

**THE IMPACT OF  
DEHYDRATION AND  
REHYDRATION ON BREWING  
YEAST**

**David Martyn Jenkins**

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## **ABSTRACT**

In the brewing industry it is standard practice to propagate a pure yeast culture and inoculate (pitch) it into the fermentation vessel. Once fermentation is complete, yeast is recovered and reused in subsequent fermentations (known as serial repitching) until a decline in performance occurs or the required number of successive fermentations has been conducted. Propagation is currently required to initiate the entire process again, which requires additional equipment, energy, water inputs and time. It has long been proposed that Active Dried Yeast (ADY) offers an alternative method of yeast supply. Adoption of this innovation by the brewing industry has been low because of perceived issues with the fermentation performance of ADY, the availability of strains and hygiene concerns.

In the current study the fermentation performance of ADY has been assessed with respect to viability, genomic stability, membrane integrity, yeast growth, attenuation, uptake of wort nutrients and aspects of flavour development. ADY requires rehydration before use and it has been demonstrated that viability is impaired in these slurries, though the extent of viability loss was dependent on strain and rehydration conditions. The source of cell death is unclear. Mitochondrial and genomic DNA integrity was assessed using a variety of techniques and shown to be unaffected by dehydration and rehydration. In contrast membrane integrity was affected. Changes in membrane fluidity, sterol content and fitness to perform could be detected in ADY. Performance of ADY in fermentation was also impaired. A lag in cell growth, attenuation and sugar and amino acid uptake were noted. Diacetyl formation occurred more rapidly and end fermentation diacetyl levels were higher for ADY. These differences were not maintained during serial repitching. It is proposed that ADY could be utilised to replace freshly propagated yeast, but direct addition to fermenters would require an improvement of performance during the first fermentation.

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## ABBREVIATIONS

%	Percentage
°C	Degrees centigrade
µg	microgram
µl	microlitre
µm	micrometer
ADY	Active Dried yeast
bp	Base pairs
cm	centimetre
Ct	Cycle threshold
dNTPs	Deoxynucleotide triphosphates
g	gram
hr	hour
Kg	kilogram
kPa	kilopascal
l	litre
LOG <sub>10</sub>	Logarithm to the base 10
m	meter
M	Molar concentration
m/v	Mass concentration
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	Millimolar
nm	nanometer
nM	Nanomolar
RCF	Relative centrifugal force
rpm	Revolutions per minute
s.g.	Specific gravity
sec	second
v/v	Volume concentration
YPD	Yeast extract, peptone and D-glucose medium

## **CHAPTER 1: INTRODUCTION**

The production of beer has been prevalent for over 8,000 years (Bamforth, 1998) and it forms an important part of the culture in many parts of the World. The beer market is a considerable one, which has led to the development of large brewing companies shifting production away from smaller regional producers. Work at these large breweries in standardising and optimising the brewing process has increased our understanding of the science underpinning brewing, which, in turn, has influenced several other aspects of science. Despite the library of work that has previously been reported, there is still much that is not known about the brewing process. This thesis will begin to address the intricacies of one relatively new technology in brewing, Active Dried Yeast (ADY), commonly referred to as simply dried yeast. To understand where ADY fits into the brewing process a brief introduction to the process is outlined here, with particular emphasis on the role of yeast. The brewing process, however, is a complex one and has been widely reviewed (Briggs *et al.*, 1981; Boulton and Quain, 2001; Briggs *et al.*, 2004; Bamforth, 2006), which the reader should consult for a more in-depth description of the process.

### **1.1 THE BREWING PROCESS**

Beer consists of four main ingredients, water, barley (other grains can be used including, but not limited to, rice, wheat, rye and oats), hops and yeast, to which additional adjuncts may be added if desired. These four ingredients undergo several stages of development to produce the final beer, which can be separated into malting (Section 1.1.1), wort production (Section 1.1.2), fermentation (Section 1.1.3), maturation (Section 1.1.4) and packaging (Section 1.1.5).

#### **1.1.1 Malting**

Barley undergoes significant processing before it is ultimately transformed into beer. The first step in this processing is the liberation of sugars from the starch based stores found in

the seed. Malting is the term given to this process, during which the maltster initiates, and subsequently halts, germination in the barley. Malting commences with a process termed steeping, during which the barley is hydrated to a water content which is often between 42-48 % (Briggs, 1998). This process initiates germination of the grains, leading to the preliminary stages of the development of a barley embryo. During its development enzymes are produced which first digest the walls of aleurone cells, the endosperm and proteins, resulting in a much softer grain which is more easily milled (Greffeuille *et al.*, 2006). Amylases integral for the hydrolysis of starch in the mashing process (Section 1.1.2), are also generated at this time. Whilst germination is a requirement of malting, the process must be stopped to prevent the conversion of the liberated sugars into a substantial shoot and rootlets (Cole *et al.*, 1998). Cessation of germination is brought about by heating and drying the grains with the use of hot air, a process termed kilning. The longer and hotter this process the darker the malt, but also the fewer enzymes which remain functioning for the subsequent mashing (Briggs, 1998). Variations in the kilning regime can produce a myriad of malts (Inns *et al.*, 2007), providing the potential for a vast array of types of beer. The type, or indeed types, of malt used in a beer have a determining effect on the final flavour and appearance of the beer. These malts, however, must first be processed to extract sugars, proteins and other compounds into a liquid form, a process which produces a sweet fermentable substrate known as wort (Section 1.1.2).

### **1.1.2 Wort production**

The sugars, nitrogen, vitamins and other nutrients which will later be utilised during fermentation (Section 1.1.3) are enclosed within the barley grain structure of the malted barley (malt) and must be freed before they can be utilised to produce beer. To achieve this, the malt is milled to produce smaller particles, termed grist. This grist is then combined with water, referred to amongst brewers as liquor, to create a mixture called mash. The mash is held at a set temperature, often 65°C, which promotes the dissolving of

sugars and encourages enzymatic conversion of residual starches into soluble carbohydrate. Temperature optimisation of this process is important as increases may result in time savings, however, reduced enzyme activity may result in less fermentable wort (Muller, 1991). The liquid containing the dissolved substances from the malt is termed wort and is collected, often strained through spent grain (un-dissolved component of the malt) to maximise the uptake of soluble substances (Briggs *et al.*, 2004).

Wort is transferred to a vessel called a kettle or copper, the latter name being a reference to the material which the vessel was traditionally made from. It is at this point when the wort is boiled. Until this point wort production is vulnerable to infection from a number of microorganisms, and boiling is an important sterilisation step which kills any contaminants. This is the last sterilisation step prior to packaging, thus hygiene is of critical importance from this point onwards. Boiling also adjusts the overall composition of the wort, notably by precipitating proteins, which subsequently form trub (protein agglomerates). The flavour of the final beer is developed with the removal of unwanted aromatic volatile compounds and the addition of hops. Hops, the third of the four essential ingredients to be added, have an important impact on the final flavour and aroma of the beer. The effect that they have is altered depending on when the hops are added during the boil, or indeed if they are added after, which is a process referred to as *dry hopping* (Briggs *et al.*, 2004; Bamforth, 2006). Hops are also thought to display antimicrobial activity (Sakamoto and Konings, 2003) as well as playing a role in the stabilisation of foam (Kunimune and Shellhammer, 2008). Adjuncts, ingredients in addition to the four major components listed earlier, such as caramels or syrups, may be added, and are dissolved during the boil. The trub and hops are removed either by filtration or in a whirlpool tank and the wort is cooled in preparation for fermentation (Briggs *et al.*, 2004; Bamforth, 2006).

### **1.1.3 Fermentation**

The key player in fermentation is yeast which, as it is the focus of this thesis, is discussed in greater detail in Section 0. Fermentation begins when yeast is inoculated or “pitched” into the wort. This is done in fermentation vessels which were traditionally varied in design, but are currently typically cylindroconical in shape (Boulton and Quain, 2001). The temperature in these vessels is maintained at a specified value depending on the yeast being used. Fermentations performed using ale strains typically occur between 15-20°C, whilst those performed using lager yeast most commonly take place at between 7-15°C and, as a consequence, normally take longer to complete (Briggs *et al.*, 2004). Oxygen is only provided while the vessels are initially filled with wort; and is important for ergosterol production (Jahnke and Klein, 1983), a requirement for yeast division (Rodriguez and Parks, 1983), in addition to other sterols and unsaturated fatty acids which are required for cell membrane structure (Parks and Casey, 1995). In the first few days of fermentation the yeast population undergoes growth whilst metabolising the wort. Key to this is the uptake of sugars and nitrogenous substances, and the production of ethanol. Ethanol is produced via the fermentative pathway as the aerobic respiratory pathway, which yields a greater amount of energy, is repressed during fermentation. Initially this repression is due to the Crabtree effect, whereby the high concentration of glucose represses aerobic respiration (Crabtree, 1929; Swanson and Clifton, 1948; De Deken, 1966). Traditionally there was very little microbiological control over the inoculation of fermentations and ethanol production is thought to have reduced competition to the yeast from other microorganisms (Verstrepen *et al.*, 2004). As oxygen and cellular levels of sterols are diminished, yeast division is halted, although growth may still occur, before yeast cells are separated from the medium by a naturally occurring phenomena termed flocculation. Yeast cells aggregate in flocs which sink to the bottom or float to the top of the medium, depending on the yeast type. This flocculation must occur in a timely manner. Premature flocculation leads to

undesired fermentation characteristics such as residual sugar content and lowered ethanol concentration (van Nierop *et al.*, 2006), whilst delayed or weak flocculation may lead to problems with yeast harvesting and filtration (van der Aar, 1995). Once flocculated, the majority of the yeast is removed or “cropped” from the vessel and can be recycled in subsequent fermentations. Fermentation is the focus of Chapters 6 and 7 and is therefore discussed in greater detail later.

#### **1.1.4 Maturation**

Once the initial/primary fermentation is complete the product is termed ‘green beer’ and normally undergoes a stand or maturation period. Diacetyl, a compound which is formed during the fermentation, can adversely affect the flavour; a stand is required to allow levels of this compound to lower due to the actions of remaining viable yeast cells. Many methods have been developed in an effort to decrease the time this process takes, for example the addition of the enzyme  $\alpha$ -acetolactate decarboxylase (Hannemann, 2002). Green beer can undergo other forms of maturation. This can be simply a period of ageing or it can be a process step whereby the beer undergoes a secondary fermentation. Regardless of the method, this maturation plays an important role in developing the flavour and appearance of the beer (Boulton and Quain, 2001; Briggs *et al.*, 2004). Once maturation is complete the beer is often pasteurised or filtered. These steps are performed to increase the stability of the final beer, removing residual yeast and contaminant microorganisms. Carbon-dioxide levels are adjusted before the beer is packaged.

#### **1.1.5 Packaging**

Once production is complete, the beer must be transferred from the large capacity storage tanks to the consumer in a way that will maximise their enjoyment of the product. The form in which it does this depends on the type of beer, and perhaps more importantly where the point of sale is. Sales in public houses or bars (termed on-trade sales) tend to

favour the use of kegs or casks and the dispense of beer on draught. These are not suitable for off-trade sales (such as supermarkets) where the consumer purchases beer to be taken away and consumed at a later date. These types of sales favour smaller containers such as aluminium cans or glass bottles. Events where there are large numbers of people often makes the use of glass bottles unsafe, but the speed of dispense of this style of beer is still appealing. For these situations plastic bottles made from polyethylene terephthalate (PET) are becoming increasingly popular. This type of packaging does suffer from oxygen ingress (Muller, 2007), which may be one reason why consumer acceptance is still relatively low (Folz and Hofmann, 2010). Although the majority of beer sold is pasteurised or sterile filtered, certain beer styles require the inoculation of yeast into the final packaging to conduct what is termed secondary fermentation or bottle conditioning (Boulton and Quain, 2001).

## 1.2 YEAST

As already alluded to, many people consider yeast to be the key factor in the production of beer. It is also the focus of this thesis and therefore deserves a thorough review. Before considering its role in brewing, its position in the wider scientific community will be addressed.

The yeast *Saccharomyces cerevisiae* has been, and indeed still is, the focus of intense scientific research in part due to its status as a model organism (Drubin, 1989; Botstein *et al.*, 1997; Zeyl, 2000; Oliver, 2001; Game, 2002; De Freitas *et al.*, 2003; Lagali *et al.*, 2003; Morgan *et al.*, 2009). It is renowned as an organism that is easily grown and manipulated within the laboratory environment. Being a eukaryote it provides a model system for other eukaryotic cells which are more problematic to work with, such as the human cell. This has led to *S. cerevisiae* being at the forefront of scientific innovation, most notably when it was the first eukaryotic organism for which the entire genome was sequenced (Goffeau *et al.*,

1996). Research is also stimulated by the reliance of industries, such as baking, wine, brewing and, more recently, bioethanol, on *S. cerevisiae* and other yeasts. This has led to a huge body of research covering all aspects of the organism and its life cycle.

