strictly required during fermentation under anaerobic conditions, it was presumed that biomass may decrease over time. This theory would also have partially supported the development of petite mutants since defective 'promitochondria' are known to develop under extended periods of anaerobiosis (Visser et al., 1994). However, the exact opposite occurred and a subsequent analysis of mtDNA content during fermentation indicated that mtDNA content followed the same trend as mitochondrial content; an increase was observed between the time points 24 hours and 28 hours (Figure 5.12). While the reasons behind these trends have still to be fully elucidated, it is possible that mitochondria are required in fermentation for the same reasons that they were required during propagation; to support cell division initially, but thereafter perhaps with a homeostatic function for structural repair and maintenance. Irrespective, the fact that mitochondrial mass and mtDNA content increase during fermentation suggests that mitochondria are essential for ensuring the success of a fermentation, even though their 'traditional' function of OXPHOS is restricted.

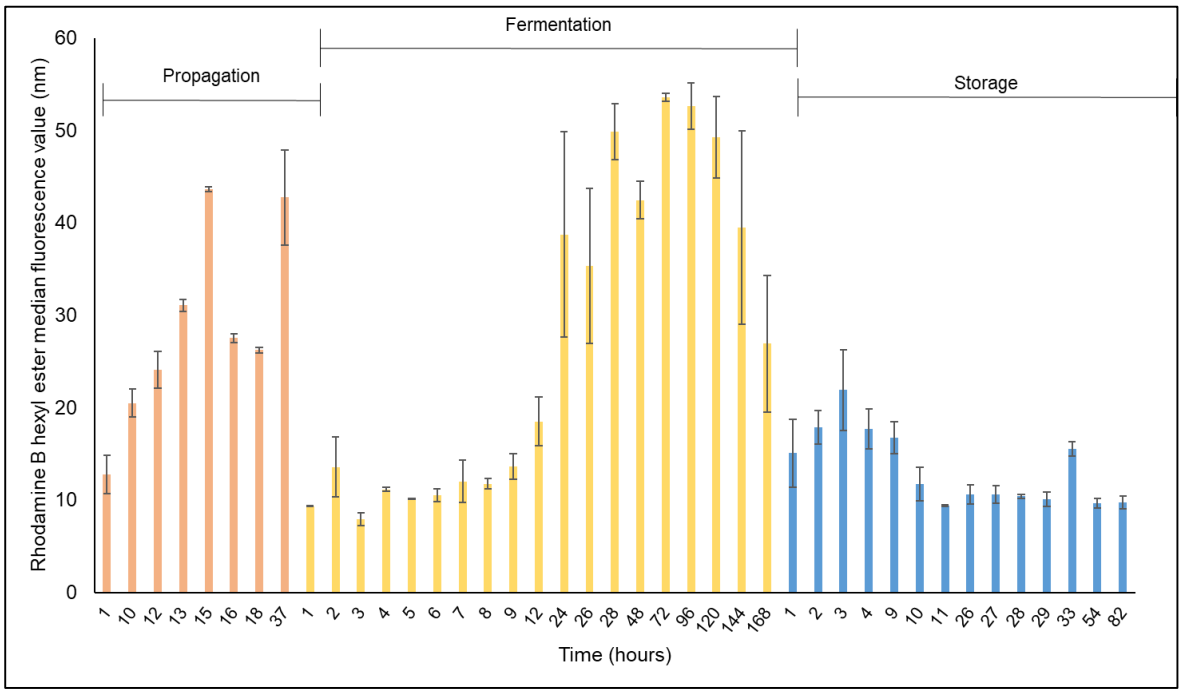


Figure 5.11 Mitochondrial content (membrane mass) of lager yeast strain SMCC100 during yeast handling. Median fluorescent values of Rhodamine B hexyl ester. Error bars showing standard deviation of the mean of triplicate samples. Note that the X- axis shows a non-linear timeline and Y- axis the fluorescent values from 20,000 cells per timepoint.

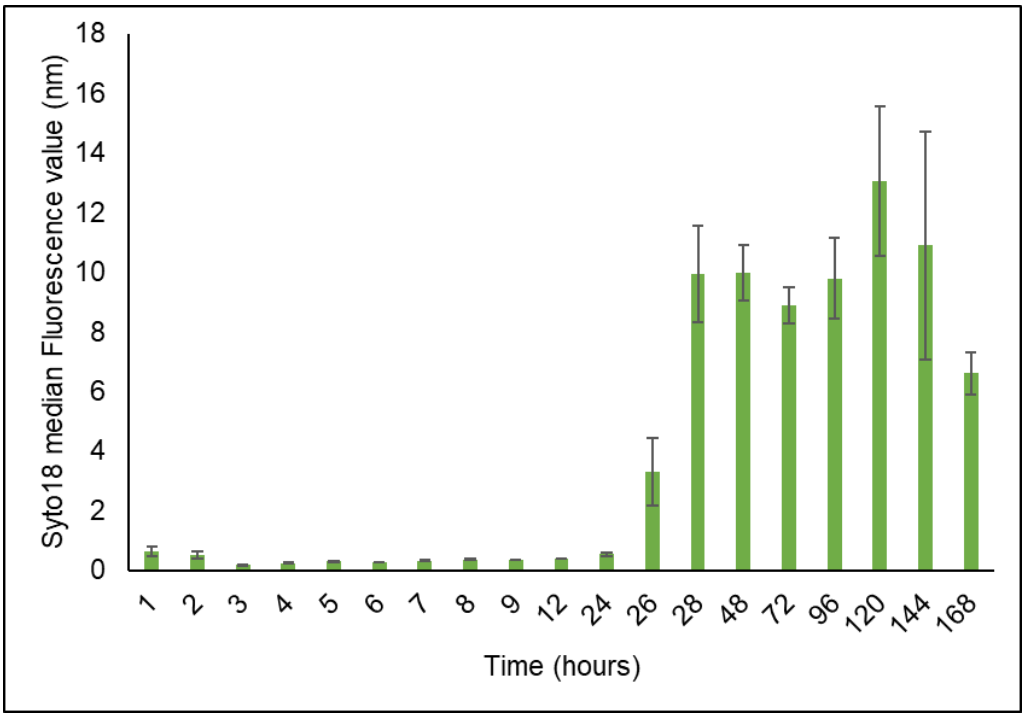


Figure 5.12 Examination of mtDNA content of lager yeast strain SMCC100 throughout yeast fermentation. Median fluorescent values of Syto18. Error bars showing standard deviation of the mean of triplicate samples. Note that the X- axis shows a non-linear timeline and Y- axis the fluorescent values from 20,000 cells per timepoint.

To support the previous data, mitochondrial morphology during fermentation was analysed using fluorescent microscopy. Samples were taken at 12h and 72h since these reflect the points where mitochondrial dynamics appeared to be the most different. It can be seen from Figure 5.13 that mitochondrial morphology varied considerably these two time points. Yeast samples taken from 13h of fermentation showed cells that contained dispersed small individual mitochondria, with little to no interconnectivity (Figure 5.13A), while samples taken after 72h revealed fused mitochondria that were interlinked and predominately located closer to the cell wall (Figure 5.13 B). It is possible that this close

proximity to the cell wall may be a result of the mitochondria donating proteins to the cell wall (O'Connor-Cox *et al*., 1995). Alternatively, it is possible that the locations of the mitochondria within the yeast cells are simply a result of intracellular CO_2 bubbles relocating / pushing the mitochondria within the cell cytoplasm towards the cell wall (Swart *et al* 2013). Either way, these images confirm an increase in mitochondrial mass between the two time points, reaffirming the previous results from Figures 5.10 and 5.11. As mentioned above, the precise reasons for this increase in mitochondrial mass is not yet known, however it seems evident that the mitochondria play a far greater role in fermentation than originally predicted, either due to its functional role in metabolic biosynthetic pathways, due to its role in nuDNA crosstalk, or perhaps due to function related to the yeast stress response. Each of these theories offers an avenue for future work.

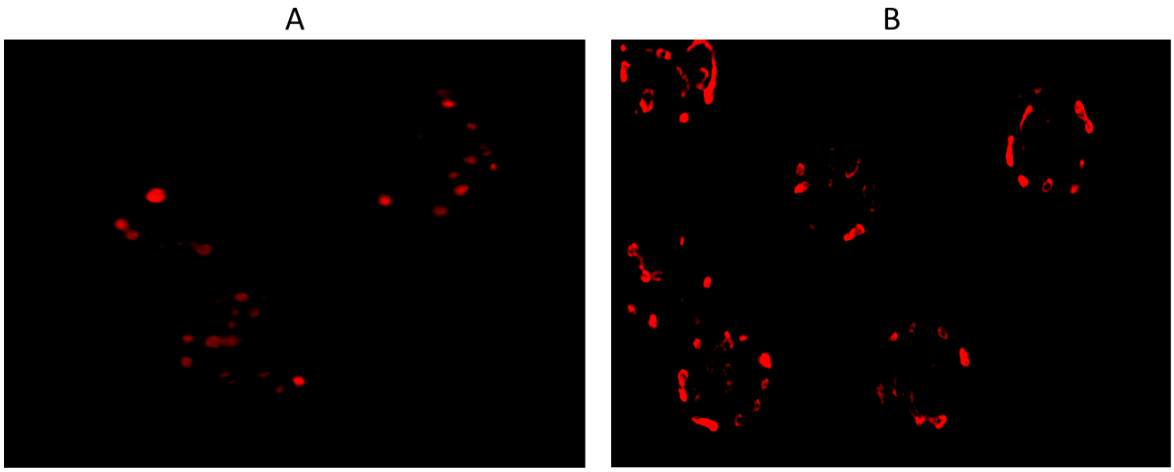


Figure 5.13 Analysis of mitochondrial membrane content during fermentation using rhodamine b hexyl ester staining in conjunction with SIM. Cells were isolated from fermentations at timepoints (A): 12 hours and (B): 72 hours

5.4 Conclusions

Within this chapter, we established methodology required to accurately visualise mitochondria and mtDNA nucleoids within living yeast cells. Once the methodology had been established, we examined differences in mitochondrial morphology between a series of grande and petite brewing strains. Initial analysis of the grande strains did not reveal any clear morphological differences, although it should be recognised that it is possible that different ratios of tubular to spherical conformations may exist within the yeast cells, which would have remained undetected. However, when comparing grande and petite cells, it was evident that petite cells consistently showed less tubular, fused mitochondria, and more globular condensed structures. When examining the quantity of mtDNA per cell, it was found that petite individuals had a much lower complement of DNA than grande cells, a phenomenon that was mirrored by the MMP of petite cells; respiratory deficient cells displayed a far lower MMP when compared to competent yeast strains.

An interesting observation was that there was considerable variation in MMP between the grande strains, something that was not expected. However, it is notable that the MMP of each strain roughly matched its propensity to form petites; strains which yielded higher numbers of petites exhibited lower MMP. This was investigated further by analysing strains most and least likely to form petites (SMCC57 and SMC100 respectively). The results showed that under increasing ethanol stress the MMP of the yeast cells increased, while the quantity of mitochondria decreased. It is possible that a combination of mitochondrial loss and increasing MMP

results in the increased occurrence of petites, especially since strain SMCC57, which naturally yielded a greater number of respiratory deficient cells, showed a more severe loss of mitochondrial mass. While these results do not provide an outright link between MMP and petite production, it is possible that strains with a higher MMP may yield more petites. One reason for this may be that an increased MMP has been shown to result in increased ROS production, linked to mtDNA damage (Suski *et al*., 2012). Although it should be noted that this study was performed in shake flasks over 24 hours in YPD, it has also been shown that mitochondria continue to generate free radicals anaerobically (Liochev, 2014)

Subsequently, changes occurring to mitochondria during yeast handling practices were investigated. It was established that mitochondrial dynamics change over the course of yeast handling, and particularly during propagation and, surprisingly, fermentation. Yeast mitochondrial mass increased during propagation and during yeast growth during fermentation, likely due to their role in cell metabolic pathways associated with the production of building blocks and lipid synthesis. In addition, morphology also changed; during the later stages of fermentation they appeared tubular / networked, while mitochondria from earlier stages remained fragmented. The location of mitochondria with the cells also varied, with the mitochondria being sequestered closer to the cell wall at the later stages, possibly due to nano CO_2 bubble formation, while the fragmented mitochondria appeared dispersed throughout the cell (Swart *et al* 2013).

Arguably the most interesting observation from this work was the increase in mitochondrial mass during fermentation, something which was unexpected. The precise reasons for this increase are not yet known, however this data does strongly suggest that mitochondria play a far greater role in fermentation than originally believed. Although this is broadly recognised from basic analysis of the fermentation performance of petites (Chapter 4), the underpinning causes remain unclear. It is possible that the mitochondria role in cell homeostasis, protein and lipid production, the yeast stress response or perhaps in nuDNA crosstalk may prove to be vital to ensure fermentation performance.

CHAPTER 6 THE IMPACT MITOTYPE ON GROWTH CHARACTERISTICS AND STRESS TOLERANCE

6.1 Introduction

Saccharomyces pastorianus is an interspecific hybrid derived from at least one hybridisation event occurring between *S. cerevisiae* and *S. eubayanus* strains (Dunn and Sherlock. 2008; Kerogus *et al*., 2017) (Section 1.1.2). This incident has given rise to many of the phenotypic differences between ale and lager yeast, including the 'cold tolerant' attribute often seen as being desirable for lager production (Rainieri *et al.,* 2007). However, there is increasing evidence that stress tolerance and other phenotypic characteristics specific to *S. pastorianus* yeast may be derived from the mitochondria, and variations between species may be due to mitotype (Baker *et al*., 2018; Hewitt *et al*., 2018) (Section 1.8). Indeed, in support of the role of mitochondria in stress tolerance, the data presented in Chapter 4 indicated that petite mutants were more sensitive to a range of stress factors than their grande counterparts.

Yeast mitochondria are inherited from the mother cell during division and it is believed that the lager yeast strains in use today contain only unilaterally inherited mtDNA from the *S. eubayanus* parent (Section 1.8). This is supported by studies investigating mitochondrial inheritance *in-vitro* which have shown that although the initial hybrid cell zygotes are heteroplasmic with both parental mitochondria, this does not last. Offspring produced over successive generations become homoplasmic containing only one of the two parents' mtDNA (Piskur *et al.,* 1994; De Vero *et al.,* 2003; Rainieri *et al.,* 2007; Solieri *et al.,* 2008; Freel *et al.,* 2015) (Section 1.8). Although mitochondrial inheritance can be directed

using GM technology (Baker *et al*., 2018), this practice gives rise to organisms that are of limited use for commercial fermentations at the current time. Consequently, in order to explore the impact of mitotype on fermentation-related characteristics, in this Chapter we aimed to produce inter-specific 'cybrid' lager yeasts containing *S. cerevisiae* mitochondria.