In the laboratory the most commonly worked with yeast is *S. cerevisiae*, the strain S288C perhaps being the most well known. Laboratory strains of *S. cerevisiae* are typically haploid or diploid, referring to a single or double compliment of DNA respectively. The haploid compliment of DNA consists of 16 chromosomes ranging from 230 Kb (chromosome I) to 1,532 Kb (chromosome IV) (Goffeau *et al.*, 1996). Haploids can be one of two mating types named  $\alpha$  and  $\alpha$ . When two haploids of opposite mating types are in close proximity they cause each other to arrest in the G1 phase of the cell cycle. Each grows a protuberance towards the mating partner forming a characteristic “schmoo”, eventually resulting in a diploid cell ( $\alpha/\alpha$ ) (Dickinson, 2004). Diploid cells can undergo mitosis and proliferate, as do the haploid cells. Although haploid cells are only capable of mitosis and vegetative growth, in the presence of a poorly-utilized carbon source such as acetate, and usually in the absence of a nitrogen source, diploid strains will switch to meiosis which leads to the formation of spores. The result of sporulation is an ascus containing four ascospores, which if returned to amenable nutrient conditions will generate new cells and commence proliferation as haploids, each with a mixture of genetic material from the original haploid cells. When haploid cells have a deficiency in nutrients, normal cell cycling is stopped and the cell becomes arrested in stationary phase. Although non-dividing in stationary phase, the yeasts remain viable and will resume proliferation once returned to complete glucose medium (Rose and Harrison, 1989). This life cycle has been well characterised and contributes to the factors which make the organism so favoured in scientific study.

### 1.3 YEAST IN BREWING

The focus of this thesis, however, is brewing yeast, which differs from laboratory yeast (Section 1.2) in several ways. Unlike haploid or diploid yeast strains, brewing yeast are typically defined as polyploid. This is due to chromosomes being present in differing numbers, producing a ploidy which cannot be described as one or two times normal compliment. Indeed, yeast may contain multiple copies of a single chromosome which may not necessarily contain identical sequences or even be the same size. Meiosis and subsequent sporulation are uncommon, as is mating between cells. Theoretically this should lead to increased genetic stability of a population, as asexual budding accounts for cell multiplication within a culture. Genetic variation has, however, been observed in the form of changes in chromosome length (Sato *et al.*, 1994), as well as altered cellular function (Bell *et al.*, 1997; Sato *et al.*, 2001).

All brewing yeasts are not the same, with two main types utilised in brewing. These are commonly referred to as lager and ale yeast named after the type of beer they are used to produce. They can also be referred to as top (ale) or bottom (lager) fermenting yeast depending on where in the fermenter the yeast types traditionally aggregated after flocculation (Boulton and Quain, 2001; Verstrepen *et al.*, 2003). Ale yeasts typically belong to the species *S. cerevisiae* whilst lager yeasts are designated *S. pastorianus*. The genetic background of lager yeast is perhaps the more complex of the two, as they were derived from a hybridisation event(s) between *S. cerevisiae* and *S. bayanus* strains (Tamai *et al.*, 1998; Rainieri *et al.*, 2006; Dunn and Sherlock, 2008; Nakao *et al.*, 2009) or a similar strain called *S. eubayanus* (Libkind *et al.*, 2011). Interestingly the mitochondrial DNA of lager yeast originates solely from *S. bayanus*, thought to be driven by the use of these yeasts at low temperatures (Rainieri *et al.*, 2008). Indeed the optimal growth temperatures for lager yeast is lower than that of ale yeast) (Guidici *et al.*, 1998) and is the basis for one of the classical methods used to differentiate the two, with lager strains unable to grow at 34°C.

However, with the advent of molecular techniques, such as PCR analysis (Legras and Karst, 2003), karyotyping (Casey, 1996) and restriction fragment length polymorphisms (Schofield *et al.*, 1995; Wightman *et al.*, 1996), traditional methods of differentiation are less widely used. Indeed as techniques probing the genotype of *S. pastorianus* have developed it has become clear that at least two distinct hybridisation events have occurred (Liti *et al.*, 2005; Dunn and Sherlock, 2008). This has led to sub-grouping of *S. pastorianus* into groups which correlate with yeast predominantly used in the production of Saaz and Frohberg type beers, respectively (Dunn and Sherlock, 2008). These two yeast types display considerable genetic variation, particularly with respect to ploidy which is lower in Saaz yeast than Frohberg. In Frohberg yeast there are twofold to threefold more *S. cerevisiae* DNA sequences than *S. bayanus*, whereas Saaz yeast generally contains similar or greater quantities of *S. bayanus* DNA sequence than *S. cerevisiae* (Dunn and Sherlock, 2008).

There are a variety of brewing yeasts in use demonstrating distinct fermentation characteristics, reflecting the spectrum of beers which they are used to create. Indeed, yeast has been referred to as the *main character in beer brewing* (Lodolo *et al.*, 2008). However, in many fermentations it may not be the only character, with traditional fermentations particularly prone to infection by non-*Saccharomyces* yeast and bacteria. Work by Hansen (1883) resulted in the isolation of pure cultures of the brewing yeast, which he called *Saccharomyces carlsbergensis* (this nomenclature has been the subject of much change in recent years). This development led to the growth of pure yeast cultures for the inoculation of fermentations, significantly reducing infection. Although certain types of beer, most notably Lambic style beers, are still fermented using mixed cultures of yeast and bacteria (Martens *et al.*, 1991), it is now standard practice in the majority of breweries to inoculate using a pure culture of yeast. In larger breweries this pure culture is typically generated using a dedicated propagation plant, of which there are several types, reviewed by Andersen (1994). Conditions in these propagators are engineered to ensure rapid

growth, using higher oxygen concentrations and temperatures than would typically be used for fermentation. The end product is checked for purity and to ensure it is the correct strain for the fermentation (Haikara *et al.*, 1990). Propagation is not required for every fermentation as yeast can be cropped and re-pitched, but in reality this is only done between 5-20 times (Boulton and Quain, 2001; Briggs *et al.*, 2004). Propagation is likely to be the mode of yeast supply for most large breweries. Smaller breweries, however, often buy in their yeast from other breweries or a specialist yeast suppliers. This supply can be unreliable and relatively expensive. One mode of yeast supply which is gaining in popularity, particularly among craft brewers, is active dried yeast.

### **1.3.1 Use of active dried yeast**

Active Dried Yeast (ADY) products consist of cells with a dry weight of 93-95 % (Bayrock and Ingledew, 1997a). They are typically packaged under nitrogen or a vacuum and are granular in appearance (Figure 1.1). ADY yeast cells are not metabolically active, but exist in a dormant state. The addition of water rehydrates the cells prompting them to regain their metabolic function, after which they regain functionalities similar to those of non-dehydrated cells.

Much of the ADY that is produced globally is utilised by the baking industry. Here the emphasis is on producing cells capable of quickly metabolising the sugars contained within the dough. Microbiological and viability control of the yeast is not the primary concern as the temperatures achieved during baking will kill any microorganism within the product. Along with the baking industry, the wine industry has also adopted the technology of ADY with some gusto. Previously wine fermentations were inoculated using the microbiological species found on the surfaces of grapes. This can lead to inconsistent fermentation, as the inoculating culture invariably differs between fermentations. As the advent of propagation systems did for the brewing industry, the use of ADY allows wine makers to inoculate with

a pure culture of yeast. It is not only the food industry which utilises ADY; the production of bioethanol has created a market for large quantities of yeast which ADY could fulfil. Here the emphasis is on high ethanol yield as well as reducing costs.

There is considerable potential for the use of ADY in the brewing industry, perhaps the most appealing being the replacement of onsite propagation for the production of pitching yeast (Gosselin and Fels, 1998; van den Berg and Van Landschoot, 2003; Powell and Fischborn, 2010). However, in addition to use for direct pitching, ADY can be used as a starter culture for the propagation process, leading to a reduction in total propagation time (Debourg and Van Nedervelde, 1999; Reckelbus *et al.*, 2000), or for bottle conditioning (Van Zandycke *et al.*, 2009). However, adoption of ADY technology by the brewing industry in general has been slower than that of others. Some sections of the industry, such as the craft brewing sector, have taken to the technology more readily than others, in particular the larger scale breweries. An explanation for this may be found in their differing methods of yeast supply. Craft breweries often lack the facilities to propagate their own yeast, thus rely on purchasing it from elsewhere. ADY therefore offers a reliable source of a pure yeast culture. Larger scale breweries tend to have dedicated propagation plants, which are viewed as a reliable method of yeast supply, thus the reasons to change practice must be compelling.

ADY can be stored at 4°C for up-to two years and can be rehydrated and ready to pitch in approximately an hour (Tobias Fischborn, personal communication), comparing favourably to conventional propagation methods which can take weeks. The rapidity of ADY allows yeast supply to better match the requirements of the brewery as it is sometimes not known when a new batch of yeast will be required. ADY may also allow larger breweries to centralise yeast supply (van den Berg and Van Landschoot, 2003), potentially making brand beers more consistent between breweries. Furthermore, ADY provides added flexibility

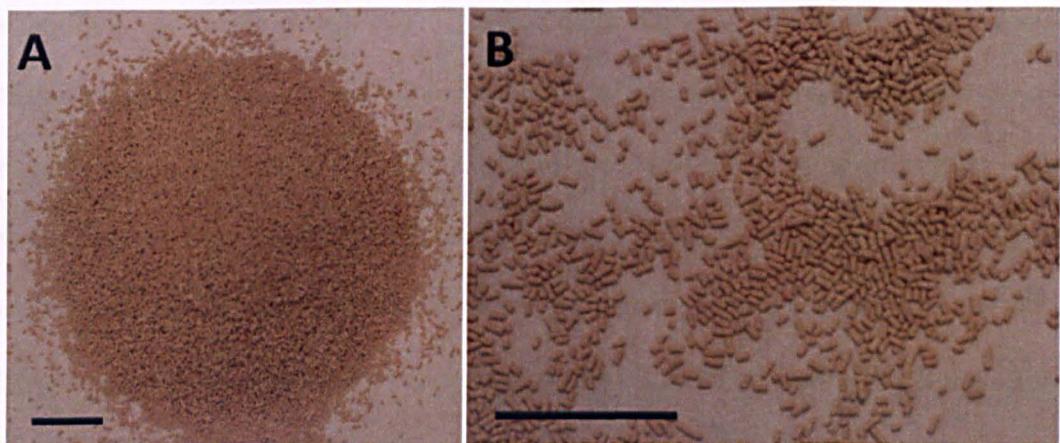
with the ability to brew on-demand and vary the yeast strain in use (of particular use to craft brewers working on a small scale). There is also a potential cost saving, with dedicated propagation plants being unnecessary, replaced by relatively simple rehydration vessels. This is more relevant for newly built breweries, as propagation plants are not yet in place. If ADY eventually supersedes onsite propagation it is likely that monetary savings will be the driving factor.

Whilst convenience and fiscal benefits are attractive to a brewer, their first concern is maintaining a consistent product; ADY must therefore show equivalency to propagated yeast before brewers' will initiate the significant change to their protocols required.

Previously, ADY has been thought to pose a contamination risk to the fermentation, due to the susceptibility of the drying process to infection by non-target organisms. However, procedural improvements have led to products which are comparable in terms of purity to propagated yeast (Quain, 2006). In addition, the absence of hops in the propagation medium used for ADY may result in increased sensitivity of contaminants during fermentation, leading to rapid death of any bacterial cells present (van den Berg and Van Landschoot, 2003).

Aroma profiles in beer produced from fermentations with ADY are comparable to those when propagated yeast is used (De Rouck *et al.*, 2007). Studies have also shown that fermentation characteristics of dried yeast show similarity to those of the wet equivalent (Debourg and Van Nedervelde, 1999; De Rouck *et al.*, 2007). However, a key concern when considering the use of ADY is the lower viability of populations compared to its stock counterparts, between 20-40 % less (lager strains tend to have a lower viability than that of ale strains, although the reasons for this are unclear). If viability is accurately assessed before pitching, the pitching rate can be adjusted accordingly to accommodate this factor without adversely effecting fermentation (Debourg and Van Nedervelde, 1999).

Significantly, a study by Finn and Stewart (2002) into the flocculation of dried brewer's yeast during fermentation found it to be abnormal when compared to wet yeast equivalent. The same authors also reported the presence of haze and less stable foam. The greater haze was surmised to be due to the presence of dead cells in suspension, which were also suggested to be responsible for reduced foam stability due the release of proteinase A. The pitching of dead cells has been associated with several of the issues encountered when using dried yeast; it is therefore clear that to make ADY a feasible alternative to propagated yeast, the low viability of its populations needs to be addressed. The causes of cell death in ADY are unclear, however, the potential for cellular damage during its production is significant (Sections 1.3.2 and 1.3.3).