Cybrids, also known as cytoplasmic hybrids, are the result of the fusion/hybridisation of a whole cell or protoplast with the cytoplast of another cell, in effect donating the cytoplasmic material without donating the nucleus. Cybridisation has found common use in plant cell culture, as a method to introduce genetic variation by fusing two protoplasts (both containing nuclei), forming a somatic hybrid (Galun and Aviv 1991). Cybrids have also seen use in human medicine and cell biology, for examining the role of mtDNA in a number of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Swerdlow 2009, Arduíno *et al* 2015). Furthermore, the production of yeast, mammalian and plant cybrids has assisted in understanding the role and contribution of mtDNA to a variety of biochemical processes, including mito-nuclear communication, reactive oxygen species production, mitochondrial calcium handling and the TCA cycle (Weinberg *et al.,* 2010; Wilkins *et al*., 2014; McKenzie *et al.,* 2016). In yeast, cybrid production has largely focused on *Saccharomyces sensu stricto* species, and such studies have been an invaluable tool in understanding the role of nucleo-mitochondrial interactions (Devarshi *et al*., 2017).

An important consideration when producing viable xenomitochondrial cybrids (cybrids produced using mitochondria isolated from different species) is the Dobzhansky-Muller incompatibility factor which was identified independently by Dobhansky in 1937 and Muller in 1942 (Welch, 2004). This describes the principle where foreign alleles can be deleterious to populations of a different background (Špírek *et al.,* 2015). The viability of cybrid cells relies on mutual communication between the nuclear and mitochondrial DNA; mitochondrial genomes from phylogenetically distant species are often unable to communicate with the receiver strains nucleus (Sulo *et al.,* 2003). However, it has been demonstrated that the action of introducing mitochondria from *Schizosaccharomyces*, *Hansenula*, and *Kluveromyces* spp. into a *S. cerevisiae* receiver was able to restore respiratory competency, with no apparent negative effects. This demonstrates that, in yeast, the dynamics which dictate the relationship between the genomic DNA and the mitochondria may be relatively conserved (Yoshida 1979; Sakanaka *et al.,* 1996; Sulo *et al.,* 2003; Špírek *et al.,* 2015).

When mitochondria are transplanted from one strain to another, it is reasonable to assume that this will directly impact their respiratory capacity; evidence has shown that some mito-types even lead to increased respiratory capacity when compared to the original strain (Procházka *et al* 2012, Albertin *et al* 2013). However, here have been few reports of the impacts of mitotype on the functionality of Crabtree positive cybrids, including aneuploid brewing yeast. In this Chapter we aim to address this by generating cybrid lager strains with *S. cerevisiae*

mitotypes, and to examine how this affects phenotypic (stress tolerance) characteristics related to industrial fermentations.

Experimental approach

In order to determine the impact of mitotype on yeast physiology, a natural petite believed to be Rho^0 derived from the lager yeast strain SMCC57 (termed SMCC57p) was selected as the host parent. This strain was chosen as it had the most distinctive characteristics in terms of petite production and mitochondrial membrane potential (Chapter 4 and Chapter 5). Simultaneously, mitochondria from the ale strain D23 were isolated and introduced to SM557p to produce a series of new cybrid yeast strains. These were then analysed for growth characteristics in wort and the impact of fermentation-related stress factors on performance using 96 well plates, as described in Section 2.4.3.

6.2 Results

6.2.1 Cybrid generation

Mitochondria derived from the ale yeast strain D23 were cybridised with a naturally produced petite generated from SMCC57 according to the principles outlined in Figure 6.2 and described in Section 2.8.1. Using this protocol a fraction of mitochondria were isolated from the respiratory competent D23 and combined with the protoplast of the respiratory deficient acceptor strain SMCC57p. Isolation of viable cybrids was achieved by growth on TSC agar (Section 2.8.1) and respiratory competence was assessed by growth on YPGly media (Section 2.2.1).

In total three lager yeast cybrids were obtained, representing a low overall yield with a limited success rate. However, their isolation demonstrated that the method represents a viable mechanism for exploring the impact of mitotype on functionality in brewing yeasts.

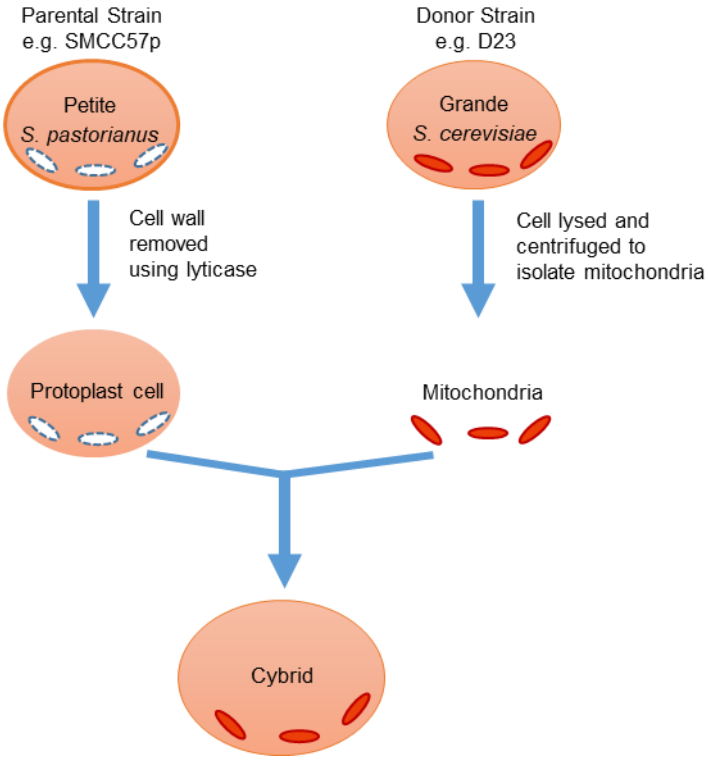


Figure 6.2 Method of cybrid production. In this instance, cybrids are derived from petite cells by inserting functional mitochondria from another species/strain of yeast in order to restore respiratory competence.

6.2.2 Genotypic confirmation of cybrid

In order to confirm that the cybrids generated comprised the original parent (strain SMCC57) genome, with mitochondria from the donor (strain D23), a series of PCR reactions were performed. Initially, inter-delta PCR was performed to confirm genome origins, as outlined in Section 2.8.2 and detailed in Chapter 3. Using this method it was possible

to confirm the identity of the cybrids as being derived from SMCC57. Two of the three cybrids matched the parental inter-delta profile (Cy1 and Cy2), and all were significantly different to the mitochondrial donor (D23) (Figure 6.3). It should be noted that cybrid 3 (Cy3) showed only a partial fingerprint, with several larger fragments absent. While it is unlikely that the cbyridisation process would have impacted the nuDNA, this isolate was nevertheless eliminated from further studies.

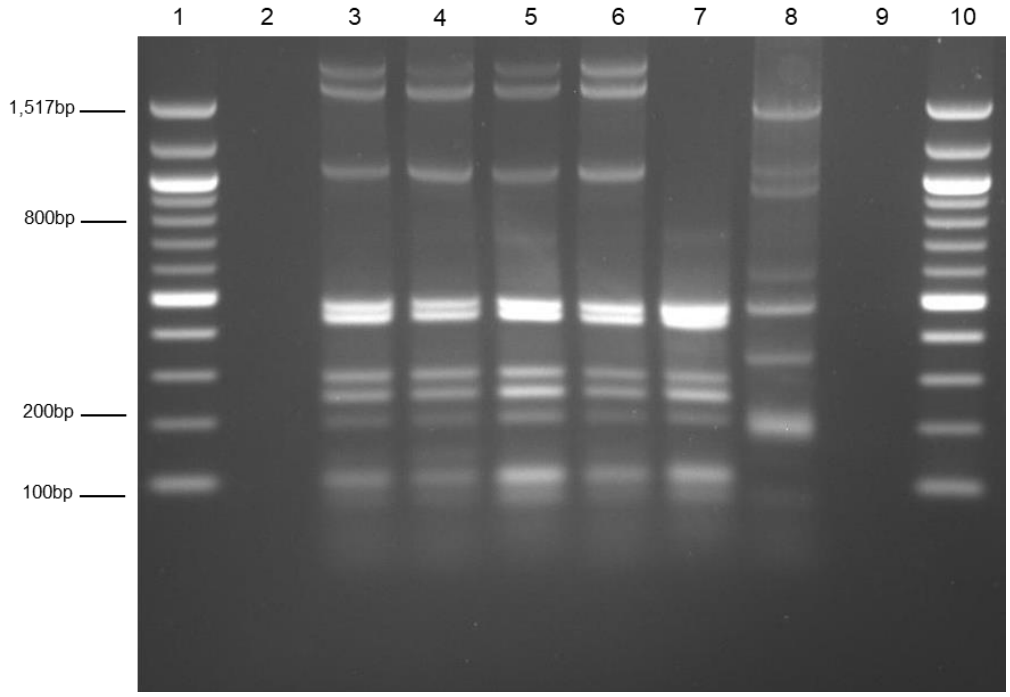


Figure 6.3 Identification of cybrid nuDNA based on inter-delta region analysis. Lanes 1: 100bp ladder; lane 2: Blank; lanes 3 and 4: Respiratory competent parental strain SMCC57; lane 5: Cybrid 1 (Cy1); lane 6: Cybrid 2 (Cy2); lane 7: Cybrid 3 (Cy3); lane 8: Donor strain D23; lane 9: Blank; lane 10: 100bp ladder.

Once the nuDNA contribution had been confirmed as being derived from SMCC57, examination of the mtDNA was required to confirm that this

matched the donor *S. cerevisiae* strain. This was achieved by examining the size of the COX1 gene in each cybrid and each parental strain as detailed in Section 2.8.3. COX1 is a mitochondrial gene which varies in size/weight between *S. cerevisiae* and *S. pastorianus* species (Špírek *et al* 2015). With the primers described in Section 2.8.2, the expected amplicon produced by *S. pastorianus* strains is ~800bp in size while the *S. cerevisiae* COX1 DNA is much smaller at ~150bp. Consequently, PCR analysis of this gene was employed as a means of quickly confirming that the mitotype of the cybrids matched the ale donor strain. It can be seen that, as expected based on the literature (Špírek *et al* 2015), PCR amplification of mtDNA from the ale donor strain (D23) and the parent lager strain (SMCC57) produced fragments of ~150bp and ~800b respectively (Figure 6.4). Subsequent analysis of all 3 cybrids (CY1-Cy3) revealed that the size of COX1 matched that of the donor strain (~150bp), confirming successful transfer of mitochondria. As noted above, even though cybrid Cy3 contained the donor mtDNA, this strain was not used in subsequent studies, due to inconsistencies in the nuDNA profile.

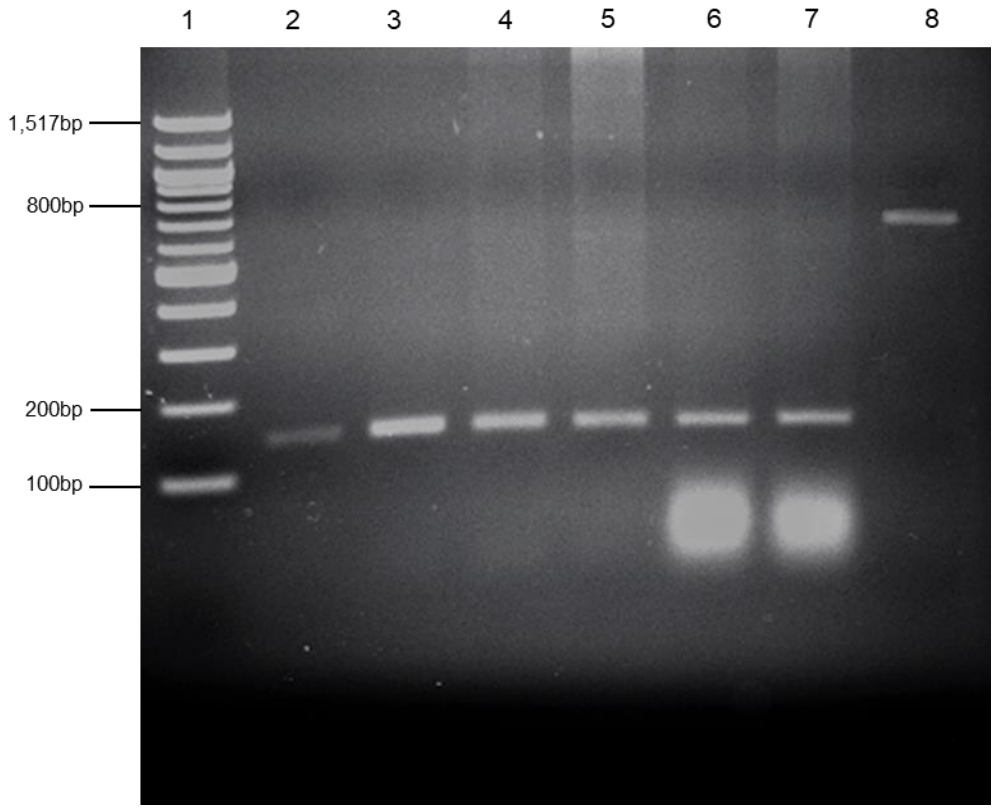


Figure 6.4 Analysis of COX1 gene length in cybrid yeast strains. Lanes 1: 100bp ladder; lanes 2 and 3: Donor strain D23; lane 4 and 5: Cybrid 1 (Cy1); lane 6 and 7: Cybrid 2 (Cy2); lane 8: Respiratory competent SMCC57 parent.

6.2.3 Growth characteristics of cybrids

Once the nuDNA and mtDNA complements of the newly generated cybrids had been confirmed, along with evidence that respiratory competence had been restored (based on the initial selection of viable colonies on YPGly; Section 6.2.1), the physiological characteristics of the new strains were evaluated. This was performed by analysing growth characteristics in an identical fashion to previously conducted (Chapter 4; Section 4.2.3). In addition, the relative responses of the cybrid strains to fermentation stress factors was also determined. Hydrogen peroxide

was applied as a mechanism for induction of oxidative stress (Section 2.4.3) and media was also supplemented with ethanol to determine the response to alcohol (Section 2.4.3).

From Figure 6.5 it can be seen that the two parental strains produced similar patterns of growth to one another over 45h. The petite mutant SMCC57p showed a reduced growth rate, as expected for a respiratory deficient strain; both respiratory competent parent strains attenuated quicker and to a higher degree. However, both of the new cybrid yeast strains showed very similar patterns of growth to SMCC57p. This data was disappointing since it indicates that despite becoming respiratory competent, the characteristics of the parent strain were maintained. The reasons for this are unknown, however it is possible that regulatory and functional communication between the mitochondria and the nucleus had become irreversibly disrupted (Antico Arciuch *et al*., 2012). It is also possible that the added complexity of the polyploid/aneuploid genomes encountered in industrial cells may be responsible for the issues arising (Špírek *et al*., 2015). An additional consideration is the establishment of mitochondria within the cell. In the current series of experiments, analysis was conducted quickly after transplantation. It would perhaps have been pertinent to allow an extended period of growth over many generations in order for mitochondrial division to occur and to ensure that the grande cybrid genotype was maintained. Irrespective, despite the failure of ‘normal’ growth characteristics to be adopted, the cybrids were subjected to stress in order to determine if other physiological characteristics were similarly affected.

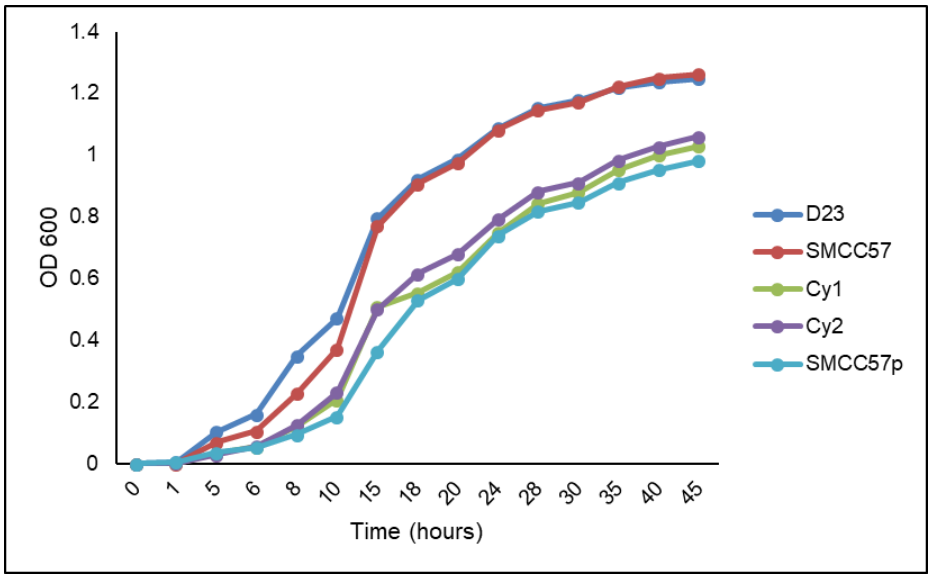


Figure 6.5 Growth profiles for strains D23, SMCC57, Cy1, Cy2 and SMCC57p. All strains were cultivated in wort for a period of 45 hours.

When cells were subjected to oxidative stress induced by hydrogen peroxide at 1mM, a sharp decline in the growth of the donor strain D23 was observed, indicating that this strain was negatively affected by this stress (Figure 6.6). In contrast, strain SMCC57 was not affected to the same extent, and growth remained similar to what would be expected if no stress was present. As above, a similar pattern was observed for both the cybrids and the petite strain SMCC57p, which continued to display reduced growth when compared to the parent strain. However, this trend did not persist as the concentration of the stressor increased. When 3mM hydrogen peroxide was applied, both cybrid yeasts showed improved performance compared to the petite strain, although still lower than the original parent (Figure 6.7). At 5mM hydrogen peroxide, growth in both the mitochondrial donor strain (D23) and the petite parent (SMCC57p) was almost completely restricted. In contrast, the cybrid yeast strains

showed similar growth characteristics to the grande parent, indicating a significant improvement in performance (Figure 6.8).

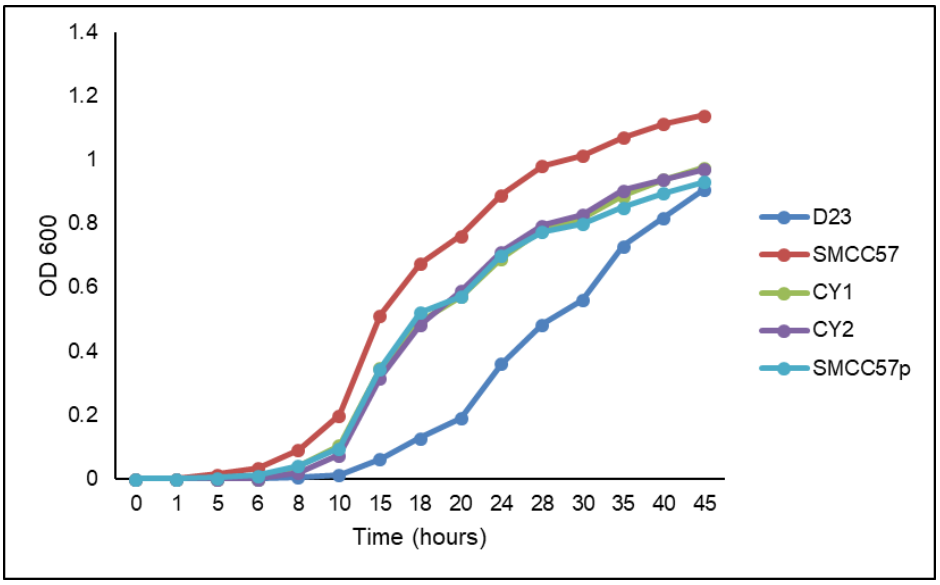


Figure 6.6 Growth profiles for strains D23, SMCC57, Cy1, Cy2 and SMCC57p in response to oxidative stress. All strains were cultivated in wort supplemented with 1 mM hydrogen peroxide for 45 hours to induce oxidative stress.

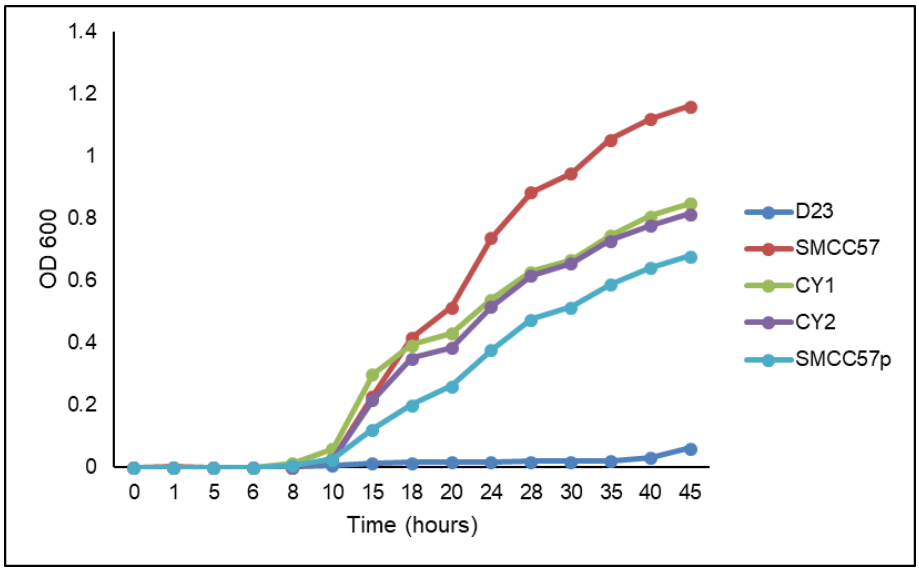


Figure 6.7 Growth profiles for strains D23, SMCC57, Cy1, Cy2 and SMCC57p in response to oxidative stress. All strains were cultivated in wort supplemented with 3 mM hydrogen peroxide for 45 hours to induce oxidative stress.

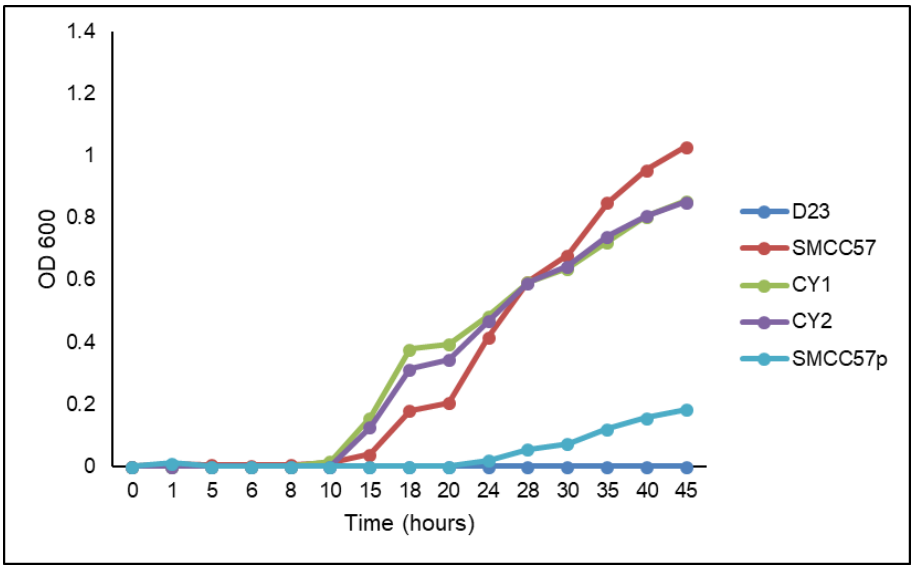


Figure 6.8 Growth profiles for strains D23, SMCC57, Cy1, Cy2 and SMCC57p in response to oxidative stress. All strains were cultivated in wort supplemented with 6 mM hydrogen peroxide for 45 hours to induce oxidative stress.

A similar pattern of results could be seen when ethanol was used to include alcohol stress. At relatively low concentrations (5% ethanol) the cybrid strains showed a poorer performance than both the donor strain and the grande strain; growth was similar to the petite parent (Figure 6.9). However, at higher concentrations (10% ethanol), the cybrids showed improved growth characteristics compared to the petite parent. In this instance the ale donor strain and the grande parent continued to out-perform all of the other strains (Figure 6.10).

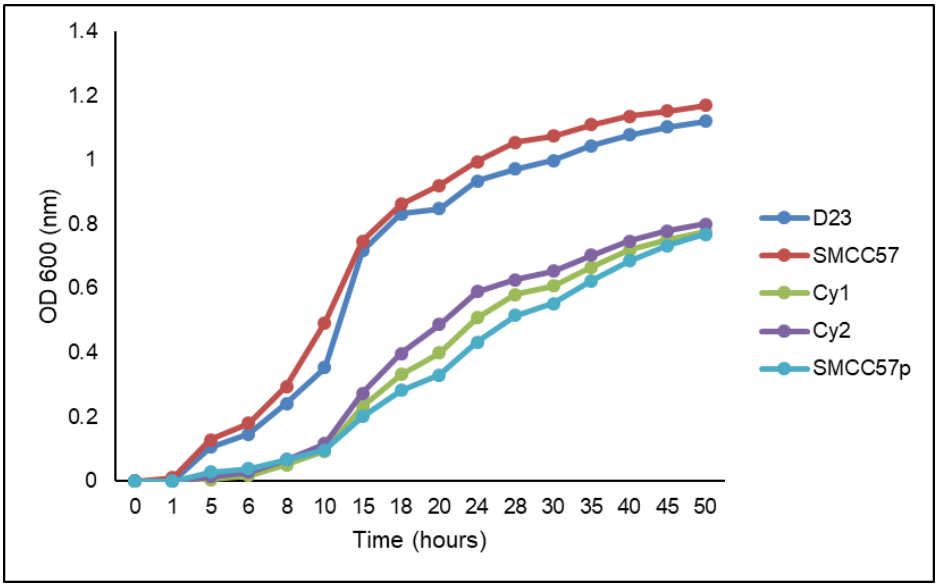


Figure 6.9 Growth profiles for strains D23, SMCC57, Cy1, Cy2 and SMCC57p in response to ethanol stress. All strains were cultivated in wort supplemented with 5% ethanol for 45 hours to induce ethanol stress.

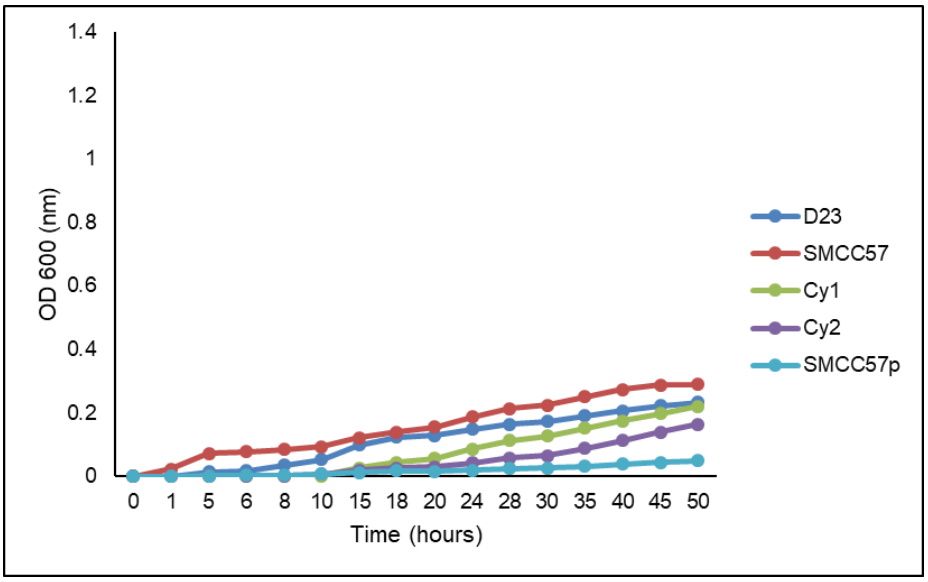


Figure 6.10 Growth profiles for strains D23, SMCC57, Cy1, Cy2 and SMCC57p in response to ethanol stress. All strains were cultivated in wort supplemented with 10% ethanol for 45 hours to induce ethanol stress.