**Figure 1.1. ADY in its dehydrated form. In each instance the size bar represents 1 cm.**

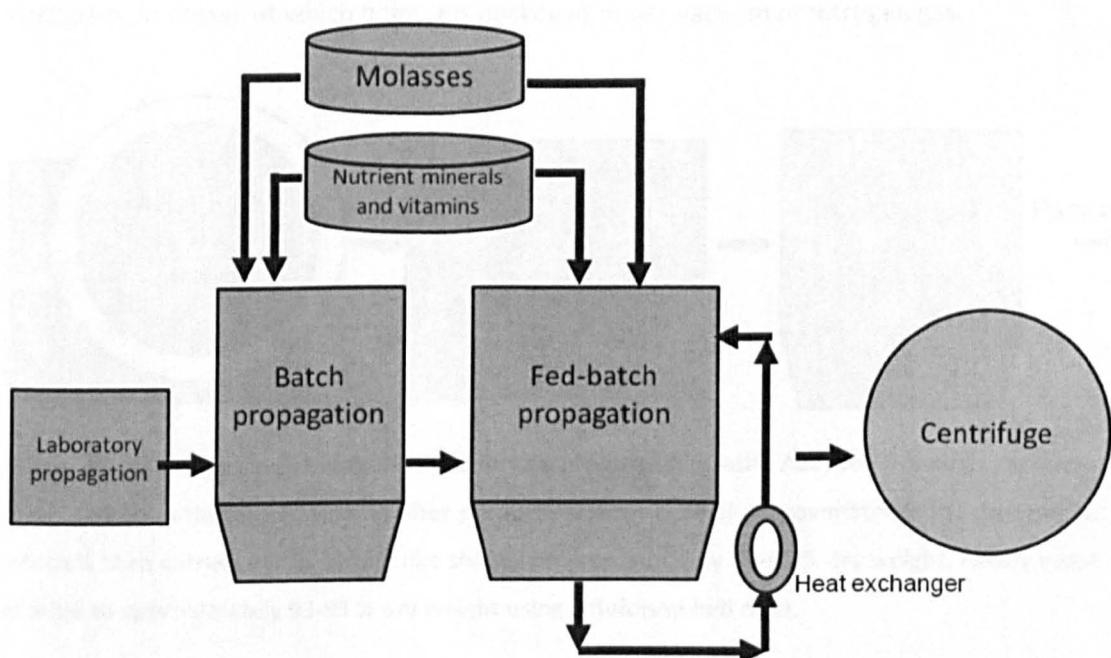
### **1.3.2 Production of ADY**

The production of ADY first requires the generation of substantial yeast biomass (Section 1.3.2.1), the early stages of which have some parallels with the propagation regimes used in brewing. The yeast population then undergoes several steps which reduce the water content (extra and intra cellular), culminating in fluidised bed drying (Section 1.3.2.2) to produce cells with a dry weight of 93-95 % (Bayrock and Ingledew, 1997a).

### 1.3.2.1 Propagation

The main purpose of propagation is to generate biomass, but there is also some consideration given to preparing yeast for the rigors of the drying process. Strains are first grown in the laboratory from frozen (-196°C) stocks to provide an inoculant (approximately 25 l) for the larger propagation vessels (Figure 1.2). Typically propagation is completed using a molasses based medium (Powell and Fischborn, In press); a by-product of beet and cane sugar production. The addition of a nitrogen source (often ammonium sulphate or ammonia) and other trace substances, such as vitamins, salts and metals, are required to make this nutrient lacking medium a suitable substrate for yeast (Quain, 2006). The medium is also adjusted to pH of 4.5-5 using sulphuric acid. Propagation then occurs in two distinct steps, a batch propagation, followed by a fed-batch propagation (Figure 1.2) (Quain, 2006; Van Zandycke *et al.*, 2009; Powell and Fischborn, In press). The key difference between these two systems, apart from the scale, is the way the medium is introduced. The batch propagation (50-80 hl) is similar to that of a brewery in that the entire medium is introduced at the beginning. Although oxygen is also introduced, the high levels of glucose result in a scenario termed the ‘Crabtree effect’, whereby glucose is degraded mainly *via* the fermentation pathway due to repression of the respiratory system, (Crabtree, 1929; Swanson and Clifton, 1948; De Deken, 1966). The fermentation of glucose to ethanol ( $C_2H_5OH$ ) and carbon-dioxide produces less ATP (two ATP molecules) than the respiration of glucose to water and carbon-dioxide (a potential 38 ATP molecules). Batch propagation lasts approximately 24 hours and the yeast is then transferred to a fed-batch propagation vessel. During fed-batch propagation (400-700 hl) the crabtree effect is avoided by gradually introducing the sugar medium, maintaining low levels (0.1 %) of available sugar (Quain, 2006; Powell and Fischborn, In press). This means that the metabolism of the yeast is mainly aerobic respiration, which is a more efficient method to create ATP, thus more efficient for cell growth. The key purpose of the propagation regime is to produce a large

amount of yeast efficiently, particular with regard to the financial cost. Given that the yeast is destined for drying, there are certain steps taken during the propagation to prepare the yeast cells for the subsequent stresses which will be encountered during dehydration and the subsequent rehydration. Towards the end of propagation, nutrient feeding is stopped to arrest cell division, and yeast is conditioned with a mild heat shock to encourage the accumulation of protectants such as trehalose (Ertugay *et al.*, 1997; Jorgensen *et al.*, 2002; Powell and Fischborn, In press).

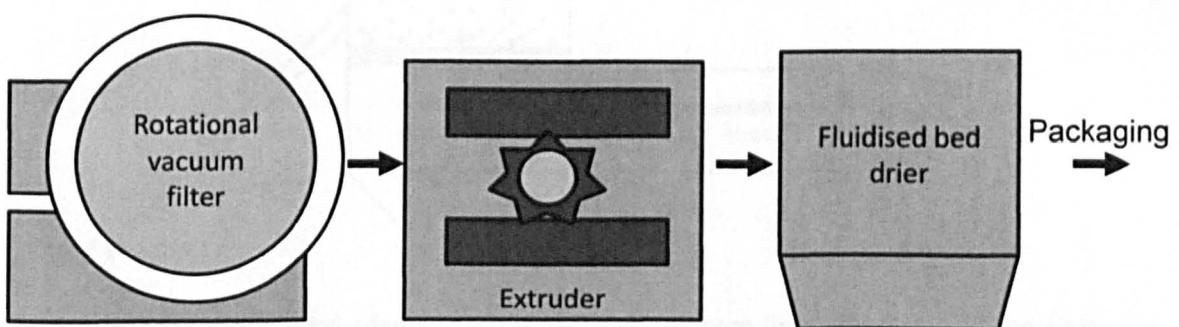


**Figure 1.2** The propagation steps typically used for the production of ADY. Laboratory propagation is followed by batch and then fed-batch propagations, before yeast is concentrated using a centrifuge. Adapted from Van Zandycke *et al.* (2009).

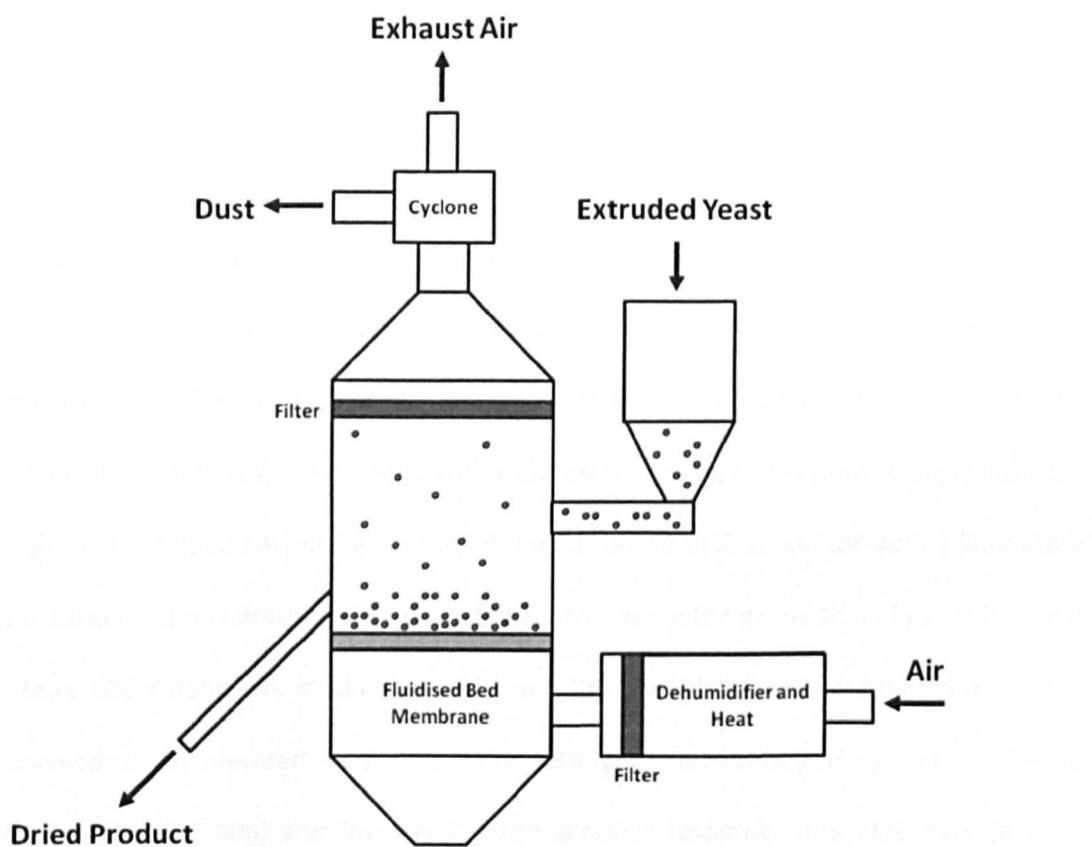
### 1.3.2.2 Drying

The first stage of the drying process is the removal of propagation broth, achieved through centrifugation and washing of the cells, resulting in a yeast concentrate of approximately 20 % dry weight. A preliminary water removal stage based on vacuum filtration is used to convert yeast cream to a cake (approximately 30-32 % dry weight), which is then extruded into noodle-like structures of approximately 0.2 mm in diameter. The remaining water is

then removed using a fluidised bed drier (Figure 1.4). In the fluidised bed drier dehumidified air is blown in from beneath a fluidised bed membrane. Extruded yeast enters from above the membrane and forms a fluid bed on its surface, resulting in the uniform drying of cell aggregates. This ensures a high level of contact between air and yeast, maintaining the product at a desired temperature ( $35\text{-}37^\circ\text{C}$ ) and promoting efficient drying (Powell and Fischborn, *In press*). Drying is continued until the dry weight of the yeast culture is approximately 93-95 % dry weight (Bayrock and Ingledew, 1997a; Powell and Fischborn, *In press*), at which point it is packaged under vacuum or nitrogen gas.



**Figure 1.3. The drying steps typically used for the production of ADY.** Adapted from Van Zandycke *et al.* (2009). A rotational vacuum filter produces a yeast cake of approximately 20 % dry weight, which is then extruded into noodle like shapes of approximately 30-32 % dry weight. Finally yeast is dried to approximately 93-95 % dry weight using a fluidised bed drier.



**Figure 1.4. Fluidised bed dried adapted from Powell and Fischborn (In press). Dehumidified air is heated and blown in from beneath a fluidised bed membrane. Extruded yeast enters from above the membrane and forms a fluid bed on its surface, resulting in the uniform drying of cell aggregates.**

### 1.3.3 Stresses associated with dehydration and rehydration

Water is essential for life, yet many organisms can survive anhydrobiosis, when the availability of water is reduced. This is because cells enter a dormant metabolic state, with cellular functions resuming once water has returned. Although the drying of yeast as a method of preservation is common, it is not a perfect technology and losses in viability (cell death) are expected. The precise cause, or causes, of this cell death are unknown, but are likely to be attributable to the plethora of stresses encountered during the production of ADY. During dehydration the loss of water is an obvious and significant stress, with some studies identifying it as the key factor responsible for a decrease in viability (Bayrock and

Ingledew, 1997b). There are, however, numerous other stresses which have been suggested to accompany the dehydration and rehydration of yeast cells.

Indicators as to what these stresses might be can be found in transcriptional studies, several of which have followed the expression of stress related genes throughout the production of ADY. Perez-Torrado et al. (2005) recorded the expression of genes during the propagation of *S. cerevisiae* used for wine making. They noted that in addition to the general stress pathway, the osmotic and oxidative stress responses were strongly induced. Singh et al. (2005) analysed the transcriptional response of *S. cerevisiae* during laboratory desiccation and rehydration. They recorded an increase in the expression of genes involved in fatty acid catabolism, gluconeogenesis, and the glyoxylate cycle, during drying, which remained at an elevated level when the cells were rehydrated (Singh et al., 2005). Rossignol et al. (2006) also investigated the genomic response of *S. cerevisiae* (a wine strain) during rehydration. They found that the general stress response genes were repressed during rehydration, whilst acid stress specific genes were induced, which it was suggested was a response to the accumulation of organic acid (Rossignol et al., 2006). Indeed rehydration appears to be more of a recovery period with genes associated with protein synthesis being induced. Although these studies are not a direct representation of the industrial propagation and drying of brewing yeast, they are a useful indicator as to the types of stresses that yeast cells are subjected to during propagation, dehydration and rehydration.