The data obtained indicated that the cybrids were weaker than the parental strains and similar to petite mutants under standard conditions. However, under more stressful environments induced by oxidative stress and ethanol stress (albeit to a lesser extent), the presence of functioning mitochondria led to improvements in growth characteristics over the petite parent. It is recognised that this analysis reflects a rather simplistic view of the complex relationship between stress, yeast health, and growth. However, it was encouraging to observe that the transferred mitochondria were able to confer some benefit to the cybrid strains, albeit not to the extent of the original parent. As alluded to briefly above, it is possible that the newly generated cybrid populations may have comprised cells with a reduced complement of mitochondria or mtDNA,

which may have affected the data collected. It is also recognised that growth characteristics are not an absolute indication of fermentation performance; it is anticipated that future analysis in this direction will allow further insight into the functionality of the cybrids generated.

6.2.4 Cybrid yeast mitochondrial morphology and membrane potential

Although mixed results were obtained from growth curve analysis of cybrid yeast under standard conditions, it was interesting to note that mitochondria were able to aid in tolerance to high levels of stress. This raised questions regarding the health and integrity of the mitochondria within cybrid strains. Consequently, it was decided that an examination of the morphology and mitochondrial membrane potential (MMP) of cybrid yeasts may be insightful in determining the 'quality' and the biomass of the transposed mitochondria, and hence it's impact on functionality. To achieve this, the mitochondrial morphology of cybrid strains Cy1 and Cy2 was examined using rhodamine b hexyl ester fluorescent staining as previously described (Section 2.7.1).

It can be seen from Figure 6.11 that the cybrid cells appeared to have mitochondrial morphologies that were similar to those of respiratory competent strains. As would be expected, the mitochondria appeared in the characteristic form of networked tubular organelles similar to shown previously (Chapter 5; Section 5.2.2), very different to the more globular appearance of the naturally produced petite SMCC57p. There were some differences in morphology between the two cybrids, although

overall they would both be described as networked, Cy2 does appear to have more aggregated sections of mitochondria.

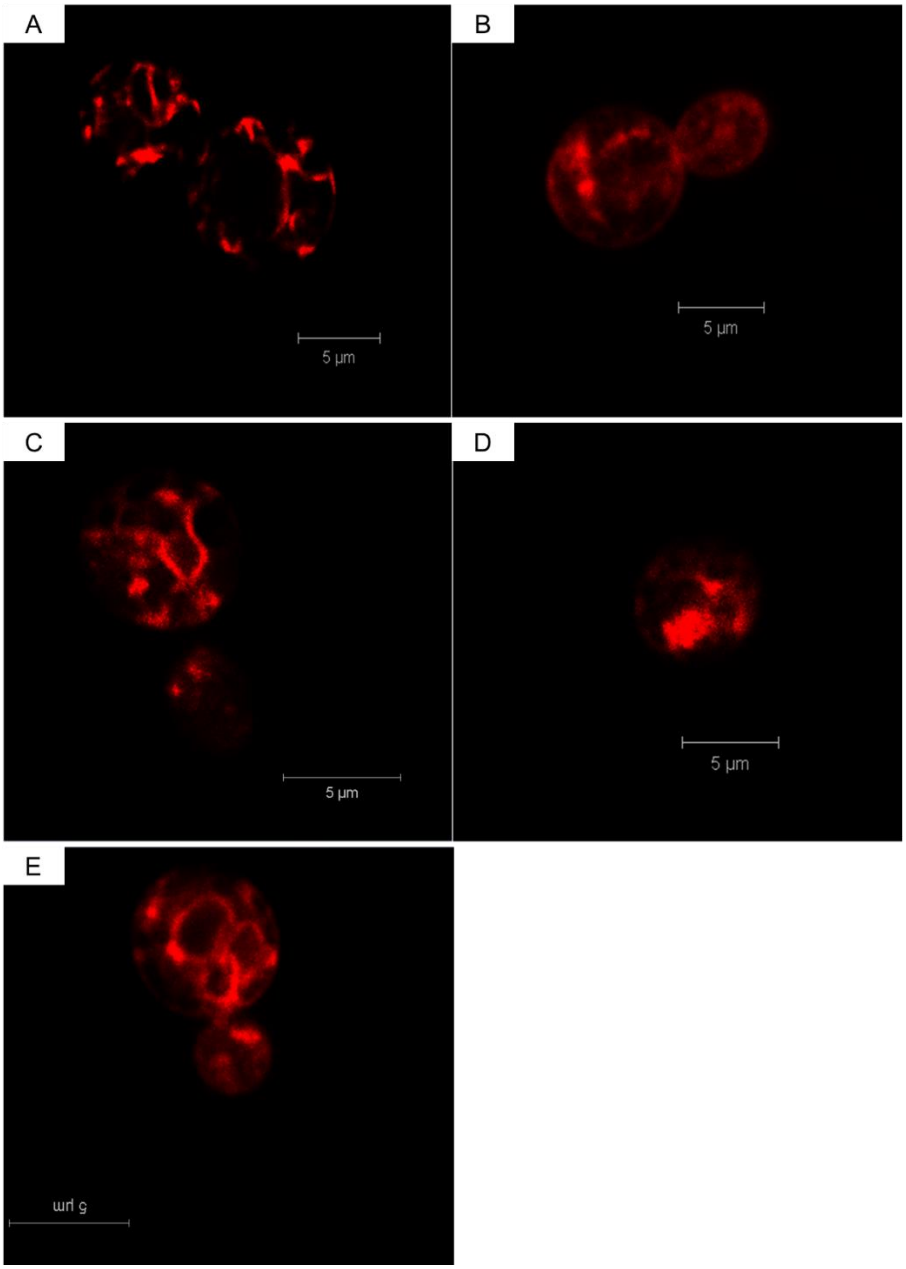


Figure 6.11 Mitochondrial morphology based on staining using rhodamine b hexyl ester visualised using fluorescent microscopy. A: Cy1; B: Cy2; C: SMCC57; D: SMCC57p; E: D23. Cells selected were representative of the entire population based on visual analysis of ~100 cells.

While mitochondrial morphology can provide some basic insight into the differences between the cybrids and their parental strains, determination of mitochondrial membrane potential can also be useful specially since this is required for effective aerobic growth and successful transmission of mitochondria to budding daughter cells (Section 1.8; Chapter 5). Interestingly, analysis of mitochondrial membrane potential between strains indicated that there was variation between cybrids (Figure 6.12), which may partially explain the differences in morphology reported above (Figure 6.11).

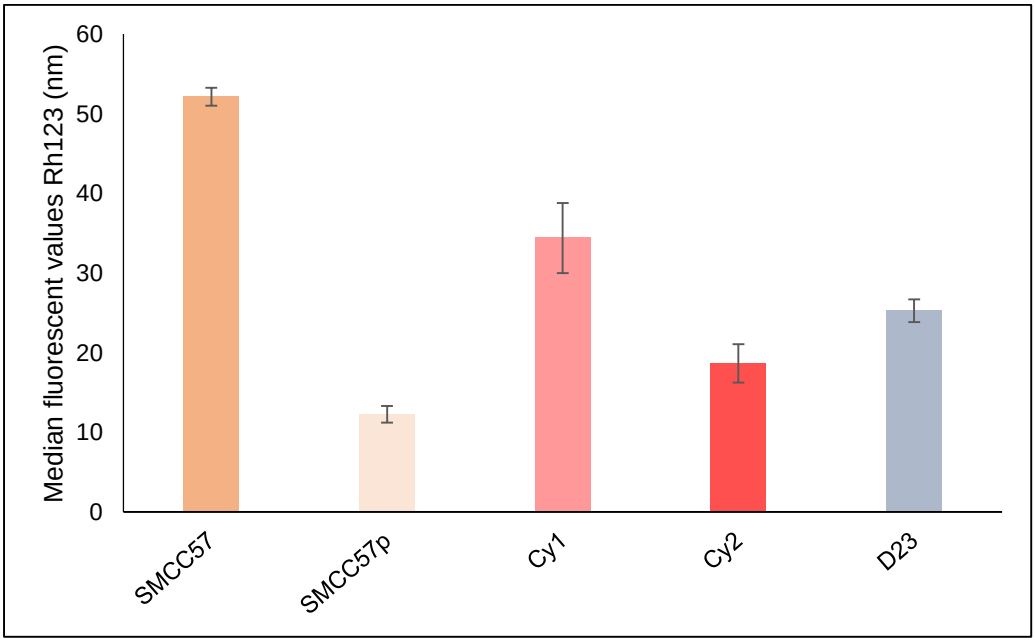


Figure 6.12 Analysis of mitochondrial membrane potential using the fluorescent stain Rhodamine 123. Data reflects the median fluorescent values obtained from analysis of populations of SMCC57, SMCC57p, Cy1, Cy2 and D23.

From Figure 6.12 it can be seen that there are clear differences in MMP between the two cybrid strains examined. Furthermore. the Cy1 strain is

significantly different to the petite SMCC57p (P value of 0.0082), while Cy2 is not (P value of 0.0731). It can be seen that the mitochondrial membrane potential of strain Cy2 is more similar to the host strain SMCC57p, and lower than the donor strain D23. In contrast, the cybrid yeast Cy1 exhibits a MMP which lies in-between the petite host and the original grande strain (Figure 6.12). Furthermore, the MMP observed in Cy1 is significantly greater than the mitochondrial donor (P value of 0.02), indicating that interpretation of the data is not straightforward. However, based on the information presented here and in Figure 6.11, it is suggested that the reduced MMP may explain why Cy2 appears to have fewer mitochondrial structures, since this is required for effective transmission from the mother cell to its daughter during budding. As mentioned above, the cybrids generated here are relatively 'young' populations and it is possible that after a number of generations, involving successful transmissions of healthy mitochondria, the MMP of both cybrids will reach that of a similar level to the original parental strain. Further work would be required to determine if this is indeed the case. Irrespective, the data does add further evidence to suggest that although initial transfer of mitochondria was successful, the characteristics of the cybrid strains remain slightly anomalous. Evidently, there are more factors at play when restoring respiratory capacity than simply moving mitochondria into the cell. Given the role of the nuDNA in mitochondrial gene regulation and mitochondrial biosynthesis (Eisenberg - Bord and Schuldiner, 2017), it is likely that investigations into crosstalk between organelles will prove to be insightful in the future.

6.4 Conclusions

This aim of the current work was to establish if it was possible to transplant functional mitochondria from *S. cerevisiae* to a petite *S. pastorianus* species using protoplast fusion, and to determine if functional traits were also transferred. Although the technique applied for cybrid generation has been proven to be a simple but effective means of moving mitochondria from a donor organism into a host cell (Sulo *et al.,* 2000), in the current study, a substantial number of experiments were required to generate very few successful cybrid colonies. Multiple reactions each comprising more than $1x10^9$ host cells were mixed with mitochondrial fractions obtained from an equal number of donor cells, and yet this generated only 3 cybrids. It should be noted that the majority of cybrids generated in the past have been constructed using haploid organisms. The current study represents the first time the technique has been applied to polyploid/aneuploid yeast strains; the fact that this was achievable offers promise for the future. However it is possible that other methods such as micro-injection of mitochondria, which has been used successfully in mammalian cells (Takeda *et al*., 2005), or cross breeding strains with functional and non-functional mitochondria may yield more success. The latter is difficult in brewing strains, due to their inability to sporulate or divide sexually (Bilinski *et al*., 1986; Gibson *et al*., 2017), however there are an increasing range of genetic techniques available which could be employed. It should be noted that this approach was not taken in the current study, due primarily to industrial concerns over consumer perception towards the use of genetically modified organisms.

Once viable cybrid colonies had been selected from non-fermentable media, PCR analysis of the COX1 gene and inter-delta PCR fingerprinting were performed to confirm the mitotype and strain identity respectively. Subsequently, each cybrid colony was analysed using the matrices previously established in Chapter 4. These allowed for the examination of strain growth characteristics under stress-induced conditions. This analysis indicated that the cybrids produced were not able to grow as effectively as the respiratory competent parent SMCC57 under standard conditions; growth patterns were similar to the petite parent strain. However, when cells were subjected to elevated levels of stress (particularly oxidative stress), the cybrid strains performed better than the petite parent SMCC57p. This can confidently be attributed to the presence of functional mitochondria, especially since these are known to be important in the oxidative stress response due to localization of manganese superoxide dismutase (mnSOD) which can serve to detoxify the cell (Grant *et al*., 1997). The fact that cybrids did not show the same characteristics as grande cells under all conditions indicates that some aspect of physiology had been compromised either during petite generation or due to transfer of mitochondria. The exact reasons are unknown, but it is possible that continually sub-culturing cybrid populations on YPGly may be required in order to ensure that the donated mitochondria had stabilised prior to phenotype analysis. It is possible that uneven distribution of mitochondria may have caused some yeast cells to show reduced respiratory competence. It would be particularly important to apply this type of protocol in future experiments

to ensure mitochondria had become ‘embedded’ into the population. These could then be used in a more direct analysis of fermentation properties, including sugar utilisation and flavour generation to provide useful insight into the suitability of such strains for industrial purposes.

Examination of the mitochondrial morphology of cybrids revealed that the cybrid yeast strain Cy2 appeared reminiscent of petite yeast cells. In contrast, strain Cy1 seemed more similar to a respiratory competent population. This observation is partially supported by subsequent analysis of MMP which indicated that the mitochondria present in this yeast were more similar to those seen in petite strains. However, this wasn’t reflected in the growth analysis, as both cybrid strains showed similar performance both in the absence and presence of stress, as already discussed. Irrespective, it is evident that there are a number of factors which impact on mitochondrial ‘health’, including the mass of mitochondria within the cell, copy number of mtDNA, overall activity, as well as their capacity to perform normal functions such as fusion and fission events both during somatic growth and during budding *(Knorre et al*., 2012; Eisenberg - Bord and Schuldiner, 2017). Since many of these are also influenced by the nuDNA (Ziaja *et al*., 1993; Eisenberg - Bord and Schuldiner, 2017), there remain a number of avenues for future research in order to fully understand the consequences of transporting mitochondria. However, it is anticipated that cybridisation does remain a viable mechanism for increasing current understanding of mitochondrial function and as a potential means of generating novel industrial yeast strains in the future.

CHAPTER 7 CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

Only recently has the role of mitochondria in brewing fermentations been closely examined. This is primarily due to the role in which mitochondria play in cellular respiration, which itself if repressed/minimised during fermentation due to the Crabtree effect and the anaerobic conditions associated with brewing. Our current understanding is that yeast mitochondria are essential for cellular function and fermentation performance, due in no small part to the number of important cellular functions they are involved in nuclear DNA signalling, amino acid synthesis, acetyl-CoA synthesis and lipid generation and desaturation of sterols and fatty acids. This is supported by the fact that cells which are lacking in mitochondrial function termed respiratory deficient or 'petites' do not ferment as efficiently or rapidly as their respiratory competent 'grande' counterparts. Fermentations conducted using cultures which predominantly consist of respiratory deficient yeast cells are usually slower with reduced growth, abnormal flocculation characteristics and poor VDK reduction profiles.