### **1.3.3.1 Osmotic stress**

Osmotic stress is clearly an important factor when considering the potential stresses of dehydration and rehydration. However, osmotic stress first occurs prior to drying during the propagation of yeast, due to the high concentration of sugars present in the growth medium during batch growth (Perez-Torrado et al., 2005). Osmotic stress can manifest

itself in several forms encompassing the movement of water and the loss of water's structural properties. Constituting a large proportion of the cell, the movement of water can have significant damaging effects. Efflux and influx of water across the membrane during dehydration and rehydration respectively can result in damage, whilst the loss of water from the membrane structure itself can also cause damage (Laroche and Gervais, 2003). Cell shrinkage, without plasmolysis, is thought to result in rupture of the cell membrane in times of osmotic stress (Morris *et al.*, 1986). Cell shrinkage also leads to cytoplasmic crowding, which may promote potentially damaging molecular interactions within the cell (Berner and Gervais, 1994; Vindelov and Arneborg, 2002). Other cell components also require water molecules to maintain their structure with water loss producing conformational changes in proteins, potentially resulting in the permanent loss of function (Hoekstra *et al.*, 2001; França *et al.*, 2007) and the dehydration of DNA molecules can result in the loss of supercoiling structure (Shirkey *et al.*, 2003).

There are, however, mechanisms within yeast cells which facilitate a degree of resistance to osmotic stress. The yeast cell membrane contains protein channels, referred to as aquaporins, which under certain circumstances can facilitate the osmotically driven efflux of water (Tanghe *et al.*, 2002), reducing potential membrane damage. In addition, yeast cells may adjust their cellular composition to reflect their changing surrounding environment. Under osmotic stress the High Osmolarity Glycerol (HOG) Mitogen-Activated Protein kinase (MAP kinase) pathway is activated (Brewster *et al.*, 1993). This name is in reference to the observation that yeast, when subjected to osmotic stress, accumulate the osmolyte glycerol (Reed *et al.*, 1987) due to the induction of *GPD1* and *GPP2* which encode enzymes involved in its synthesis (Rep *et al.*, 1999). Glycerol is accumulated in an attempt to counterbalance external osmotic pressure (Reed *et al.*, 1987). Cells under osmotic stress may also accumulate the disaccharide trehalose (Eleutherio *et al.*, 1997), which is thought to exhibit various protecting functions (discussed in Section 1.3.4).

In addition to compositional changes, osmotic stress can cause cells to exhibit transient structural modifications including thickening of the cell wall (Morris *et al.*, 1986), reorganisation of the actin cytoskeleton (Chowdhury *et al.*, 1992) and changes to membrane fluidity (Laroche *et al.*, 2005). Indeed changes with respect to the cell membrane are thought to be vital to the cell's osmotic response due to the presence of the transmembrane protein Sln1. Sln1 is an osmosensor histidine kinase, which monitors changes in turgor pressures (Reiser *et al.*, 2003) and is responsible for the activation of HOG MAP kinase pathway (Posas *et al.*, 1996).

### **1.3.3.2 Oxidative stress**

Although osmotic stress clearly has a significant impact during dehydration and rehydration, it is by no means the only stress which yeast are subject to. Oxidative stress, due to the production of reactive oxygen species (ROS), is perhaps the most significant of these other stresses. Indeed, Shima *et al.* (2008) have identified, through genome-wide screening (using deletion mutants), correlations between air-drying sensitivity and oxidative stress sensitivity suggesting significant relatedness between the mechanisms or targets of the two stresses.

ROS are not only produced during ADY production; they are generated by mitochondria during normal aerobic metabolism (Trancikova *et al.*, 2004). Indeed, the method of biomass production (fed-batch propagation discussed in Section 1.3.2.1) is the first major source of ROS in the production of ADY. The process is specifically engineered to maintain maximum aerobic respiration throughout the process (Perez-Torrado *et al.*, 2005). Upon dehydration yeast cells can show more than a 10-fold increase in intracellular oxidation, assessed using the fluorescent probe 2',7'-dichlorofluorescein. The origins of the ROS that appear during dehydration are unclear (Pereira *et al.*, 2003), but the targets of oxidative damage are identifiable. Lipids, a key constituent of the plasma membrane, are known to suffer

peroxidation during dehydration (França *et al.*, 2005; Herdeiro *et al.*, 2006; Garre *et al.*, 2010). Although other cell components are also susceptible to ROS damage, DNA (Leroy *et al.*, 2001) and proteins (Herdeiro *et al.*, 2006) for example, it is the damage to the plasma membrane an organelle, known to be integral to survival during osmotic stress (Simonin *et al.*, 2007a), which is perhaps the most significant. The oxidative stresses encountered during ADY production are in addition to the potential oxidative stress which yeast may encounter during fermentation, particular in the initial stages (Higgins *et al.*, 2003). The accumulative effect of these stresses is not known.

As with osmotic stress, the yeast cell has a selection of protective, or damage limiting, responses which are elicited by oxidative stress. Glutathione (GSH) functions as one of these responses, acting as a ROS scavenger reacting to produce its oxidised form (GSSG) (Grant, 2001). Indeed Espindola *et al.* (2003) identified glutathione as playing a significant role in the maintenance of intracellular redox balance during dehydration. Thioredoxin serves a similar scavenging function as GSH and has also been suggested to improve dehydration tolerance (Perez-Torrado *et al.*, 2009). In addition to protection from osmotic damage, the accumulation of trehalose may also protect from oxidative damage (Pereira *et al.*, 2003; Herdeiro *et al.*, 2006). The yeast oxidative response also encompasses the activity of several enzymes. One such enzyme, the cytoplasmic catalase (Ctt1), catalyses the breakdown of hydrogen peroxide to oxygen and water (França *et al.*, 2005). Through the analysis of mutants lacking this enzyme it has been shown that its actions improve dehydration tolerance in laboratory strains (França *et al.*, 2005).

### **1.3.3.3 Additional contributory stresses**

Although two key stresses have been highlighted, osmotic (Section 1.3.3.1) and oxidative (Section 1.3.3.2), there other stresses present during ADY production. Although they may

be considered of little significance on their own, these stresses may combine to cause significant damage and should not be ignored.

Like all organisms, yeasts have optimum temperatures for metabolic activity at which they exhibit maximal growth. *S. cerevisiae* has a growth optimum of approximately 31°C, whilst the lager yeast *S. pastorianus* has a lower optimum of approximately 27°C (Guidici *et al.*, 1998). Above these optimal temperatures yeast cell components may become damaged. Yeast are subject to heat stress during the drying phase, where a temperature of approximately 35-37°C is reached (Powell and Fischborn, In press). This is considerably lower than some other drying techniques, such as spray drying which can impart a temperature in excess of 100°C (Luna-Solano *et al.*, 2000), but is still likely to have an impact on the yeast cells. Like many organisms, yeast generate protective proteins in response to heat shock (Lindquist and Craig, 1988). The heat shock response in yeast is intimately associated with the osmotic stress response, with the *HSP12* gene being activated by HOG MAP kinase pathway (Varela *et al.*, 1995).

Additional stress may also be derived from the presence of damaged cells that leach cellular compounds during rehydration. Weak organic acids can leak from damaged cells, eliciting induction of the weak acid stress regulon during rehydration (Rossignol *et al.*, 2006). There are, however, benefits associated with leaching/leaking, as some products can be beneficial to cells, such as amino acids and nucleotides which are made freely available to cells (Rossignol *et al.*, 2006). It is therefore possible that improvements to aspects of damage brought about to cells during rehydration may not be wholly beneficial, as the decreased non-viable cell concentration may be detrimental.

During propagation, cells undergo intentional nutrient limitation (Section 1.3.2.1) to encourage the accumulation of protectants. This starvation, although common in laboratory and industrial cultures, may also contribute to the overall stress of the yeast.

Indeed entry into stationary phase due to nutrient limitation requires significant changes to the biochemistry of the cell such as the accumulation of storage carbohydrates, most notably glycogen (Rothmand-Denes and Cabib, 1970) and trehalose (Jorgensen *et al.*, 2002), alteration of the cell wall structure (de Nobel *et al.*, 1990) and changes in membrane structure (Takeo *et al.*, 1976).

#### **1.3.4 Process steps to optimise ADY viability**

The potential stresses which may damage yeast during ADY production, and a description of some of the methods by which yeast may resist damage has been described above. Some of these inherent protective responses have been exploited in the propagation of ADY to increase cellular stress resistance.

Stationary phase cells cope better during osmotic stress than exponential phase cells (Hounsa *et al.*, 1998), therefore towards the end of propagation nutrient supply is restricted, forcing the cells to cease dividing and enter stationary phase. This nutrient limitation is also accompanied by a mild heat shock (Ertugay *et al.*, 1997; Jorgensen *et al.*, 2002). This acts to stimulate a molecular response which results in the accumulation of protectants which can provide resistance to subsequent heat or other stress (Piper, 1993; Li *et al.*, 2009). The major protectant accumulated is trehalose, a disaccharide which can be found in industrially produced ADY at around 10 % of its dry weight (Van Dijck *et al.*, 1995). Trehalose is of importance as it may help protect cells from osmotic, oxidative and heat stress which are all encountered during the drying process. However, whether levels of this sugar increase cell survival during dehydration, and its mechanism are still unclear. Alexandre *et al.* (1998) demonstrated that there was a lack of correlation between the accumulation of cellular trehalose and viability maintenance under induced stresses. In contrast, Ratnakumar and Tunnacliffe (2006) showed that a high intracellular trehalose concentration can improve stress tolerance, but is neither necessary nor sufficient for

survival in desiccation. These authors further postulated that trehalose's main function in desiccation involved minimising protein inactivation by acting as a chemical chaperone. Hounsa *et al.* (1998) concluded that trehalose did have an important protective function for the survival of yeast under severe osmotic stress which would occur during rehydration.

As well as confusion over the extent of protection which trehalose provides during dehydration, there is also debate about the mode in which it may provide protection. The transition temperature of the amorphous phase of trehalose is relatively high and makes trehalose more stable than other cell components, thus providing a stabilising presence in the cell. The formation of a stable and highly viscous glass may hold biomolecules in a form which allows them to return to their native structure and thereby be fully functional following rehydration (França *et al.*, 2007). Trehalose may have more specific interactions with some cellular components, such as proteins and lipids. Trehalose and other sugars, such as sucrose, have been shown to preserve structure and function of proteins when water is removed (Singer and Lindquist, 1998). It also suppresses the aggregation of denatured proteins, maintaining them in a partially-folded state from which they can be reactivated by molecular chaperones (Singer and Lindquist, 1998; Hoekstra *et al.*, 2001). In vitro, trehalose has been demonstrated to significantly reduce the oxidation of fatty acids, another key cell component (Oku *et al.*, 2003). Furthermore, it has been demonstrated that the presence of trehalose lowers the phase transition temperature of dry to liquid crystal phase and thus makes it possible to avoid a transition during rehydration (Leslie *et al.*, 1994) a key point of damage in osmotically stressed yeast (Laroche *et al.*, 2005; Simonin *et al.*, 2007a). It is suggested that this is achieved by the disaccharide directly replacing water molecules in the structure of the lipid membrane. Trehalose shows a direct interaction with the phospholipid headgroups during drying, reducing the van der Waals interactions among the hydrocarbon chains (Patist and Zoerb, 2005). Hydrogen bonding between sugars and the polar headgroups of the lipids contribute to preserving the integrity of biological

structures. As water is removed or lost, the interactions that occur between the sugars and polar headgroups act as replacement, maintaining the membrane fluid interface (Crowe *et al.*, 1998; Patist and Zoerb, 2005). It is not clear as to the precise role trehalose plays in industrial strains, where the sugar is often present in much higher concentrations than those commonly investigated in the laboratory.

There should also be a word of caution as although trehalose may protect against stresses, it may also be detrimental to normal metabolism. In keeping with its role of maintaining chemical stability, trehalose inhibits the reactivation of denatured proteins, once the stress has been removed. Thus, the continued presence of trehalose interferes with refolding (Singer and Lindquist, 1998; Herdeiro *et al.*, 2006) and directly inhibits enzyme action (Sampedro *et al.*, 2002; Sebollela *et al.*, 2004). This may be detrimental to brewing yeast pitched into fermentation medium where the population is required to be highly metabolically active, thus it is likely to be advantageous if the sugar is able to be rapidly removed from cells.

#### **1.4 AIMS AND OBJECTIVES**

Despite the extensive innovation which has occurred within the brewing industry, improvements to the processes involved are still actively sought. Yeast supply is one area which has potential for further optimisation. Replacing the propagation of the inoculating yeast culture with ADY provides potential savings in time and money. Although ADY use has been prevalent in the baking and wine industries the specific requirements for pitching quality brewing yeast may limit the technology's adoption. The plethora of stresses involved in ADY production pose a significant threat to yeast integrity and in particular its viability. Therefore, before ADY is likely to be adopted as the industrial standard the extent of damage caused by drying the yeast and the effect this has on its fermentation performance needs to be demonstrated. This thesis will first assess the extent of cell death

when three brewing yeast are dehydrated and rehydrated, determining the extent of the problem. Cellular components which are both key to cell function and potential sources of stress damage will then be probed to determine if damage has occurred. Finally, ADY will be utilised in a fermentation to determine its suitability for the process, comparing its profiles to a propagated yeast culture.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 YEAST STRAINS AND STORAGE

LAL1 lager yeast, LAL2 ale yeast and LAL4 ale yeast were provided by Lallemand Inc. (Montreal, Canada) as ADY and in non-dry form. ADY was stored at 4°C. Non-dried (control) laboratory-grown yeast was stored on YPD slants at 4°C. YPD was produced by dissolving Yeast extract (1 %), Peptone (2 %) and D-Glucose (2 %) in RO (reverse osmosis) water. If required the medium was solidified using agar (1.2 %) (all media supplied by Fisher Scientific, UK). Stock cultures of each strain were cryogenically maintained in cryovials (Nalgene Nunc International, UK). A loop full of yeast grown on YPD agar was re-suspended in 1ml YPD containing 20 % (v/v) glycerol (Sigma, UK) as a cryoprotectant to maintain cell viability. The yeast were stored at -80°C.

**Table 2.1. Batch numbers of ADY used throughout the project**

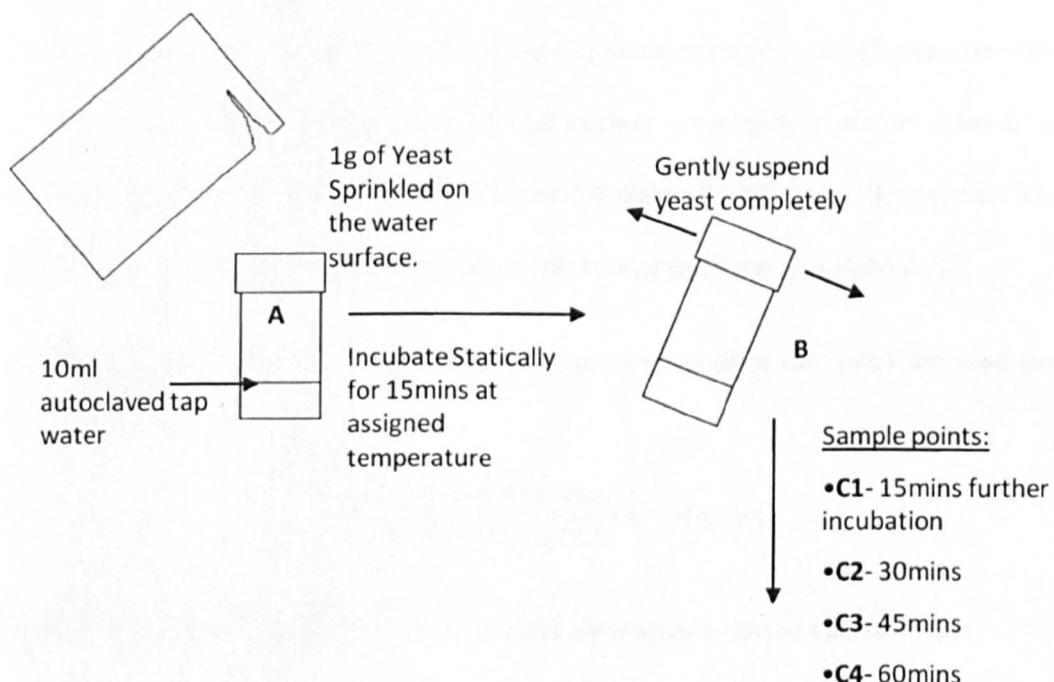
Strain	Batch Number	Source	Expiry Date
LAL1	NOT SUPPLIED	Lallemand Inc. Canada.	03-2008
	22801120	Lallemand Inc. Canada.	04-2009
	22810611090300v	Lallemand Inc. Canada	10-2009
	22809501180627v	Lallemand Inc. Canada	11-2009
	22810611290627v	Lallemand Inc. Canada	10-2010
LAL2	10804280377711v	Lallemand Inc. Canada.	04-2009
	1087117102	Lallemand Inc. Canada.	06-2011
LAL4	22901891277711v	Lallemand Inc. Canada.	12-2009
	2290011019v	Lallemand Inc. Canada.	02-2011

Table 2.2. The name, source, genotype and reference of the yeast strains used throughout the project.

Strain	Source	Genotype	Notes	References
LAL1	Lallemand Inc. Montreal, Canada	Not analysed	Lager strain	(Jenkins <i>et al.</i> , 2010)
LAL2	Lallemand Inc. Montreal, Canada	Not analysed	Ale strain	(Jenkins <i>et al.</i> , 2010)
LAL4	Lallemand Inc. Montreal, Canada	Not analysed	Ale strain	(Jenkins <i>et al.</i> , 2010)
S288C	Professor Stephen Oliver, University of Cambridge, UK	MATa: gal2;mal	Haploid lab strain	(Goffeau <i>et al.</i> , 1996)
Y00000	European <i>Saccharomyces cerevisiae</i> archive for functional analysis (EUROSCARF)	MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0	Synonym: BY4741 Haploid lab strain	(Thakur and Chakrabarti, 2010)
Y02667	EUROSCARF	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLR056w::kanMX4	Non-functioning YLR056w/ <i>ERG3</i>	(Schuller <i>et al.</i> , 2004)
Y00788	EUROSCARF	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YMR202w::kanMX4	Non-functioning YMR202w/ <i>ERG2</i>	(Schuller <i>et al.</i> , 2004)

## 2.2 PREPARATION AND REHYDRATION OF ADY

ADY was prepared, using the manufacturers' standard protocol, by propagation on molasses based media and dried using a fluidized bed drier (Van Zandycke *et al.*, 2009; Powell and Fischborn, 2010). ADY was provided by Lallemand Inc (Montreal, Canada) in sealed packets under vacuum (LAL1) or nitrogen (LAL2 and LAL4) and stored at 4°C prior to use. Rehydration was performed based on the manufacturer's guidelines (figure 2.1); 1 g dried yeast was sprinkled onto 10 ml attemperated tap water in a 30 ml universal tube unless stated. ADY was incubated at the desired rehydration temperature for 15 min, mixed gently to separate any clumps and incubated for a further 45 min.



**Figure 2.1. Rehydration procedure for ADY based on the instruction provided by the manufacturer.**  
A, B, and C1-4 represent the points at which samples were removed for viability analyses.

## 2.3 CELL DENSITY DETERMINATION AND ESTIMATION OF VIABILITY IN ADY POPULATIONS

For estimation of viability in ADY, yeast was rehydrated prior to analysis as described in section 2.2. Samples for viability testing were recovered in triplicate before rehydration

(Sample Point A), after initial mixing (Sample Point B) and subsequently at 15 min intervals (Sample Points C1-C4) (Figure 2.1).

Cell suspensions were diluted with water (purified using reverse osmosis) to the required density (approximately  $1 \times 10^7$  cells/ml). Cell density was measured using an Improved Neubauer counting chamber (Weber Scientific International Ltd, UK) and standard light microscope (BH-2, Olympus, U.S.A.) at a magnification of x400. At least 200 cells were counted to calculate cell density (Equation 2.1).

The number of live cells in each population was estimated by microscopy using brightfield (Section 2.3.1 methylene blue) and fluorescent stains (Sections 2.3.2 Mg-ANS and 2.3.3 Oxonol). In addition, a direct evaluation of the replicative capacity of cells was determined by slide culture (2.3.4). Irrespective of the method employed, triplicate samples were assessed and a minimum of 100 cells per sample were enumerated. The number of live (viable) cells is expressed as a percentage of the total population (Equation 2.2).

**Equation 2.1. The calculation of cell density in yeast cultures using the values obtained from a counting chamber.**

$$\frac{\text{cells} * \text{dilution factor}}{\text{volume of chamber}} = \text{cells/ml}$$

**Equation 2.2. Viability assessment of yeast cell populations expressed as a percentage.**

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

### **2.3.1 Methylene blue viability assessment**

Methylene blue (Sigma-Aldrich, UK) was dissolved in sodium citrate solution (2 % W/V) to a final concentration of 0.01 % (Pierce, 1970). Yeast cells were enumerated using a haemocytometer and diluted to a concentration of approximately  $1 \times 10^7$  cells/ml. Yeast suspension (0.5 ml) was mixed with methylene blue solution (0.5 ml) and after a static

incubation of 5 min at room temperature examined microscopically (Olympus BH-2 microscope at x400 magnification). Non-viable cells were stained blue and viable cells remained unstained.

### **2.3.2 MgANS viability assessment**

MgANS (8-Anilino-1-naphthalene-sulfonic acid hemi-magnesium salt hydrate) staining was performed according to the method of McCaig *et al.* (1990). 0.3 g MgANS (Sigma-Aldrich, U.K.) was dissolved in 2 ml of absolute ethanol (Fisher Scientific, U.K.) and diluted in 98 ml of sterile water producing a final concentration of 0.3 % (w/v). This was used as a stock concentration and stored at 4°C in a light protected container. Yeast cells were enumerated using a haemocytometer and diluted to a concentration of approximately  $1 \times 10^7$  cells/ml. Yeast suspension (0.5 ml) was mixed with MgANS solution (0.5 ml) and incubated statically in a dark environment for 5 min at room temperature. Cells were examined using a fluorescence microscope (Optiphot-2, 100 W mercury lamp light source and a triple pass filter set for DAPI-FITC-TRITC, Nikon, Japan). Non-viable cells appeared green and viable cells remained un-stained (McCaig, 1990). Fluorescent images were taken using a Leica (Germany) DM5000 B microscope with L5 filter cube (excitation BP 480/40 nm, dichromatic mirror 505 nm, and suppression filter BP 527/30 nm).

### **2.3.3 Oxonol viability assessment**

Oxonol (bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Dibac<sub>4</sub>(3))) viability assessment was determined following the method of Lloyd and Dinsdale (2000). 1 mg oxonol dye (Invitrogen Ltd., U.K.) was dissolved in 1 ml absolute ethanol and stored at -20°C in a dark environment. 10 µL of the stock solution was diluted in 1 ml water to produce a working solution of 10 µg/ml.  $1 \times 10^7$  cells/ml yeast suspension (0.9 ml) was mixed with 100 µL of working solution of oxonol and incubated in a dark environment at room temperature for 5 min. Cells were examined using a fluorescence microscope (Optiphot-2, 100 W mercury

lamp light source and a triple pass filter set for DAPI-FITC-TRITC, x400 magnification). Non-viable cells appeared green and viable cells remained unstained (Lloyd and Dinsdale, 2000).

#### **2.3.4 Slide culture viability assessment**

The slide culture technique used to assess viability was adapted from the American Society of Brewing Chemists Methods of Analysis (Russell *et al.*, 1984). In this instance YPD agar was used in preference to MYGP (malt extract, yeast extract, glucose and peptone) media. Using a pipette, 1 ml molten YPD agar was dispersed evenly over a 3 x 1 inch slide, previously sterilized by flaming. Once agar had solidified, 10 µl yeast cell suspension ( $1 \times 10^6$  cells/ml) were pipetted onto the surface and a cover slip placed on top. Slides were incubated at 25°C for approximately 18 hr. Cells were examined using a light microscope and individuals giving rise to microcolonies were deemed viable, while single cells were scored as non-viable.

#### **2.3.5 Statistical analysis of the factors determining viability estimation**

The statistical significance of temperature and method of assessment on viability estimation was determined using multi-factorial ANOVA based on a nested experimental design using Minitab (Version 16, Minitab Inc., U.S.A.). The experiment was balanced, with triplicate estimates of viability for each combination of variable (temperature of rehydration and method of assessment). In each instance the null hypothesis was that no significant difference existed between data sets. If the P value generated by the test was less than 0.05 then the null hypothesis of no significant difference was rejected.

## **2.4 BUDDING INDEX**

The percentage of cells exhibiting a bud, termed budding index, was also calculated (Equation 2.3).