The primary goals of this work was to garner a greater understanding of mitochondria, mitochondrial DNA, mitochondrial function and how this can relate to fermentation. In chapter 3, the strains were characterised based on their ITS and Inter-delta profiles, sorting them into species and strain. With the strains identified, the mtDNA genome of the strains were firstly examined through conventional means using RFLP to produce fingerprints of extracted mtDNA. These RFLP profiles generated in this manner suggested no clear differences between the *S. pastorianus*

strains, while it was effective for *S. cerevisiae* strains. In order to better scrutinise the mtDNA genomes of the *S. pastorianus* strains a more powerful molecular tool was required. With the sequencing data we were able to better analyse the strains and examine for any subtle differences occurring in the *S. pastorianus* genomes in the form of SNPs. Using genome maps, the genes and complexes were identified and annotated. Additionally a phylogenetic trees were constructed to examine the evolutionary relationship between sequenced strains and the reference mitochondrial genomes available on NCBI website.

With the identities of the strains established in Chapter 3, Chapter 4 examined in greater detail the propensity of each strain to produce the respiratory deficient phenotype. Evidence was provided that supported the long held truth that strains produce petites at different rates. It should be noted that even though the values may appeared minor, all of the strains produced petites under conditions which were not at all severe. It is reasonable then to assume that this capacity to form petites may be problematic, especially since it is likely that the quantity of mutant cells within a yeast slurry would only increase through successive fermentations due to associated stress factors (Powell *et al*., 2007; Gibson *et al*., 2008; Lawrence *et al*., 2012). The mtDNA profiles of these naturally occurring petite strains were examined as well as examining their fermentation profiles and characteristics verses their respiratory competent counterparts. Previous studies particularly focused on the differences between Rho^{-} and Rho^{0} petite strains which had often been generated artificially through use of ethidium bromide

We examined how the strains grew and fermented under standard condition with results revealing that the petites were able to grow initially as well as the grande strains but failed to attenuate at the same level. The storage sugars trehalose and glycogen were examined for these strains both pre and post fermentation, with results suggesting that petites are less likely to survive storage induced stresses. Further to the examination of the fermentations, the organoleptic profiles of both grande and petite strains through using GC-MS headspace analysis focusing specifically on volatile compounds and VDKs. The results indicated a great diversity across the lager strains with many of the compounds being produced at varying quantities. On average however, the petites produced far greater quantities of isobutanol and diacetyl compounds, further suggesting the importance of respiratory competency in achieving a consistent final product.

Chapter 5 examined in greater detail some key matrices of mitochondria specifically examining the morphology, content and mitochondrial membrane potential. This chapter relied on the development of a staining protocol which could effectively and reproducibly examine mitochondria within living yeast cells.

In conjunction with fluorescent microscopy, flow cytometry played a key role in establishing the mitochondrial content and MMP of the yeast strains. While work has been done previously examining mitochondria within yeast cells, there is little work examining differences between grandes and petites in terms of morphology, content and MMP. There is

some work examining petites using fluorescent staining methodologies (Lawrence *et al*., 2012) but these predominately focused on differentiating between the two petite types Rho^- and Rho^0.

Some results were surprising, in particular the lack of variation between the grande and petites in terms of overall mitochondrial content. Differences were seen however, between grande and petite in total mtDNA content and mitochondrial membrane potential. What was also noted was a variation of MMP which occurred within the grande grouping. Some strains having a far higher MMP than others in a similar fashion to the rate of petite production across the strains. Closer examination of the differences in MMP between the lager strains SMCC100 and SMCC57 was conducted, revealing similar mitochondrial content but very different mitochondrial membrane potentials. These strains were additionally exposed to ethanol stress at concentrations reminiscent of those that would be seen in a brewery and there was a noticeable trend where MMP increases and mitochondrial content decreases. Higher MMP may be linked with higher rates of mitochondrial damage and possibly petite production.

It was established that mitochondrial dynamics change over the course of yeast handling, and particularly during propagation and, surprisingly, fermentation. Yeast mitochondrial mass increased during propagation and during yeast growth during fermentation, likely due to their role in cell metabolic pathways associated with the production of building blocks and lipid synthesis. In addition, morphology also changed; during the later

stages of fermentation they appeared tubular / networked, while mitochondria from earlier stages remained fragmented. The location of mitochondria with the cells also varied, with the mitochondria being sequestered closer to the cell wall at the later stages, possibly due to nano CO_2 bubble formation, while the fragmented mitochondria appeared dispersed throughout the cell (Swart *et al* 2013).

In the final chapter, a method to produce cytoplasmic hybrids was used in order to transplant functional mitochondria of *S. cerevisiae* origin into a respiratory deficient *S. pastorianus* strain. While only three respiratory competent colonies produced using this method. The cybrids growth characteristics were examined under aerobic conditions in wort additionally they were exposed to additional stress factors both ethanol and oxidative (hydrogen peroxide). The results from these experiments did not appear to be too promising with the cybrids displaying similar growth profiles to the petite. However, when exposed to higher levels of ethanol and hydrogen peroxide the cybrids performed better than the petite strain. There is a possibility that the methodology did not equally distribute the mitochondria with some cells resulting in phenotypes more similar to that of petites.

In addition to examining the growth characteristics of these cybrids we also examined the mitochondrial morphology and membrane potential using the methodology established in Chapter 5. The results revealed mitochondrial morphology similar to respiratory competent yeast cells, forming tubular networks. When examining the mitochondrial membrane

potential, we found that Cy2 did not yield the same MMP as the grande strain SMCC57, but appeared to be more similar to the petite, while Cy1 was significantly different to the petite and being more similar to SMCC57. While initial growth experiments did not yield impressive results it is anticipated that cybridisation does remain a viable mechanism for increasing current understanding of mitochondrial function and as a potential means of generating novel industrial yeast strains in the future.

7.2 Future Work

The following are experiments which could expand on and complement data produced during this PhD.

I. Examination of natural petite verses artificially produced petite

Much of the work which has been conducted previously examining petite samples has looked at petites which have been artificially produced through the use of ethidium bromide. While as method of petite production it has clear advantages over other methods, it is possible the petites produced are less representative than produced naturally. It would be interesting to see what differences there are when examining these petites versus petites which appear naturally. Closer examination of their mtDNA profiles may reveal similar profiles, further supporting the idea of 'hotspots' or structurally weak areas within the mtDNA genome. Additionally, a closer examination of fermentation characteristics and organoleptic profiles in order to ascertain if naturally occurring petites are indeed any better or worse than those produced through artificial means.

It is possible that the petites produced through artificial means are far worse than those produced naturally or there may be no difference between the two types due to the presence of hotspots and ethidium bromide only encourages the mutation.

II. Impact of process on petite production

As previously established in Chapter 1 and Chapter 4, petites naturally occur within yeast slurry population with brewers reporting different percentages of petites ranging from 1 % - 3 % and higher (Morrison and Suggett, 1983; Gibson *et al*., 2007). We established in Chapter 4 that stress induced conditions can cause increases in petite frequency across the strains. While the rate of petites has been examined when cropping yeast (Lawrence *et al*., 2012) based on cone residence (Smart, 2013), and during storage (Gibson *et al*., 2008), examination of petites produced during fed batch fermentation, it has been shown previously that respiratory deficient mutants occur when exposed to ethanol for prolonged periods of time (Chi and Arneborg, 1999; Voordeckers *et al*., 2015). Additionally, as oxygen requirements vary between strains (Boulton and Quain, 2001) a closer examination of propagation and how these requirements impact a strains propensity to produce petites i.e. are strains which require more oxygen more or less prone to producing petites. It is possible that yeast with higher oxygen demands have higher levels of cellular ROS which in turn could result in additional mtDNA damage.

III. Impact of petite percentage on fermentation

Throughout this thesis the petite cultures used were usually 100 % petite populations. This is unlikely to be the case in most breweries, so understanding the impact of various concentrations of petite on other yeast cells and the fermentation as a whole would be useful to know. This would need to be done with a number of different strains as it was established in Chapter 4 and 5 that not all petite yeast cells act the same, with some strains petites having similar profiles to respiratory competent strains. Additionally examination of how different processes such as fermenting with VHG wort will have on the petites.

IV. Cross-talk between mitochondria and nucleus

Cross talk between mitochondria and the nucleus is important for cellular homeostasis, and this cross talk relies on continuous feedback and metabolic pathways or signal transduction pathways to fuction across the mitochondrial inner membrane (Poyton and McEwen, 1996; Cagin and Enriquez, 2015). This crosstalk gives essential information to the nucleus and other organelles and allows for appropriate expression programs to be triggered (Cagin and Enriquez, 2015). Some work has suggested that when this communication is lost that there is an increase in the expression of genes responsible for the biogenesis of mitochondria and others suggest these genes are unaffected or down regulated (Epstein *et al*., 2001; Butow and Avadhani, 2004). With petites being a prevalent factor in brewing strains it is important to understand what communication if any is taking place the nucleus and dysfunctional mitochondria and

what impact these signals have on overall cell functionality, i.e. to better understand how petites persist through successive fermentations. Using RNAseq technology and gene microarray analysis could identify differences in key gene expression between both respiratory competent and incompetent strains and may reveal which mitochondrial genes are triggered by nuclear activity (Singh *et al*., 2005). Examination of mRNA or gene expression across the strains which are more likely to produce petites may reveal why the mutation is more likely to occur in some strains over others. Additionally, examination of how foreign/xeno mitochondria present within petite cells communicate with the receiver strain.

V. Additional methods for the production of cybrids

While the scope wasn't present within this PhD to examine numerous avenues of cybrid generation, it would be something worth considering either bio-ballistics or microinjection as methods of producing more successful cybrid cells. While method used within this project while effective did not yield great numbers of cybrids, the methodology required to produce protoplasts would be required in order to negate the cell wall. Perhaps the use of microinjection with the cytoplasm of the donor may help with the uptake of mitochondria into the new host cell. The downside in producing cybrids this way may be that it's not possible due the size of the needle, most microinjection techniques described are used on larger mammalian cells with some cells being too small or too fragile for the process which may be the case for the protoplasts (Kole *et al.*, 2011).

7.3 Final remarks

Fermentation characteristics of petite populations are well documented, but little was known about the basic cytology of brewing yeast mitochondria. The work completed in this thesis adds to our understanding of brewing yeast mitochondria. While many aspects of mitochondrial cytology and functionality are strain dependent, it should be recommended that brewers better understand their yeasts requirements and sensitivities to stress. A better understanding of the brewer's yeast strain will in turn reduce the incidence of petites and improve consistency of the product.

References

Abdulkhair, W.M.H., 2018. The yeast role in medical applications. https://doi.org/10.5772/intechopen.69408

Aguilera, A., Benítez, T., 1985. Role of mitochondria in ethanol tolerance of *Saccharomyces cerevisiae*. Arch. Microbiol. 142, 389–392.

Albertin, W., da Silva, T., Rigoulet, M., Salin, B., Masneuf-Pomarede, I., de Vienne, D., Sicard, D., Bely, M., Marullo, P., 2013. The mitochondrial genome impacts respiration but not fermentation in interspecific *Saccharomyces* Hybrids. PLoS ONE 8.

Alexeyev, M., Shokolenko, I., Wilson, G., LeDoux, S., 2013. The Maintenance of mitochondrial DNA integrity—Critical analysis and update. Cold Spring Harb. Perspect. Biol. 5, a012641.

Altmann, K., Westermann, B., 2005. Role of essential genes in mitochondrial morphogenesis in *Saccharomyces cerevisiae*. Mol. Biol. Cell 16, 5410–5417.

Alves, S.L., Herberts, R.A., Hollatz, C., Trichez, D., Miletti, L.C., de Araujo, P.S., Stambuk, B.U., 2008. Molecular analysis of maltotriose active transport and fermentation by *Saccharomyces cerevisiae* reveals a determinant role for the AGT1 permease. Appl. Environ. Microbiol. 74, 1494–1501.

Antico Arciuch, V.G., Elguero, M.E., Poderoso, J.J., Carreras, M.C., 2012. Mitochondrial regulation of cell cycle and proliferation. antioxid. redox signal. 16, 1150–1180.

Aung-Htut, M.T., Lam, Y.T., Lim, Y.-L., Rinnerthaler, M., Gelling, C.L., Yang, H., Breitenbach, M., Dawes, I.W., 2013. Maintenance of mitochondrial morphology by autophagy and its role in high glucose effects on chronological lifespan of *Saccharomyces cerevisiae*. Oxid. Med. Cell. Longev. 2013.

Austriaco, O. P., N., 2012. Endoplasmic reticulum involvement in yeast cell death. Front. Oncol. 2.

Bahafid, W., Joutey, N.T., Asri, M., Sayel, H., Tirry, N., Ghachtouli, N.E., 2017. Yeast biomass: An alternative for bioremediation of heavy metals. Yeast - Ind. Appl.

Baker, E., Wang, B., Bellora, N., Peris, D., Hulfachor, A.B., Koshalek, J.A., Adams, M., Libkind, D., Hittinger, C.T., 2015. The genome sequence of *Saccharomyces eubayanus* and the domestication of lager-brewing yeasts. Mol. Biol. Evol. 32, 2818–2831.

Baker, E.P., Peris, D., Moriarty, R.V., Li, X.C., Fay, J.C., Hittinger, C.T., 2018. Mitochondrial DNA and temperature tolerance in lager yeasts.

Bandara, A., Fraser, S., Chambers, P.J., Stanley, G.A., 2009. Trehalose promotes the survival of *Saccharomyces cerevisiae* during lethal ethanol stress, but does not influence growth under sublethal ethanol stress. FEMS Yeast Res. 9, 1208–1216.

Barnett, J.A., 2000. A history of research on yeasts 2: Louis Pasteur and his contemporaries, 1850–1880. Yeast 16, 755–771.

Battersby, B.J., Richter, U., 2013. Why translation counts for mitochondria - retrograde signalling links mitochondrial protein synthesis to mitochondrial biogenesis and cell proliferation. J. Cell Sci. 126, 4331–4338.

Bendiak, B., Fang, T.T., 2000. End-group determination of oligosaccharides: a gas chromatography-mass spectrometry/mass spectrometry method for distinguishing between all D-aldohexoses and D-ketohexoses. Carbohydr. Res. 327, 463–481.

Berger, K.H., Yaffe, M.P., 2000. Mitochondrial DNA inheritance in *Saccharomyces cerevisiae*. Trends Microbiol. 8, 508–513.

Berlowska, J., Kregiel, D., Rajkowska, K., 2014. Biodiversity of brewery yeast strains and their fermentative activities: Biodiversity of brewery yeast strains. Yeast 32(1), 289–300.

Bilinski, C.A., Russell, I., Stewart, G.G., 1986. Analysis of sporulation in brewer's yeast: Induction of tetrad formation*. J. Inst. Brew. 92, 594–598.

Bliek, A.M. van der, Shen, Q., Kawajiri, S., 2013. Mechanisms of mitochondrial fission and fusion. Cold Spring Harb. Perspect. Biol. 5, a011072.

Bokulich, N.A., Bamforth, C.W., 2013. The Microbiology of malting and brewing. Microbiol Mol Biol Rev 77, 157–172.

Bonet-Ponce, L., Saez-Atienzar, S., da Casa, C., Flores-Bellver, M., Barcia, J.M., Sancho-Pelluz, J., Romero, F.J., Jordan, J., Galindo, M.F., 2015. On the mechanism underlying ethanol-induced mitochondrial dynamic disruption and autophagy response. Biochim. Biophys. Acta BBA - Mol. Basis Dis. 1852, 1400–1409.

Borzani, W., Vairo, M.L., 1958. Quantitative adsorption of methylene blue by dead yeast cells. J. Bacteriol. 76, 251–255.

Boulton, C., Quain, D., 2001. Brewing yeast and fermentation. Blackwell Science, Oxford.

Boulton, C.A., 1991. Developments in brewery fermentation. Biotechnol. Genet. Eng. Rev. 9, 127–182.

Boyd, A.R., Gunasekera, T.S., Attfield, P.V., Simic, K., Vincent, S.F., Veal, D.A., 2003. A flow-cytometric method for determination of yeast viability and cell number in a brewery. FEMS Yeast Res. 3, 11–16.

Brányik, T., Vicente, A.A., Dostálek, P., Teixeira, J.A., 2008. A review of flavour formation in continuous beer fermentations*. J. Inst. Brew. 114, 3–13.

Briggs, D.E. (Ed.), 2004. Brewing: science and practice, Woodhead Publishing in food science and technology. CRC Press ; Woodhead Pub. Ltd, Boca Raton : Cambridge, England.

Butow, R.A., Avadhani, N.G., 2004. Mitochondrial signalling: The retrograde response. Mol. Cell 14, 1–15.

Cagin, U., Enriquez, J.A., 2015. The complex crosstalk between mitochondria and the nucleus: What goes in between? Int. J. Biochem. Cell Biol. 63, 10–15.

Casey, G.P., Xiao, W., Rank, G.H., 1988. Application of pulsed field chromosome electrophoresis in the study of chromosome XIII and the electrophoretic karyotype of industrial strains of *Saccharomyces* yeasts. J. Inst. Brew. 94, 239–243.

Chen, P., Dong, J., Yin, H., Bao, X., Chen, L., He, Y., Chen, R., Wan, X., Zhao, Y., Hou, X., 2016. Genome comparison and evolutionary analysis of different industrial lager yeasts (*Saccharomyces pastorianus*): Comparative analysis of genomic characteristics of lager yeasts. J. Inst. Brew. 122, 42–47.

Chi, Z., Arneborg, N., 1999. Relationship between lipid composition, frequency of ethanol-induced respiratory deficient mutants, and ethanol tolerance in *Saccharomyces cerevisiae*. J. Appl. Microbiol. 86, 1047–1052.

Contamine, V., Picard, M., 2000. Maintenance and integrity of the mitochondrial genome: A plethora of nuclear genes in the budding yeast. Microbiol Mol Biol Rev 64, 281–315.

Coruzzi, G., Kaye Trembath, M., Tzagoloff, A., 1979. [9] The isolation of mitochondrial and nuclear mutants of Saccharomyces cerevisiae with specific defects in mitochondrial functions, in: Methods in enzymology, biomembranes. Part G: Bioenergetics: Biogenesis of mitochondria, organization, and transport. Academic Press, pp. 95–106.

D'Amore, T., Russell, I., Stewart, G.G., 1989. Sugar utilization by yeast during fermentation. J. Ind. Microbiol. 4, 315–323.

Dasari, S., Kölling, R., 2011. Cytosolic localization of acetohydroxyacid synthase Ilv2 and its impact on diacetyl formation during beer fermentation. Appl. Environ. Microbiol. 77, 727–731.

Datta, S., Annapure, U.S., Timson, D.J., 2017. Different specificities of two aldehyde dehydrogenases from *Saccharomyces cerevisiae var. boulardii.* Biosci. Rep. 37.

Day, M., 2013. Yeast petites and small colony variants: for everything there is a season. Adv. Appl. Microbiol. 85, 1–41.

De Vecchis, D., Cavellini, L., Baaden, M., Hénin, J., Cohen, M.M., Taly, A., 2017. A membrane-inserted structural model of the yeast mitofusin Fzo1. Sci. Rep. 7.

Dieckmann, C.L., Gandy, B., 1987. Preferential recombination between GC clusters in yeast mitochondrial DNA. EMBO J. 6, 4197–4203.

Dimmer, K.S., Fritz, S., Fuchs, F., Messerschmitt, M., Weinbach, N., Neupert, W., Westermann, B., 2002. Genetic basis of mitochondrial function and morphology in *Saccharomyces cerevisiae*. Mol. Biol. Cell 13, 847–853.

Duarte, A.W.F., Dayo-Owoyemi, I., Nobre, F.S., Pagnocca, F.C., Chaud, L.C.S., Pessoa, A., Felipe, M.G.A., Sette, L.D., 2013. Taxonomic assessment and enzymes production by yeasts isolated from marine and terrestrial Antarctic samples. Extrem. Life Extreme Cond. 17, 1023–1035.

Dunn, B., Levine, R.P., Sherlock, G., 2005. Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. BMC Genomics 6, 53.

Dunn, B., Sherlock, G., 2008. Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. Genome Res. 18, 1610–1623.

Ehlers, R.A., Hernandez, A., Bloemendal, L.S., Ethridge, R.T., Farrow, B., Evers, B.M., 1999. Mitochondrial DNA damage and altered membrane potential (delta psi) in pancreatic acinar cells induced by reactive oxygen species. Surgery 126, 148–155.

Eisenberg-Bord, M., Schuldiner, M., 2017. Ground control to major TOM: mitochondria–nucleus communication. FEBS J. 284, 196–210.

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., Weng, S., Wong, E.D., Lloyd, P., Skrzypek, M.S., Miyasato, S.R., Simison, M., Cherry, J.M., 2013. The reference genome sequence of *Saccharomyces cerevisiae*: Then and now. G3 GenesGenomesGenetics 4, 389–398.

Ephrussi, B., Hottinguer, H., Chimenes, A., 1949. Action de lacriflavine sur les levures .1. La Mutation Petite Colonie. Ann. Inst. Pasteur (Paris) 76, 351–367.

Epstein, C.B., Waddle, J.A., Hale, W., Davé, V., Thornton, J., Macatee, T.L., Garner, H.R., Butow, R.A., 2001. Genome-wide responses to mitochondrial dysfunction. Mol. Biol. Cell 12, 297–308.

Ernandes, J.R., Williams, J.W., Russell, I., Stewart, G.G., 1993. Respiratory deficiency in brewing yeast strains: effects on fermentation, flocculation, and beer flavor components. J. Am. Soc. Brew. Chem. USA.

Ernst, D.C., Downs, D.M., 2018. Mmf1p couples amino acid metabolism to mitochondrial DNA maintenance in *Saccharomyces cerevisiae*. mBio 9.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49 Pt 1, 329–337.

Feldmann, H. (Ed.), 2012a. Front Matter, in: Yeast. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp. I–XIX.

Foury, F., Roganti, T., Lecrenier, N., Purnelle, B., 1998. The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. FEBS Lett. 440, 325–331.

Fox, T.D., 2012. Mitochondrial protein synthesis, import, and assembly. Genetics 192, 1203–1234.

François, J., Parrou, J.L., 2001. Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 25, 125–145.

Fritsch, E.S., Chabbert, C.D., Klaus, B., Steinmetz, L.M., 2014. A genome-wide map of mitochondrial DNA recombination in yeast. Genetics 198, 755–771.

Gafni, A., Calderon, C.E., Harris, R., Buxdorf, K., Dafa-Berger, A., Zeilinger-Reichert, E., Levy, M., 2015. Biological control of the cucurbit powdery mildew pathogen *Podosphaera xanthii* by means of the epiphytic fungus *Pseudozyma aphidis* and parasitism as a mode of action. Front. Plant Sci. 6.

Gallone, B., Mertens, S., Gordon, J.L., Maere, S., Verstrepen, K.J., Steensels, J., 2018. Origins, evolution, domestication and diversity of *Saccharomyces* beer yeasts. Curr. Opin. Biotechnol., Food biotechnology • Plant biotechnology 49, 148–155.

Geissler, A., Krimmer, T., Bömer, U., Guiard, B., Rassow, J., Pfanner, N., 2000. Membrane potential-driven protein import into mitochondria. Mol. Biol. Cell 11, 3977–3991.

Generoso, W.C., Schadeweg, V., Oreb, M., Boles, E., 2015. Metabolic engineering of Saccharomyces cerevisiae for production of butanol isomers. Curr. Opin. Biotechnol., Environmental biotechnology • Energy biotechnology 33, 1–7.

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

Gibson, B.R., Lawrence, S.J., Leclaire, J.P.R., Powell, C.D., Smart, K.A., 2007. Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol. Rev. 31, 535–569.

Gibson, B. R., Prescott, K.A., Smart, K.A., 2008. Petite mutation in aged and oxidatively stressed ale and lager brewing yeast. Lett. Appl. Microbiol. 46, 636–642.

Goffeau, A., 1996. 1996: A vintage year for yeast and Yeast. Yeast 12, 1603–1606.

Gomez, M., Pérez-Gallardo, R.V., Sánchez, L.A., Díaz-Pérez, A.L., Cortés-Rojo, C., Carmen, V.M., Saavedra-Molina, A., Lara-Romero, J., Jiménez-Sandoval, S., Rodríguez, F., Rodríguez-Zavala, J.S., Campos-García, J., 2014. Malfunctioning of the iron–sulfur cluster assembly machinery in *Saccharomyces cerevisiae* produces oxidative stress via an iron-dependent mechanism, causing dysfunction in respiratory complexes. PLOS ONE 9, e111585.

Granchi, L., Bosco, M., Messini, A., Vincenzini, M., 1999. Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR–RFLP analysis of the rDNA ITS region. J. Appl. Microbiol. 87, 949–956.

Grant, C.M., MacIver, F.H., Dawes, I.W., 1997. Mitochondrial function is required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. FEBS Lett. 410, 219–222.

Gray, M.W., Burger, G., Lang, B.F., 2001. The origin and early evolution of mitochondria. Genome Biol. 2, reviews1018.1.

Guaragnella, N., Coyne, L.P., Chen, X.J., Giannattasio, S., 2018. Mitochondria–cytosol–nucleus crosstalk: learning from *Saccharomyces cerevisiae*. FEMS Yeast Res. 18.

Guaragnella, N., Palermo, V., Galli, A., Moro, L., Mazzoni, C., Giannattasio, S., 2014. The expanding role of yeast in cancer research and diagnosis: insights into the function of the oncosuppressors p53 and BRCA1/2. FEMS Yeast Res. 14, 2–16.

Guaragnella, N., Ždralević, M., Antonacci, L., Passarella, S., Marra, E., Giannattasio, S., 2012. The role of mitochondria in yeast programmed cell death. Front. Oncol. 2.

Guillamón, J.M., Sabaté, J., Barrio, E., Cano, J., Querol, A., 1998. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch. Microbiol. 169, 387–392.

Guillaume, C., Delobel, P., Sablayrolles, J.-M., Blondin, B., 2007. Molecular basis of fructose utilization by the wine yeast *Saccharomyces cerevisiae*: A mutated HXT3 allele enhances fructose fermentation. Appl Env. Microbiol 73, 2432–2439.

Gupta, M., Abu-Ghannam, N., Gallaghar, E., 2010. Barley for brewing: Characteristic changes during malting, brewing and applications of its by-Products. Compr. Rev. Food Sci. Food Saf. 9, 318–328.

Hatoum, R., Labrie, S., Fliss, I., 2012. Antimicrobial and probiotic properties of yeasts: From fundamental to novel applications. Front. Microbiol. 3.

Hazelwood, L.A., Daran, J.-M., van Maris, A.J.A., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* Metabolism. Appl. Environ. Microbiol. 74, 2259–2266.

Hebert, S.L., Lanza, I.R., Nair, K.S., 2010. Mitochondrial DNA alterations and reduced mitochondrial function in aging. Mech. Ageing Dev. 131, 451–462.

Hewitt, C.J., Nebe-Von-Caron, G., 2001. An industrial application of multiparameter flow cytometry: Assessment of cell physiological state and its application to the study of microbial fermentations. Cytometry 44, 179–187.

Hittinger, C.T., Steele, J.L., Ryder, D.S., 2018. Diverse yeasts for diverse fermented beverages and foods. Curr. Opin. Biotechnol. 49, 199–206.

Höhr, A.I.C., Straub, S.P., Warscheid, B., Becker, T., Wiedemann, N., 2015. Assembly of β-barrel proteins in the mitochondrial outer membrane. Biochim. Biophys. Acta 1853, 74–88.

Hori, A., Yoshida, M., Shibata, T., Ling, F., 2009. Reactive oxygen species regulate DNA copy number in isolated yeast mitochondria by triggering recombination-mediated replication. Nucleic Acids Res. 37, 749–761.

Horowitz, M.P., Greenamyre, J.T., 2010. Mitochondrial iron metabolism and its role in neurodegeneration. J. Alzheimers Dis. JAD 20, S551–S568.

Ibeas, J.I., Jimenez, J., 1997. Mitochondrial DNA loss caused by ethanol in *Saccharomyces* flor yeasts. APPL Env. MICROBIOL 63(1), 7–12.

Jacobson, J., Duchen, M.R., Heales, S.J.R., 2002. Intracellular distribution of the fluorescent dye nonyl acridine orange responds to the mitochondrial membrane potential: implications for assays of cardiolipin and mitochondrial mass. J. Neurochem. 82, 224–233.