**Equation 2.3. Calculation to determine the budding index of cell populations.**

$$\frac{\text{cells exhibiting a bud}}{\text{total number of cells}} = \text{budding index}$$

## **2.5 ANALYSIS OF YEAST GENETIC STABILITY**

### **2.5.1 Quantification of DNA**

DNA content of extractions was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., U.S.A.). 1.5 µl sample was pipetted onto the pedestal of the instrument and the measurement taken. Calculations were performed automatically, using ND-1000 software (Version 3.1.0, NanoDrop Technologies Inc., U.S.A.) and a value provided for the quantity of DNA based on the absorbance at 260 nm (Gallagher and Desjardins, 2006).

### **2.5.2 Assessment of genomic DNA**

#### **2.5.2.1 Pulsed Field Gel Electrophoresis (PFGE)**

Yeast DNA, from whole cell populations, was embedded in agarose using the CHEF yeast genomic DNA plug kit (Bio-Rad Laboratories, U.S.A.). YPD grown and rehydrated dried yeast cells were washed and resuspended in cold 50 mM ethylenediaminetetraacetic acid (EDTA), pH 8 (Sigma-Aldrich, U.K.). The cells in suspension were enumerated and  $1.8 \times 10^8$  cells were harvested by centrifugation at 1,500 g and 4°C for 5 min. The cells were then resuspended in 150 µl cell suspension medium (Bio-Rad Laboratories, U.S.A.) and equilibrated to 50°C. 2 % CleanCut agarose solution (Bio-Rad Laboratories, U.S.A.) was heated using a microwave until liquefied and then equilibrated to 50°C. 9 µl lyticase solution was added to the cell suspension immediately prior to the addition of 150 µl agarose solution. The suspension was mixed by gentle pipetting and approximately 100 µl transferred to a plug mould. Agarose was allowed to solidify at 4°C for 30 min before the

plugs were pushed into a 15 ml conical centrifuge tube. 750 µl lyticase buffer and 25.5 µl lyticase solution (Bio-Rad Laboratories, U.S.A.) were pipetted over the plugs. The plugs, suspended in lyticase solution, were then incubated at 37°C and 120 rpm for 2 hr. Lyticase solution was then removed by gentle pouring. The plugs were rinsed in water, before the addition of 750 µl proteinase buffer and 30 µl proteinase K stock (Bio-Rad Laboratories, U.S.A.). The plugs and proteinase K solution were incubated statically at 50°C overnight. Proteinase K solution was then removed and the plugs washed in 1 ml washing buffer (Bio-Rad Laboratories, U.S.A.) for 1 hr at 120 rpm. This washing step was repeated and the plugs then stored at 4°C until use. Approximately 2 mm of each plug was used for chromosome separation. Yeast chromosomes were separated through a 1 % agarose gel (1 x TAE buffer) using a CHEF II Pulsed Field Gel Electrophoresis system (Bio-Rad Laboratories Ltd., U.K.) with a 60 sec switch time for 15 hr and 90 sec switch time for 9 hr. A yeast chromosome PFG marker was used for fragment length reference (New England BioLabs, U.S.A.). Gels were stained using 0.5 µg/ml ethidium bromide TAE solution (Sigma-Aldrich, U.K.) and visualised using a gel imaging system (Gel Doc, Bio-Rad Laboratories, U.S.A.).

#### **2.5.2.2 Delta Primer pair 12/21 polymerase chain reaction (PCR)**

Rehydrated ADY samples and control laboratory yeast populations were inoculated onto YPD plates and incubated at 25°C for 48 hr. DNA was extracted from five individual colonies of each sample according to the method described by Powell and Diacetis (2007). 50TE buffer containing 7.44 g/L EDTA (Sigma-Aldrich, U.K.), 6.06 g/L TRIS (Sigma-Aldrich, U.K.), adjusted to pH 7.5 with HCL (Fisher Scientific, U.K.) and sterilised by autoclaving at 121°C and 103 kPa for 15 min. 10 % SDS (Sigma-Aldrich, U.K.) solution was also produced and sterilised by filtration through a 0.45 µM pore size filter (Millipore, U.S.A.). 50TE-SDS buffer was produced by mixing 200 ml of 50TE buffer with 20 ml 10 % SDS solution. A colony was transferred to 660 µL 50TE-SDS buffer. Suspended cells were vortexed and incubated at 65°C for 10 min to lyse the cells. Subsequently 340 µL of 5M potassium acetate (Sigma-

Aldrich, U.K.) was added and samples were maintained at 4°C for approximately 15 min and occasionally inverted, until proteins were precipitated. Proteins were removed by centrifugation for 10 min at 13,000 rpm and 600 µL of supernatant was transferred into a fresh tube with 600 µL isopropanol. Samples were maintained at room temperature for 10 min to precipitate DNA. DNA was isolated by centrifugation at 10,000 rpm for 10 min and the aqueous phase disposed of. DNA pellets were washed in 100 µL of 95 % cold (-20°C) ethanol before being air-dried and resuspended in 80 µL TE buffer (Powell and Diacetis, 2007). DNA was quantified (Section 2.5.1), diluted to 250 ng/µL and stored at -20°C until required.

The primer pair delta12 (5'-TCAACAATGGAATCCAAC-3') and delta21 (5'-CATCTAACACCGTATATGA-3') (Legras and Karst, 2003) were obtained from Eurofins MWG Operon (Germany). Reactions, using Phusion High-fidelity DNA polymerase (New England Biolabs, U.S.A) (Table 2.3), were amplified using a TC-512 thermal cycler (Techne, U.K.) (

Table 2.4). PCR products were resolved on a 1 % agarose gel (Sigma-Aldrich, U.K.) at 100 mV.

**Table 2.3. PCR mixture content for delta primer pair 12/21 analysis of genomic DNA of individual yeast colonies.**

Component	Volume ( $\mu$ l)/ 20 $\mu$ l reaction	12 Reaction master mix volume ( $\mu$ l)	Final Concentration
H <sub>2</sub> O	11.5	138	N/A
5 x Phusion HF Buffer (New England Biolabs, U.S.A)	4	48	1 X
2mM dNTPs (New England Biolabs, U.S.A)	2	24	200 $\mu$ M
Delta 12 primer (Eurofins MWG Operon, Germany)	0.4	4.8	1 $\mu$ M
Delta 21 primer (Eurofins MWG Operon, Germany)	0.4	4.8	1 $\mu$ M
DNA 250 ng/ $\mu$ L	1.5	N/A	18.75 ng/ $\mu$ L
Phusion DNA Polymerase (NEB)	0.2	2.4	0.02 U/ $\mu$ L

**Table 2.4. Program for delta primer pair 12/21 PCR analysis.**

Cycle Step	Temp °C	Time	Cycles
Initial denaturation	98	30 seconds	x1
Denaturation	98	10 seconds	x35
Annealing	48	30 seconds	
Extension	72	90 seconds	
Final extension	72	10 minutes	x1

### 2.5.3 Assessment of mitochondrial DNA

#### 2.5.3.1 Analysis of mtDNA by restriction fragment length polymorphism (RFLP)

DNA was extracted from whole cell populations based on the mitochondrial extraction method of Defontaine *et al.* (1991) as modified by Nguyen *et al.* (2000). Approximately 0.5 g (wet weight) of yeast cells were washed with 4 ml of cold (4°C) 50 mM EDTA (Fisher

Scientific, U.K.) and pelleted by centrifugation (1,000 RCF for 5 min). Cells were then resuspended in 5 ml of buffer A (1.2 M sorbitol and 50 mM EDTA, both from Fisher Scientific, U.K.) and 100 µl of β-mercaptoethanol (Sigma-Aldrich, U.S.A.), prior to incubation at 37°C for 10 min. Cells were pelleted by centrifugation (1,000 RCF for 5 min) and resuspended in 5 ml buffer B (0.5 M sorbitol, 10 mM EDTA and 50 mM tris-HCl, all Fisher Scientific, U.K.) with approximately 10,000 units of lyticase enzyme (Sigma-Aldrich, U.S.A.), prior to incubation at 37°C and 120 rpm for 1 hr. Cell suspensions were then sonicated using a Soniprep 150 plus (MSE, U.K.) at 19 KHz for 30 sec .Cells were pelleted by centrifugation (1,000 RCF for 5 min) and the supernatant retained. The supernatant, containing the isolated mitochondria, was then centrifuged (16,000 RCF at 4°C for 10 min) and the supernatant discarded. The mitochondria were then suspended in 0.5 ml buffer B. 10 units of DNAase were added and the solution was incubated at room temperature for 10 min. The suspension was washed with buffer B to remove the DNAase and any residual genomic DNA. Mitochondria were again pelleted by centrifugation (16,000 RCF at 4°C for 10 min) and the supernatant discarded. The pellet was resuspended in 0.6 ml lysis buffer (100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, 4 mM Sodium lauroyl sarcosinate, all from Fisher Scientific, U.K.) to which 0.5 ml phenol-chloroform (Sigma-Aldrich, U.S.A) was added, and mixed by pipetting. The suspension was centrifuged (16,000 RCF at 4°C for 5 min) and the supernatant retained, to which 0.6 ml chloroform was added. The suspension was centrifuged (16,000 RCF 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 RCF at 4°C for 30 min) and subsequently washed with 0.6 ml of 75 % ethanol, before finally being resuspended in 30 µl RO water.DNA was quantified using a NanoDrop ND-1000 spectrophotometer and the concentration was adjusted to 500 ng/µL. mtDNA was cut using the enzyme *Hinf*I (New England Biolabs, U.S.A) with the appropriate buffer (supplied

with the enzyme). 1.5 µL of buffer was added to a 12 µL aliquot of DNA before addition of 1.5 µL enzyme. Digests were incubated at 37°C for 30 min and profiles separated using a 1 % (w/v) agarose gel at 70 mV.

#### **2.5.3.2 Relative quantification of ACT1 and COX2 using Real-Time PCR analysis**

Quantitative Real-Time PCR (qPCR) analysis was used to assess the relative abundance of nuclear DNA and mtDNA. An entire genome extraction, adapted from Lee *et al.* (Lee *et al.*, 1985), was performed using whole cell populations of rehydrated ADY and control cultures. Cells were harvested by centrifugation (1,000 RCF for 5 min) and re-suspended in 5 ml of washing buffer (1 M Sorbitol (Sigma-Aldrich, U.S.A.), 50 mM KH<sub>2</sub>PO<sub>4</sub> (Fisher Scientific U.K.)). The cells were again harvested and then weighed. Two volumes of washing buffer were used to re-suspend the cells, assuming that 1 g wet weight was equivalent to 1 ml volume. 0.5 ml cell suspension was transferred to a 1.5 ml polypropylene micro-centrifuge tube. 0.5 ml washing buffer and 4 µL β-mercaptoethanol (Sigma-Aldrich, U.S.A.) were also added to the tube which was mixed and incubated for 10 min at room temperature weakening the cell wall. 200 U of lyticase enzyme (Isolated from *Arthrobacter luteus*, Sigma-Aldrich, U.S.A.) solution were added, mixed well and left for 45 min at room temperature to digest the yeast cell wall and form spheroplasts. The suspension was centrifuged for 1 min at 16,000 RCF and the supernatant resuspended in 1 ml 50 mM EDTA pH 8.5 (Sigma-Aldrich, U.S.A.), a chelating agent which binds to DNase cofactors thus protecting DNA. Cells were lysed by the addition of 40 µL SDS solution (10 % w/v) and heating at 65°C for 30 min. The preparation was cooled on ice and 200 µL high-salt solution (3 M Potassium acetate and 2 M glacial acetic acid (Fisher Scientific U.K.)) added, prior to a further 15 min incubation on ice. The lysate was then centrifuged (16,000 RCF for 30 min) removing cell debris, precipitated proteins and SDS, which reacts with the potassium acetate to form an insoluble product. DNA was precipitated when 0.8 ml supernatant was removed to which 0.6 ml iso-propanol (Sigma-Aldrich, U.S.A.) was added and incubated for 5 min at room

temperature. The precipitate was centrifuged (2 min 16,000 RCF) and washed in 0.5 ml 70 % ethanol. The ethanol was removed and the pellet was dissolved in 200 µL of water. RNA was removed by the addition of 5 µL 10 mg/ml RNase and incubation for 60 min at 37°C. DNA was then purified using a phenol-chloroform extraction, removing any residual proteins. 0.5 ml mixture of Phenol – chloroform – isoamyl alcohol (25:24:1) (Sigma-Aldrich, U.S.A.), added to 200 µL of DNA with 100 µL of water in phase lock gel microfuge tubes (Helena Bioscience, U.K.). Tubes were centrifuged (16,000 RCF for 5 min) and the aqueous phase removed and pippetted into a fresh tube with 250 µL chloroform (Sigma-Aldrich, U.S.A.) alone. Tubes were again centrifuged (16,000 RCF for 5 min) and the aqueous phase removed to a fresh tube. 20 µL of 3 M sodium acetate was added followed by 660 µL 100 % ethanol. This neutralised the charge of the phosphate backbone of DNA, making the molecules less soluble, thus the DNA could be harvested by centrifugation (16,000 RCF for 5 min). The DNA pellet that was collected was then washed with 70 % ethanol to remove any residual salt and resuspended in water. DNA was quantified (2.5.1) and adjusted to 10 ng/µL.