Jenkins, Cheryl L., Lawrence, S.J., Kennedy, A.I., Thurston, P., Hodgson, J.A., Smart, K.A., 2009. Incidence and formation of petite mutants in lager brewing yeast *Saccharomyces Cerevisiae* (Syn. *S. Pastorianus*) Populations. J. Am. Soc. Brew. Chem. 67, 72–80.

Jornayvaz, F.R., Shulman, G.I., 2010a. Regulation of mitochondrial biogenesis. Essays Biochem. 47, 69–84.

Kaniak-Golik, A., Skoneczna, A., 2015. Mitochondria–nucleus network for genome stability. Free Radic. Biol. Med. 82, 73–104.

Kayikci, Ö., Nielsen, J., 2015. Glucose repression in *Saccharomyces cerevisiae*. FEMS Yeast Res. 15.

Kitagaki, H., Araki, Y., Funato, K., Shimoi, H., 2007. Ethanol-induced death in yeast exhibits features of apoptosis mediated by mitochondrial fission pathway. FEBS Lett. 581, 2935–2942.

Klionsky, D.J., Herman, P.K., Emr, S.D., 1990. The fungal vacuole: composition, function, and biogenesis. Microbiol. Rev. 54, 266–292.

Klis, F.M., Mol, P., Hellingwerf, K., Brul, S., 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 26, 239–256.

Knorre, D.A., Sokolov, S.S., Zyrina, A.N., Severin, F.F., 2016. How do yeast sense mitochondrial dysfunction? Microb. Cell 3, 532–539.

Kobayashi, G.S., 1996. Disease Mechanisms of fungi, in: Baron, S. (Ed.), Medical Microbiology. University of Texas Medical Branch at Galveston, Galveston (TX) Chapter 74.

Kole, A.J., Knight, E.R.W., Deshmukh, M., 2011. Activation of apoptosis by cytoplasmic microinjection of cytochrome c. J. Vis. Exp. JoVE.

Kolesnikov, A.A., Gerasimov, E.S., 2012. Diversity of mitochondrial genome organization. Biochem. Mosc. 77, 1424–1435.

Kováč, L., Varečka, L., 1981. Membrane potentials in respiring and respiration-deficient yeasts monitored by a fluorescent dye. Biochim. Biophys. Acta 637, 209–216.

Krogerus, K., Magalhães, F., Vidgren, V., Gibson, B., 2015. New lager yeast strains generated by interspecific hybridization. J. Ind. Microbiol. Biotechnol. 42, 769–778.

Lawrence, S.J., Wimalasena, T.T., Nicholls, S.M., Box, W.G., Boulton, C., Smart, K.A., 2012. Incidence and characterization of petites isolated from lager brewing yeast s*accharomyces cerevisiae* populations. J. Am. Soc. Brew. Chem. 70, 268–274.

Lawrence, S.J., Nicholls, S., Box, W.G., Sbuelz, R., Bealin-Kelly, F., Axcell, B., Smart, K.A., (2013). The relationship between yeast cell age, fermenter cone environment, and petite mutant formation in lager fermentations. J. Am. Soc. Brew. Chem.71:2, 90-96,

Legras, J.-L., Karst, F., 2003. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. FEMS Microbiol. Lett. 221, 249–255.

Lemasters, J.J., Nieminen, A.L., Qian, T., Trost, L.C., Elmore, S.P., Nishimura, Y., Crowe, R.A., Cascio, W.E., Bradham, C.A., Brenner, D.A., Herman, B., 1998. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta 1366, 177–196.

Lewis, S.C., Joers, P., Willcox, S., Griffith, J.D., Jacobs, H.T., Hyman, B.C., 2015. A rolling circle replication mechanism produces multimeric lariats of mitochondrial DNA in *Caenorhabditis elegans*. PLOS Genet 11, e1004985.

Lill, R., Hoffmann, B., Molik, S., Pierik, A.J., Rietzschel, N., Stehling, O., Uzarska, M.A., Webert, H., Wilbrecht, C., Mühlenhoff, U., 2012. The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism. Biochim. Biophys. Acta 1823, 1491–1508.

Ling, F., 2000. A role for MHR1, a gene required for mitochondrial genetic recombination, in the repair of damage spontaneously introduced in yeast mtDNA. Nucleic Acids Res. 28, 4956–4963.

Lipinski, K.A., Kaniak-Golik, A., Golik, P., 2010. Maintenance and expression of the *S. cerevisiae* mitochondrial genome—From genetics to evolution and systems biology. Biochim. Biophys. Acta BBA - Bioenerg., 16th European Bioenergetics Conference 2010 1797, 1086–1098.

Ljungman, M., Hanawalt, P.C., 1992. Efficient protection against oxidative DNA damage in chromatin. Mol. Carcinog. 5, 264–269.

Lockshon, D., 2002. A heritable structural alteration of the yeast mitochondrion. Genetics 161, 1425–1435.

Lodolo, E.J., Kock, J.L.F., Axcell, B.C., Brooks, M., 2008. The yeast *Saccharomyces cerevisiae*– the main character in beer brewing. FEMS Yeast Res. 8, 1018–1036.

MacAlpine, D.M., 2001. Replication and preferential inheritance of hypersuppressive petite mitochondrial DNA. EMBO J. 20, 1807–1817.

Maclean, M.J., Aamodt, R., Harris, N., Alseth, I., Seeberg, E., Bjør\a as, M., Piper, P.W., 2003. Base excision repair activities required for yeast to attain a full chronological life span. Aging Cell 2, 93–104.

Maleszka, R., Skelly, P.J., Clark-Walker, G.D., 1991. Rolling circle replication of DNA in yeast mitochondria. EMBO J. 10, 3923–3929.

Mallett, J.R., Stuart, M.S., Quain, D.E., 2018. Draught beer hygiene: a forcing test to assess quality. J. Inst. Brew. 124, 31–37.

Maresova, L., Urbankova, E., Gaskova, D., Sychrova, H., 2006. Measurements of plasma membrane potential changes in *Saccharomyces cerevisiae* cells reveal the importance of the Tok1 channel in membrane potential maintenance. FEMS Yeast Res. 6, 1039–1046.

Marques, W.L., Raghavendran, V., Stambuk, B.U., Gombert, A.K., 2016. Sucrose and *Saccharomyces cerevisiae*: a relationship most sweet. FEMS Yeast Res. 16.

Mashimo, K., Ohno, Y., 2006. Ethanol hyperpolarizes mitochondrial membrane potential and increases mitochondrial fraction in cultured mouse myocardial cells. Arch. Toxicol. 80, 421–428.

Mell, J.C., Burgess, S.M., 2003. Yeast as a Model Genetic Organism, in: John Wiley & Sons, Ltd (Ed.), Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester, UK.

Miceli, M.V., Jiang, J.C., Tiwari, A., Rodriguez-Quiñones, J.F., Jazwinski, S.M., 2012. Loss of mitochondrial membrane potential triggers the retrograde response extending yeast replicative lifespan. Front. Genet. 2, 102.

Mitra, K., Lippincott-Schwartz, J., 2010. Analysis of mitochondrial dynamics and functions using imaging approaches, in: Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J., Yamada, K.M. (Eds.), Current Protocols in Cell Biology. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Mitsuya, D., Hayashi, T., Wang, Y., Tanaka, M., Okai, M., Ishida, M., Urano, N., 2017. Isolation of aquatic yeasts with the ability to neutralize acidic media, from an extremely acidic river near Japan's Kusatsu-Shirane Volcano. J. Biosci. Bioeng. 124, 43–46.

Mohammadi, S., Saberidokht, B., Subramaniam, S., Grama, A., 2015. Scope and limitations of yeast as a model organism for studying human tissue-specific pathways. BMC Syst. Biol. 9.

Mustacchi, R., Hohmann, S., Nielsen, J., 2006. Yeast systems biology to unravel the network of life. Yeast Chichester Engl. 23, 227–238.

Nagley, P., Mattick, J.S., 1977. Mitochondrial DNA replication in petite mutants of yeast: resistance to inhibition by ethidium bromide, berenil and euflavine. Mol. Gen. Genet. MGG 152, 277–283.

Nakao, Y., Kanamori, T., Itoh, T., Kodama, Y., Rainieri, S., Nakamura, N., Shimonaga, T., Hattori, M., Ashikari, T., 2009. Genome sequence of the lager brewing yeast, an interspecies hybrid. DNA Res. Int. J. Rapid Publ. Rep. Genes Genomes 16, 115–129.

Nielsen, J., 2013. Production of biopharmaceutical proteins by yeast. Bioengineered 4, 207–211.

Nielsen, O., 2005. Control of the yeast propagation process--how to optimize oxygen supply and minimize stress. Tech. Q. MBAA Commun.

Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., Falcone, S., Valerio, A., Cantoni, O., Clementi, E., Moncada, S., Carruba, M.O., 2005. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310, 314–317.

Nordström, K., 1963. Formation of ethyl acetate in fermentation with brewer's yeast: IV. Metabolism of acetyl-coenzyme A. J. Inst. Brew. 69, 142–153.

Nunnari, J., Marshall, W.F., Straight, A., Murray, A., Sedat, J.W., Walter, P., 1997. Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. Mol. Biol. Cell 8, 1233–1242.

O'Connor-Cox, E.S., Lodolo, E.J., Axcell, B.C., 1995. The relative importance of mitochondrial protein synthesis to brewing yeast performance. J. Am. Soc. Brew. Chem. 53, 128–135.

Okamoto, K., Perlman, P.S., Butow, R.A., 1998. The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud. J. Cell Biol. 142, 613–623.

Okamoto, K., Shaw, J.M., 2005. Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu. Rev. Genet. 39, 503–536.

O'Rourke, B., Cortassa, S., Aon, M.A., 2005. Mitochondrial ion channels: gatekeepers of life and death. Physiol. Bethesda Md 20, 303–315.

O'Rourke, T.W., Doudican, N.A., Mackereth, M.D., Doetsch, P.W., Shadel, G.S., 2002. Mitochondrial dysfunction due to oxidative mitochondrial DNA damage is reduced through cooperative actions of diverse proteins. Mol. Cell. Biol. 22, 4086–4093.

Osman, C., Noriega, T.R., Okreglak, V., Fung, J.C., Walter, P., 2015. Integrity of the yeast mitochondrial genome, but not its distribution and inheritance, relies on mitochondrial fission and fusion. Proc. Natl. Acad. Sci. 112, E947–E956.

Otsuga, D., Keegan, B.R., Brisch, E., Thatcher, J.W., Hermann, G.J., Bleazard, W., Shaw, J.M., 1998. The dynamin-related GTPase,

Dnm1p, controls mitochondrial morphology in Yeast. J. Cell Biol. 143, 333–349.

Paradies, G., Paradies, V., De Benedictis, V., Ruggiero, F.M., Petrosillo, G., 2014. Functional role of cardiolipin in mitochondrial bioenergetics. Biochim. Biophys. Acta 1837, 408–417.

Parone, P.A., Da Cruz, S., Tondera, D., Mattenberger, Y., James, D.I., Maechler, P., Barja, F., Martinou, J.-C., 2008. Preventing mitochondrial fission impairs mitochondrial function and leads to loss of mitochondrial DNA. PLoS ONE 3.

Paschen, S.A., Neupert, W., Rapaport, D., 2005. Biogenesis of β-barrel membrane proteins of mitochondria. Trends Biochem. Sci. 30, 575–582.

Pérez-Gallardo, R.V., Briones, L.S., Díaz-Pérez, A.L., Gutiérrez, S., Rodríguez-Zavala, J.S., Campos-García, J., 2013. Reactive oxygen species production induced by ethanol in *Saccharomyces cerevisiae* increases because of a dysfunctional mitochondrial iron–sulfur cluster assembly system. FEMS Yeast Res. 13, 804–819.

Pham, T., Wimalasena, T., Box, W.G., Koivuranta, K., Storgårds, E., Smart, K.A., Gibson, B.R., 2011. Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery ‘wild’ yeast contaminants. J. Inst. Brew. 117, 556–568.

Picard, M., Shirihai, O.S., Gentil, B.J., Burelle, Y., 2013. Mitochondrial morphology transitions and functions: implications for retrograde signaling? Am. J. Physiol. - Regul. Integr. Comp. Physiol. 304, R393–R406.

Pierce, J.S., 1970. Institute of Brewing: Analysis Committee Measurement of Yeast Viability. J. Inst. Brew. 76, 442–443.

Pincus, D.H., Orenga, S., Chatellier, S., 2007. Yeast identification – past, present, and future methods. Med. Mycol. 45, 97–121.

Pires, E., Brányik, T., 2015. Biochemistry of Beer Fermentation. Springer.

Pires, E.J., Teixeira, J.A., Brányik, T., 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. Appl. Microbiol. Biotechnol. 98, 1937–1949.

Piskur, J., 1997. The transmission disadvantage of yeast mitochondrial intergenic mutants is eliminated in the mgt1 (cce1) background. J. Bacteriol. 179, 5614–5617.

Piskur, J., Smole, S., Groth, C., Petersen, R.F., Pedersen, M.B., 1998. Structure and genetic stability of mitochondrial genomes vary among yeasts of the genus *Saccharomyces*. Int. J. Syst. Bacteriol. 48, 1015–1024.

Pohl, C.H., Kock, J.L.F., van Wyk, P.W.J., Albertyn, J., 2006. Cryptococcus anemochoreius sp. nov., a novel anamorphic basidiomycetous yeast isolated from the atmosphere in central South Africa. Int. J. Syst. Evol. Microbiol. 56, 2703–2706.

Powell, C.D., Diacetis, A.N., 2007. Long term serial repitching and the genetic and phenotypic stability of brewer's yeast. J. Inst. Brew. 113, 67–74.

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

Poyton, R.O., Dagsgaard, C.J., 2002. Mitochondrial-nuclear crosstalk is involved in oxygen-regulated gene expression in yeast, in: Lahiri, S., Prabhakar, N.R., Forster, R.E. (Eds.), Oxygen sensing: Molecule to man, Advances in Experimental Medicine and Biology. Springer US, Boston, MA, pp. 177–184.

Poyton, R.O., McEwen, J.E., 1996. Crosstalk between nuclear and mitochondrial genomes. Annu. Rev. Biochem. 65, 563–607.

Prakash, A., Doublié, S., 2015. Base excision repair in the mitochondria. J. Cell. Biochem. 116, 1490–1499.

Rainieri, S., Kodama, Y., Nakao, Y., Pulvirenti, A., Giudici, P., 2008. The inheritance of mtDNA in lager brewing strains: Lager brewing strains mtDNA. FEMS Yeast Res. 8, 586–596.

Rautio, J., Londesborough, J., 2003. Maltose transport by brewer's yeasts in brewer's wort. J. Inst. Brew. 109, 251–261.

Reed, G., Nagodawithana, T.W., 1990. Use of yeasts in the dairy industry, in: Reed, G., Nagodawithana, T.W. (Eds.), Yeast Technology. Springer Netherlands, Dordrecht, pp. 441–445.

Rosenfeld, E., Schaeffer, J., Beauvoit, B., Salmon, J.-M., 2004. Isolation and properties of promitochondria from anaerobic stationary-phase yeast cells. Antonie Van Leeuwenhoek 85, 9–21.

Saerens, S.M.G., Duong, C.T., Nevoigt, E., 2010. Genetic improvement of brewer's yeast: current state, perspectives and limits. Appl. Microbiol. Biotechnol. 86, 1195–1212.

Samp, E.J., Foster, R.T., Edelen, C., 2010. Influence of cardiolipin on lager beer dimethyl sulfide levels: a possible role involving mitochondria? J. Am. Soc. Brew. Chem. 68, 204–209.

Santos, J.H., Hunakova, L., Chen, Y., Bortner, C., Houten, B.V., 2003. Cell sorting experiments link persistent mitochondrial DNA damage with loss of mitochondrial membrane potential and Apoptotic Cell Death. J. Biol. Chem. 278, 1728–1734.

Sawada, K., Kitagaki, H., 2016. Residual mitochondrial transmembrane potential decreases unsaturated fatty acid level in sake yeast during alcoholic fermentation. PeerJ 4, e1552.

Scarpulla, R.C., 2012. Nucleus-encoded regulators of mitochondrial function: integration of respiratory chain expression, nutrient sensing and metabolic stress. Biochim. Biophys. Acta 1819, 1088–1097.

Schuller, D., Valero, E., Dequin, S., Casal, M., 2004. Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiol. Lett. 231, 19–26.

Sesaki, H., Southard, S.M., Yaffe, M.P., Jensen, R.E., 2003. Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial Outer Membrane. Mol. Biol. Cell 14, 2342–2356.

Shadel, G.S., 1999. Yeast as a Model for Human mtDNA Replication. Am. J. Hum. Genet. 65, 1230.

Shaw, J.M., Nunnari, J., 2002. Mitochondrial dynamics and division in budding yeast. Trends Cell Biol. 12, 178–184.

Shepard, K.A., Yaffe, M.P., 1999. The yeast dynamin-like protein, Mgm1p, functions on the mitochondrial outer membrane to mediate mitochondrial inheritance. J. Cell Biol. 144, 711–720.

Shimotsu, S., Asano, S., Iijima, K., Suzuki, K., Yamagishi, H., Aizawa, M., 2015. Investigation of beer-spoilage ability of *Dekkera*/*Brettanomyces* yeasts and development of multiplex PCR method for beer-spoilage yeasts. J. Inst. Brew. 121, 177–180.

Shiota, T., Traven, A., Lithgow, T., 2015. Mitochondrial biogenesis: Cell-cycle-dependent investment in making mitochondria. Curr. Biol. 25, R78–R80.

Sibirny, A.A., 2016. Yeast peroxisomes: structure, functions and biotechnological opportunities. FEMS Yeast Res. 16.

Simon, V.R., Karmon, S.L., Pon, L.A., 1997. Mitochondrial inheritance: cell cycle and actin cable dependence of polarized mitochondrial movements in *Saccharomyces cerevisiae*. Cell Motil. Cytoskeleton 37, 199–210.

Singh, K.K., Kulawiec, M., Still, I., Desouki, M.M., Geradts, J., Matsui, S.-I., 2005. Inter-genomic cross talk between mitochondria and the nucleus plays an important role in tumorigenesis. Gene 354, 140–146.

Smart, K., 2008. Brewing Yeast Fermentation Performance. John Wiley & Sons.

Smart, K.A., Whisker, S., 1996. Effect of serial repitching on the fermentation properties and condition of brewing yeast. J. Am. Soc. Brew. Chem. 54, 41–44.

Solieri, L., 2010. Mitochondrial inheritance in budding yeasts: towards an integrated understanding. Trends Microbiol. 18, 521–530.

Špírek, M., Poláková, S., Jatzová, K., Sulo, P., 2015. Post-zygotic sterility and cytonuclear compatibility limits in *S. cerevisiae* xenomitochondrial cybrids. Front. Genet. 5.

Stuart, J.A., 2005. Localization of mitochondrial DNA base excision repair to an inner membrane-associated particulate fraction. Nucleic Acids Res. 33, 3722–3732.

Suski, J.M., Lebiedzinska, M., Bonora, M., Pinton, P., Duszynski, J., Wieckowski, M.R., 2012. Relation between mitochondrial membrane potential and ROS formation, in: mitochondrial Bioenergetics, Methods in Molecular Biology. Humana Press, pp. 183–205.

Taddei, A., Gasser, S.M., 2012. Structure and Function in the Budding Yeast Nucleus. Genetics 192, 107–129.

Takeda, K., Tasai, M., Iwamoto, M., Onishi, A., Tagami, T., Nirasawa, K., Hanada, H., Pinkert, C.A., 2005. Microinjection of cytoplasm or mitochondria derived from somatic cells affects parthenogenetic development of murine oocytes. Biol. Reprod. 72, 1397–1404.

Takpho, N., Watanabe, D., Takagi, H., 2018. Valine biosynthesis in *Saccharomyces cerevisiae* is regulated by the mitochondrial branched-chain amino acid aminotransferase Bat1. Microb. Cell 5, 293–299.

Tamura, Y., Onguka, O., Itoh, K., Endo, T., Iijima, M., Claypool, S.M., Sesaki, H., 2012. Phosphatidylethanolamine biosynthesis in mitochondria. J. Biol. Chem. 287, 43961–43971.

Torriani, S., Zapparoli, G., Suzzi, G., 1999. Genetic and phenotypic diversity of *Saccharomyces sensu stricto* strains isolated from Amarone wine. Diversity of *Saccharomyces* strains from Amarone wine. Antonie Van Leeuwenhoek 75, 207–215.

Trevisol, E.T.V., Panek, A.D., Mannarino, S.C., Eleutherio, E.C.A., 2011. The effect of trehalose on the fermentation performance of aged cells of *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 90, 697–704.

Trumbly, R.J., 1992. Glucose repression in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. 6, 15–21.

Tubb, R.S., Liljestrom, P.L., 1986. A colony-colour method which differentiates alpha-galactosidase-positive strains of yeast. J. Inst. Brew. 92(6), 588–590.

Turk, E.M., Das, V., Seibert, R.D., Andrulis, E.D., 2013. The Mitochondrial RNA Landscape of *Saccharomyces cerevisiae*. PLoS ONE 8, e78105.

Tzagoloff, A., Dieckmann, C.L., 1990. PET genes of *Saccharomyces cerevisiae*. Microbiol. Rev. 54, 211–225.

Van Houten, B., Hunter, S.E., Meyer, J.N., 2016. Mitochondrial DNA damage induced autophagy, cell death, and disease. Front. Biosci. Landmark Ed. 21, 42–54.

Van Leeuwen, C.C., Weusthuis, R.A., Postma, E., Van den Broek, P.J., Van Dijken, J.P., 1992. Maltose/proton co-transport in *Saccharomyces cerevisiae*. Comparative study with cells and plasma membrane vesicles. Biochem. J. 284, 441–445.

Verbelen, P.J., Depraetere, S.A., Winderickx, J., Delvaux, F.R., Delvaux, F., 2009. The influence of yeast oxygenation prior to brewery fermentation on yeast metabolism and the oxidative stress response. FEMS Yeast Res. 9, 226–239.

Verma, A.S., Agrahari, S., Rastogi, S., Singh, A., 2011. Biotechnology in the realm of history. J. Pharm. Bioallied Sci. 3, 321–323.

Verstrepen, Kevin J., Derdelinckx, G., Dufour, J.-P., Winderickx, J., Thevelein, J.M., Pretorius, I.S., Delvaux, F.R., 2003. Flavor-active esters: adding fruitiness to beer. J. Biosci. Bioeng. 96, 110–118.

Verstrepen, K. J., Derdelinckx, G., Verachtert, H., Delvaux, F.R., 2003. Yeast flocculation: what brewers should know. Appl. Microbiol. Biotechnol. 61, 197–205.

Vevea, J.D., Swayne, T.C., Boldogh, I.R., Pon, L.A., 2014. Inheritance of the fittest mitochondria in yeast. Trends Cell Biol. 24, 53–60.

Vidgren, V., Multanen, J.-P., Ruohonen, L., Londesborough, J., 2010. The temperature dependence of maltose transport in ale and lager strains of brewer's yeast. FEMS Yeast Res. 10, 402–411.

Vieira, É.D., da Graça Stupiello Andrietta, M., Andrietta, S.R., 2013. Yeast biomass production: a new approach in glucose-limited feeding strategy. Braz. J. Microbiol. 44, 551–558.

Visser, W., van Spronsen, E.A., Nanninga, N., Pronk, J.T., Gijs Kuenen, J., van Dijken, J.P., 1995. Effects of growth conditions on mitochondrial morphology in *Saccharomyces cerevisiae*. Antonie Van Leeuwenhoek 67, 243–253.

Voordeckers, K., Kominek, J., Das, A., Espinosa-Cantú, A., Maeyer, D.D., Arslan, A., Pee, M.V., Zande, E. van der, Meert, W., Yang, Y., Zhu, B., Marchal, K., DeLuna, A., Noort, V.V., Jelier, R., Verstrepen, K.J., 2015. Adaptation to high ethanol reveals complex evolutionary pathways. PLOS Genet. 11, e1005635.

Wallis, O.C., Whittaker, P.A., 1974. Induction of petite mutation in yeast by starvation in glycerol. J. Gen. Microbiol. 84, 11–18.

Walther, A., Hesselbart, A., Wendland, J., 2014. Genome sequence of Saccharomyces carlsbergensis, the world's first pure culture lager yeast. G3 GenesGenomesGenetics 4, 783–793.

Wang, X., Mann, C.J., Bai, Y., Ni, L., Weiner, H., 1998. Molecular cloning, characterization, and potential roles of cytosolic and mitochondrial

aldehyde dehydrogenases in ethanol metabolism in Saccharomyces cerevisiae. J. Bacteriol. 180, 822–830.

Westermann, B., 2014. Mitochondrial inheritance in yeast. Biochim. Biophys. Acta BBA - Bioenerg., 18th European Bioenergetics Conference 2014 Lisbon, Portugal 1837, 1039–1046.

Westermann, B., 2012. Bioenergetic role of mitochondrial fusion and fission. Biochim. Biophys. Acta BBA - Bioenerg., 17th European Bioenergetics Conference 1817, 1833–1838.

White, Bruns, T., Lee, S., Taylor, J., 1990. White, T. J., T. D. Bruns, S. B. Lee, and J. W. Taylor. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. pp. 315–322.

Wightman, P., Quain, D.E., Meaden, P.G., 1996. Analysis of production brewing strains of yeast by DNA fingerprinting. Lett. Appl. Microbiol. 22, 90–94.

Williamson, D.H., Fennell, D.J., 1979. [62] Visualization of yeast mitochondrial dna with the fluorescent stain “DAPI,” in: Methods in Enzymology, Biomembranes Part G: Bioenergetics: Biogenesis of Mitochondria, Organization, and Transport. Academic Press, pp. 728–733.

Wisnovsky, S., Jean, S.R., Kelley, S.O., 2016. Mitochondrial DNA repair and replication proteins revealed by targeted chemical probes. Nat. Chem. Biol. 12, 567–573.

Wolters, J.F., Chiu, K., Fiumera, H.L., 2015. Population structure of mitochondrial genomes in *Saccharomyces cerevisiae*. BMC Genomics 16, 451.

Woo, D.K., Poyton, R.O., 2009. The absence of a mitochondrial genome in rho0 yeast cells extends lifespan independently of retrograde regulation. Exp. Gerontol. 44, 390–397.

Xufre, A., Albergaria, H., Gírio, F., Spencer-Martins, I., 2011. Use of interdelta polymorphisms of *Saccharomyces cerevisiae strains* to monitor population evolution during wine fermentation. J. Ind. Microbiol. Biotechnol. 38, 127–132.

Yoon, Y.G., Koob, M.D., Yoo, Y.H., 2010. Re-engineering the mitochondrial genomes in mammalian cells. Anat. Cell Biol. 43, 97–109.

Youle, R.J., Bliek, A.M. van der, 2012. Mitochondrial fission, fusion, and Stress. Science 337, 1062.

Zelenaya-Troitskaya, O., Perlman, P.S., Butow, R.A., 1995. An enzyme in yeast mitochondria that catalyzes a step in branched-chain amino acid biosynthesis also functions in mitochondrial DNA stability. EMBO J. 14, 3268–3276.

Zhou, Z.-X., Zhang, M.-J., Peng, X., Takayama, Y., Xu, X.-Y., Huang, L.-Z., Du, L.-L., 2013. Mapping genomic hotspots of DNA damage by a single-strand-DNA-compatible and strand-specific ChIP-seq method. Genome Res. 23, 705–715.

Ziaja, K., Michaelis, G., Lisowsky, T., 1993. Nuclear control of the messenger RNA expression for mitochondrial ATPase subunit 9 in a new yeast mutant. J. Mol. Biol. 229, 909–916.

Zinovkina, L.A., 2018. Mechanisms of Mitochondrial DNA Repair in Mammals. Biochem. Biokhimiia 83, 233–249.

Zorov, D.B., Juhaszova, M., Sollott, S.J., 2014. Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. Physiol. Rev. 94, 909–950.

Zorova, L.D., Popkov, V.A., Plotnikov, E.Y., Silachev, D.N., Pevzner, I.B., Jankauskas, S.S., Babenko, V.A., Zorov, S.D., Balakireva, A.V., Juhaszova, M., Sollott, S.J., Zorov, D.B., 2018. Mitochondrial membrane potential. Anal. Biochem., Mitochondrial Biochemistry and Bioenergenetics 552, 50–59.

Zuo, X., Xue, D., Li, N., Clark-Walker, G.D., 2007. A functional core of the mitochondrial genome maintenance protein Mgm101p in Saccharomyces cerevisiae determined with a temperature-conditional allele. FEMS Yeast Res. 7, 131–140.

Zweifel, S.G., Fangman, W., 1991. A nuclear mutation reversing a biased transmission of yeast mitochondrial DNA 9.