Reactions were then prepared following the manufacturers recommendations using the StepOne™ Real-Time PCR System software (Applied Biosystems) (Table 2.5). The relative amounts of COX2 were compared using an ACT1 control sample as the endogenous control; thus control samples were assigned a relative quantification of one for COX2, and the value for dried samples was determined relative to this value. Primers were produced for the nuclear marker ACT1 (5' CGCTCCTCGTGCTGTCTT 3' and 5' TTGACCCATACCGACCATGA 3') based on previously published sequences (Taylor *et al.*, 2005) and the mtDNA marker COX2 (5' GTAACAGCTGCAGATGTTATTCA 3' and 5' CCATAGAAAACACCTTCTCTTG 3') designed based on mtDNA sequence data (Rainieri *et al.*, 2008).

The efficiencies of the primers were determined by running real time PCR on serial dilutions of the sample DNA (10, 5, 1, 0.25, 0.10, 0.05 and 0.01 ng/ $\mu$ l were run in triplicate). Mean Ct values were plotted against the  $\text{LOG}_{10}$  of the concentration of DNA, the straight line equation calculated (Equation 2.4) which was used to calculate the subsequent efficiency values (Equation 2.5) (Rebrikov and Trofimov, 2006).

**Equation 2.4. Formula used to describe linear graphs generated using Microsoft Excel. (a represents the gradient of the line, b the intercept, y the mean Ct value and x  $\text{LOG}_{10}$  of the concentration of DNA)**

$$y = ax + b$$

**Equation 2.5. Efficiency calculation for real-time PCR primers (a represents the gradient of the linear graph) (Rebrikov and Trofimov, 2006).**

$$E = 10^a$$

**Table 2.5. Real-Time PCR reaction mix (\*adjusted for calculation of primer efficiencies).**

COMPONENT	CONCENTRATION	SINGLE REACTION AMOUNT (10 $\mu$ l)	SINGLE REACTION CONCENTRATION
MASTER MIX (APPLIED BIOSYSTEMS)	2X	5 $\mu$ l	1X
FORWARD PRIMER	6.25 pmol/ $\mu$ l	0.2 $\mu$ l	125 nM
REVERSE PRIMER	6.25 pmol/ $\mu$ l	0.2 $\mu$ l	125 nM
SAMPLE DNA	10 ng/ $\mu$ l*	1 $\mu$ l	1 ng/ $\mu$ l*
H <sub>2</sub> O	N/A	3.6 $\mu$ l	N/A

### 2.5.3.3 Triphenyltetrazolium chloride (TTC) overlay

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. YPD plates (20 for each sample) were inoculated with rehydrated ADY and non-dried control cultures of yeast for each strain at a concentration of approximately 50 viable cells per plate (assessed using citrate methylene

blue staining, 2.3.1), thus approximately 1000 cells per sample were assayed in total. Plates were incubated for three days at 25°C. Batches of solution A were prepared by combining 1.26 g sodium phosphate ( $\text{NaH}_2\text{PO}_4$ , Fisher Scientific, U.K.) and 3 g agar, dissolved in water and made up to 100 ml. Batches of solution B were prepared by dissolving 0.2g 2,3,5- triphenyltetrazolium chloride (Sigma-Aldrich, U.K.) in 100 ml water. Both solutions were autoclaved separately and combined in equal amounts to produce TTC agar. The YPD plates were then overlaid with TTC agar and left at room temperature for a further 4 hr, before the percentage of petites was scored. Red colonies were deemed respiratory competent and white colonies were designated as petite mutants.

#### **2.5.3.4 Induction of respiratory deficient cells**

A protocol based on the previous methods described by Rickwood (1991) and Schneider-Berlin *et al.* (2005) was used to induce petite formation using the mutagen ethidium bromide. Yeast cultures derived from dried populations were prepared as described in section 2.2. Non-dried populations were grown using YPD at 25°C and 120 rpm. Cells were washed with water and the viability estimated using the citrate methylene blue staining technique (Pierce, 1970) (section 2.3.1).  $2 \times 10^6$  viable cells per ml were inoculated into triplicate flasks containing 25 ml YPD medium and 100  $\mu\text{M}$  ethidium bromide (Sigma-Aldrich, U.S.A.). Flasks were protected from light using aluminium foil and incubated at 25°C at 120 rpm for 5 hr. Samples were removed at 0 hr and 5 hr for viability assessment (methylene blue) (Section 2.3.1) and petite enumeration (Section 2.5.3). Respiratory deficient cells were also generated during growth on starvation media (1 % yeast extract, 2 % peptone and 0.1M phosphate buffer (Sigma-Aldrich, U.S.A.)) for 15 hr, with samples taken at 0 hr and 15 hr. All samples were examined for petites using the TTC overlay technique previously outlined.

## **2.6 MEMBRANE FUNCTION ANALYSIS**

### **2.6.1 Selection and verification of yeast mutants displaying defective membrane function**

Candidate genes, for various membrane fitness phenotypes, were identified through searches of the *Saccharomyces* Genome Database (SGD) and deletion mutants procured from Euroscarf (Frankfurt, Germany). Deletion mutants obtained from EUROSCARF had the gene of interest interrupted with *kanMX* module, containing a gene conferring kanamycin resistance in *Escherichia coli* and G418 disulphate salt resistance in *S. cerevisiae* (Jimenez and Davies, 1980). Deletion mutants were grown in 100 ml YPD with the addition of 400 µl (50 mg/ml) G418 disulphate salt (Sigma-Aldrich, U.S.A.) for a final concentration of 200 mg/l (Wach *et al.*, 1994). G418 resistance was confirmed by growth of the yeast on YPD plates in which G418 was present for 48 hr at 25°C.

The deletion was also confirmed by PCR analysis. Primers were designed, with the assistance of Vector NTI (Invitrogen Ltd, U.K.), to amplify regions flanking the gene of interest (DNA sequence obtained from Saccharomyces Genome Database <http://www.yeastgenome.org/>). The expected fragment size was calculated based on the position of the primers and the size of the *kanMX4* insert (1634 bp). DNA extracted from cells, according to the method described by Powell and Diacetis (2007) and outlined previously (Section 2.5.2.2), was quantified and diluted to 250 ng/µL. Amplification reactions were performed with Phusion High-fidelity DNA polymerase (New England Biolabs, U.S.A.) (Table 2.7) and using a TC-512 thermal cycler (Techne, U.K.) (Table 2.8). PCR products from the mutant and wild type (Y00000) cells were resolved on a 1 % (w/v) agarose gel at 100 mV for 1.5 hr. Once the deletions were confirmed aliquots were stored at -80°C and working slopes stored at 4°C, with addition of G418 disulphate salt to the media. When cultures were grown for subsequent analysis, there was no addition of G418 disulphate salt.

**Table 2.6. The primer sequence and expected amplification fragment size for confirmation of mutants**

Strain	Forward Primer	Reverse Primer	Annealing temperature (°C)	Amplification fragment size (bp)
Y02667	<i>gcattatTCggTcgTTAG</i>	<i>gcactaacgtgaggTgtaca</i>	56	2116
Y00000	<i>gcattatTCggTcgTTAG</i>	<i>gcactaacgtgaggTgtaca</i>	56	1580
Y00788	<i>gccactagcagtctgctatG</i>	<i>cttatacgcatacacccgctt</i>	65	1203
Y00000	<i>gccactagcagtctgctatG</i>	<i>cttatacgcatacacccgctt</i>	65	669

**Table 2.7. PCR mixture content for confirmation of EUROSCARF strain inserts.**

Component	Volume ( $\mu$ l)/ 20 $\mu$ l reaction	Master Mix Volume ( $\mu$ l)	Final Concentration
H <sub>2</sub> O	10.5	63	N/A
5 x Phusion HF Buffer (New England Biolabs, U.S.A.)	4	24	1x
MgCl <sub>2</sub> (New England Biolabs, U.S.A.)	0.5	3	1.25 mM
2mM dNTPs (New England Biolabs, U.S.A.)	2	12	200 $\mu$ M each
Primer A (Eurofins MWG Operon, Germany)	0.4	2.4	0.5 $\mu$ M
Primer B (Eurofins MWG Operon, Germany)	0.4	2.4	0.5 $\mu$ M
DNA 250 ng/ $\mu$ l	2	N/A	25 ng/ $\mu$ l
Phusion DNA Polymerase (New England Biolabs, U.S.A.)	0.2	1.2	0.02 U/ $\mu$ l

**Table 2.8. PCR cycle for confirmation of EUROSCARF strains. The annealing temperature was dependent on the primer and these specified in Table 2.6.**

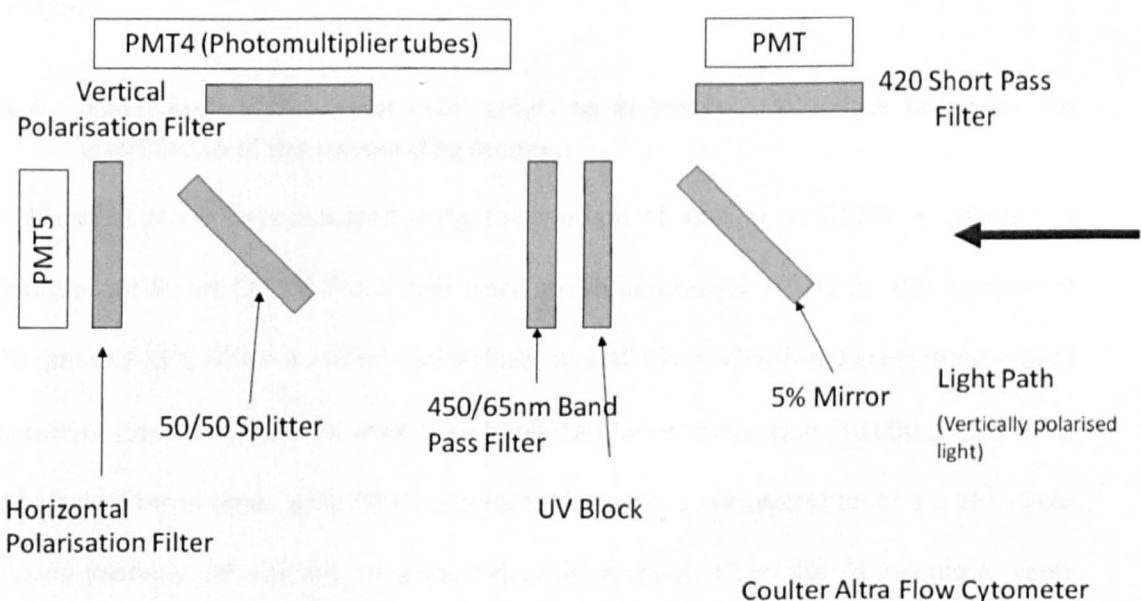
Cycle Step	Temp °C	Time	Cycles
Initial denaturation	98	30 sec	x1
Denaturation	98	10 sec	
Annealing	Primer dependent	30 sec	
Extension	72	60 sec	x35
Final extension	72	10 min	x1

### **2.6.2 Assessment of membrane fluidity using the stains DPH and TMA-DPH**

ADY cultures were rehydrated at 30°C as outlined previously (Figure 2.1). Control cells were grown in 100 ml YPD for 48 hours at 25°C and 120 rpm, contained within a 250 ml conical flask. Cell suspensions were centrifuged (1,500 RCF for 5 min) and washed twice in phosphate buffered saline (PBS, pH 7.4, Oxoid, U.K.), before dilution in PBS to approximately  $1 \times 10^7$  cells/ml. Stock solutions of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Invitrogen Ltd, U.K.) and N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium *p*-toluenesulfonate (TMA-DPH) (Invitrogen Ltd, U.K.) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, U.S.A.) to 200 µM and 500 µM respectively. 10 µL stock solution were added to 990 µl cell suspension (final concentrations of 2 and 5 µM (Kaur and Bachhawat, 1999)) and incubated at room temperature for 1 hour before analysis using a flow cytometer. DPH and TMA-DPH stained cells were analysed separately.

The cytometer used for this analysis was the Beckman-Coulter Altra (U.S.A.) with a laser providing violet excitation (407nm), the excitation maxima of the dyes being approximately 360 nm. The filters of were arranged as shown in Figure 2.1. A 420 nm short pass filter was used to isolate scattered light, with both forward scatter and side scatter recorded. A UV block and 450/65 nm BP filter used to isolate the emission fluorescence of the respective dyes (maxima ≈430 nm). After filtering, the emission fluorescence was split using a 50/50

splitter. Half the emission was filtered by a horizontally polarised filter and half by a vertically polarised filter and signals intensity recorded. Beads were used to calibrate the stream alignment. 100,000 events were recorded. Flow cytometry standard (FCS) data files were analyzed using WEASEL v2.5 (The Walter and Eliza Hall Institute of Medical Research, Australia). Staining was visualised using an Axiovert 135 TV (Carl Zeiss International, Germany) microscope. Magnification was 1000 times the actual size, excitation wavelength 358 nm and emission wavelength 461 nm.



**Figure 2.2. The filter set-up of the flow cytometer used to assess DPH and TMA-DPH staining of yeast cells.**

### 2.6.3 Determination of the sterol content of LAL1 by Organo Balance (Germany)

Control cells were grown following a laboratory propagation regime using YPD medium (Section 2.7.1). Fed-batch yeast cells were grown by Lallemand Inc. (Montreal, Canada) following the propagation regime utilised in the production of dried yeast (Section 0). Fed-batch yeast was subsequently dried, by Lallemand Inc., using a fluidised-bed drier producing dried yeast samples. Cell samples were then dispatched to an Organo Balance, Germany, for sterol content determination. The following is the method utilised by Organo Balance to determine the concentration of sterols in the cell samples described.

Stigmasterol was added to each sample for use as an internal standard. Sterols were saponified using methanolic potassium hydroxide and extracted using n-hexane. Samples were evaporated and the residues dissolved in chloroform. Sterols were then derivatised with N-Methyl-N-trimethylsilyltrifluoro-acetate before being separated using gas-chromatography (HP-5MS column, Agilent, U.S.A.) and detected using mass spectrometry (6890N-5975B, Agilent, U.S.A.). Sterols were quantified using external standards of ergosterol, cholesterol and squalene. Sterols, which are labelled “unknown” could not be identified.

#### **2.6.4 Assessment of the yeast cells' ability to assimilate a substrate based on the acidification of the surrounding medium**

Acidification power was assessed using the method of Kara et al (1988) as adapted in Siddique and Smart (2000). Stock cells were grown aerobically in 100 ml YPD medium at 120 rpm and 25°C within a 250 ml conical flask, and ADY was rehydrated using the standard procedure (Section 2.1). Cells were then harvested by centrifugation (16,000 g for 5 min) and washed three times with RO H<sub>2</sub>O before dilution to a concentration of  $1 \times 10^9$  viable cells/ml (viability determined using methylene blue stain). 19 ml sterile deionised water was dispensed into a universal bottle and continuously stirred. A pH probe was used to measure the pH of the water before the addition of 1 ml yeast slurry and the subsequent recording of the pH at minute intervals, indicating the spontaneous proton efflux of the yeast. At 10 min 5 ml 20.2 % (w/v) filter sterilised (0.45 µM pore size filter (Millipore, U.S.A.)) glucose solution was added and the proton efflux monitored for a further 10 min. The water acidification power test (WAP) was performed using the same method, with the substitution of 5ml of water for the 5ml of glucose solution. The water acidification power, glucose acidification power and glucose induced acidification were then calculated as in Table 2.9.

**Table 2.9. The calculations performed for the measurement of proton efflux from yeast cell populations.**

Arbitrary Term	Calculation
Passive Acidification Power (WAP)	Water pH - pH <sub>20</sub> (pH after 20 minutes) after the addition of water
Substrate Induced Acidification Power (GAP)	Water pH - pH <sub>20</sub> after addition of glucose as a substrate
Glucose Induced Acidification Power (GIPE)	GAP - WAP

#### **2.6.5 The inhibitory effects of membrane stresses assessed using Phenotypic Microarray (PM) technology**

Phenotype microarrays were conducted using the OmniLog system (Biolog Inc., U.S.A.). The Omnilog system is commonly used to identify microorganisms based on cell activity in 10 96-well plates containing various nutrients or inhibitory substances. However, this system can be adapted to allow bespoke plates to be designed by the user. Cells are inoculated into the wells and the respiratory activity is recorded via the reduction of a tetrazolium dye (colourless) to formazan (purple). In this study we used PM technology to investigate the effect of sodium dodecyl sulphate (SDS) and ethanol on cell growth. For the technology to work reaction wells need to contain a buffer called IFY-0 and a dye which were both supplied by the manufacturer (Biolog Inc., U.S.A.). To mimic fermentations, wort was included as the substrate for yeast cells. As these were bespoke plates a degree of optimisation was required to maximise the signal recorded from the dye by the recording instrument (Omnilog, Biolog Inc., U.S.A.), therefore various concentrations of IFY-0, wort, dye and cell concentrations were tested based on the manufacturers recommendations for their premade PM plates. LAL1 and Y00000 were initially tested using 80 µl of various wort:IFY-0 ratios (100 % wort, 75:25, 50:50 and 25:75) as well as 20 µl of differing dye and cell concentrations (2x, 1x and 0.5x the recommended concentrations ). Based on this preliminary work, cells were then tested for their susceptibility to ethanol (Fisher Scientific,

U.K.) and sodium dodecyl sulphate (SDS) (Sigma-Aldrich, U.S.A.). A master mix was produced and aliquoted ensuring that wells were filled with 20 µl wort, 50 µl IFY-0, 2.7 µl dye mix D, 4.2 µl cell suspension (0.62 transmittance) and 3.2 µl H<sub>2</sub>O. 20 µl of various ethanol solutions (filter sterilised, 0.45 µM pore size, Millipore, U.S.A.) were then added to produce a range of ethanol concentrations from 3 % to 14 %. Separate plates were produced in which 20 µl of various SDS solutions (filter sterilised, 0.45 µM pore size, Millipore, U.S.A.) were added to produce a range of SDS concentrations from 100 µg/ml to 2000 µg/ml. LAL1, LAL2 and LAL4 (both ADY and control YPD grown cultures), Y00000 and the two mutants Y02667 and Y00788 were tested for growth in both the inhibitors. Plates were then incubated in an Omnilog instrument (Biolog Inc., U.S.A.) at 25°C for 72 hours. Colorimetric readings were taken every 15 minutes by the Omnilog. Individual Biolog data files were analysed using Omnilog file management/kinetic plot (version OL\_PM\_FM/KIN 1.20.02). Files were compared using parametric analysis (version OL\_PM\_PAR 1.20.02) or Excel (Microsoft, U.S.A.).

## **2.7 MINIATURE SCALE FERMENTATIONS**

### **2.7.1 Control yeast propagation**

10 ml YPD media was inoculated with a loop of yeast stock from a YPD agar slope and incubated for 48 hr at 25°C and 120 rpm. It was then sequentially transferred to 100 ml YPD (48 hr) and 1000 ml YPD (72 hr). After cultivation in this fashion, the yeast slurry was centrifuged at 1,500 RCF for 5 min and the resulting yeast concentrate resuspended 50:50 with spent YPD media. The number of viable cells per ml was established using methylene blue staining (Section 2.3.1) and fermentation media (brewery produced wort) inoculated at  $1.5 \times 10^7$  viable cells per ml.

## **2.7.2 Fed-batch yeast propagation**

Fed-batch propagation was completed in the research laboratories of Lallemand Ltd. (Montreal, Canada) according to the protocol detailed below and provided by Tobias Fischborn.

Working slopes were used to inoculate 20 ml of 10 % (w/v) malt extract medium (Sigma-Aldrich, U.S.A.). These were incubated at 30°C for 48 hr. This 20 ml inoculum was then used to inoculate 1 L of batch medium; consisting of 170 g of pure molasses (50:50 mixture of cane and beet), 2.5 g of Mono Ammonium Phosphate(MAP) (Sigma-Aldrich, U.S.A.), 10 g of Fermaid (Lallemand Inc., Canada), 10 mg Pyridoxine (Sigma-Aldrich, U.S.A.) and was made up to 1Kg with hot tap water. The MAP, pyridoxine, and Fermaid were prepared and sterilized separately and added aseptically at the time of yeast inoculation. To initiate the fed-batch propagation, the 1L batch grown yeast was added aseptically to 5 L of sterilised tap water. 1.16 ml 85 % phosphoric acid (A&C Chemical, Canada), 13.3 mg calcium pantothenate (Sigma-Aldrich, U.S.A.), 10.5 mg thiamine (Sigma-Aldrich, U.S.A.), 167 µg biotin (Sigma-Aldrich, U.S.A.) and 1.67 mg pyridoxine (Sigma-Aldrich, U.S.A.) were added at the start of the propagation. The fed-batch propagation was supplied with a 50 % (w/v) molasses solution and 5 % (w/v) ammonia solution (Sigma-Aldrich, U.S.A.), as determined throughout the propagation. The propagation was completed in a 15 L capacity vessel and was incubated at 28°C and the pH maintained at 5.5 by use of an acid balance (2M sulphuric acid (Sigma-Aldrich, U.S.A.)). Air was supplied to the propagation at an initial rate of 10 l/min.

At the end of the propagation there was a maturation period, prior to harvesting, cooling, and separation. This maturation involved elevating the temperature of the propagation from 28°C to 32°C for 2 hours. At the same time ammonia feeding had ceased and the molasses feeding was reduced to zero. The yeast was then separated and stored cold (4°C),

prior to transportation in a chilled contained (approximately 4°C). The yeast was used within 48 hours of production.

### **2.7.3 Dried yeast rehydration**

Dried yeast rehydration was scaled up from the previous protocol (Section 2.2), although the ratio of water to yeast (10:1) remains the same. A 500 ml capacity Duran bottle was filled with 200 ml tap water and autoclaved (121°C and 103 kPa for 15 min). This was then allowed to attemperate to 25°C in an incubator. 20 g freshly opened ADY was sprinkled onto the water surface and left for 15 min. A magnetic stirrer was then used to fully suspend the ADY, at which point it was left static for a further 45 min. Attemperation is recommended by the manufacturer for temperature shifts between rehydration and fermentation of greater than 10°C. As these fermentations were at 15°C no attemperation was required. The number of viable cells per ml was established using methylene blue staining. Two pitching rates were used. The first, recommended Lallemand Inc. (Montreal, Canada), was 2 g ADY per L medium ( $\approx 1.8 \times 10^7$  cells per ml). The second was inoculated at  $1.5 \times 10^7$  viable cells per ml.

**Table 2.10. The growth parameters and pitching rate of yeast used during miniature scale fermentation experiments**

	YPD Grown- Control	ADY pitched on viable cell number	ADY pitched on cell mass	Fed-batch Produced
Preparation	YPD grown (Section 2.7.1)	Molasses based media and dried using a fluidized bed drier	Molasses based media and dried using a fluidized bed drier	Molasses based medium (Section 0)
Temperature of rehydration/growth	25°C	25°C	25°C	28/32°C
Pitching Rate cells/ml	$1.5 \times 10^7$	$1.5 \times 10^7$	$1.8 \times 10^7$	$1.5 \times 10^7$

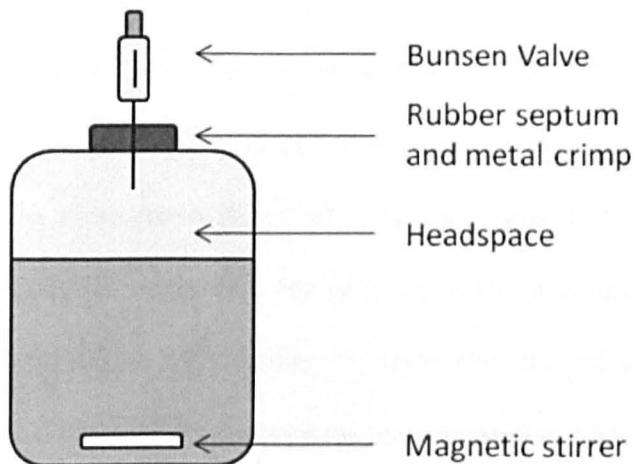
#### 2.7.4 Wort

Industrially produced hopped wort (1.060 s.g.) was collected from the hot paraflow system located in Molson Coors Brewery (Burton-on-Trent, U.K.). Wort was subsequently sterilised (121°C and 103 kPa for 15 min).

#### 2.7.5 Miniature fermenter set-up

Miniature fermentation vessels (FVs) were set-up based on the method described by Quain *et al.* (1985). 100 ml aliquots of brewery produced wort (1.060 s.g.) were transferred to 150 ml sterile Wheaton glass serum bottles (Sigma-Aldrich, U.K.). A foam stopper was placed in the neck, and the FVs were incubated at 15°C to acclimate to fermentation temperature. Magnetic stirrers (approximately 120 rpm) were used to agitate the wort allowing aeration over a period of 24 hours. Yeast was pitched (according to the pitching rates outlined in Table 2.10) and the fermentation vessels were sealed with a rubber septum and a metal crimp. A hypodermic needle was then inserted through the septum. A Bunsen

valve, attached to the needle, allowed the exit of gas from the vessel (Figure 2.3). Fermentations were performed at 15°C and lasted for 6 days monitored by measuring the weight lost by the vessels.



**Figure 2.3. Miniature fermentation vessel schematic**

### 2.7.6 Sampling

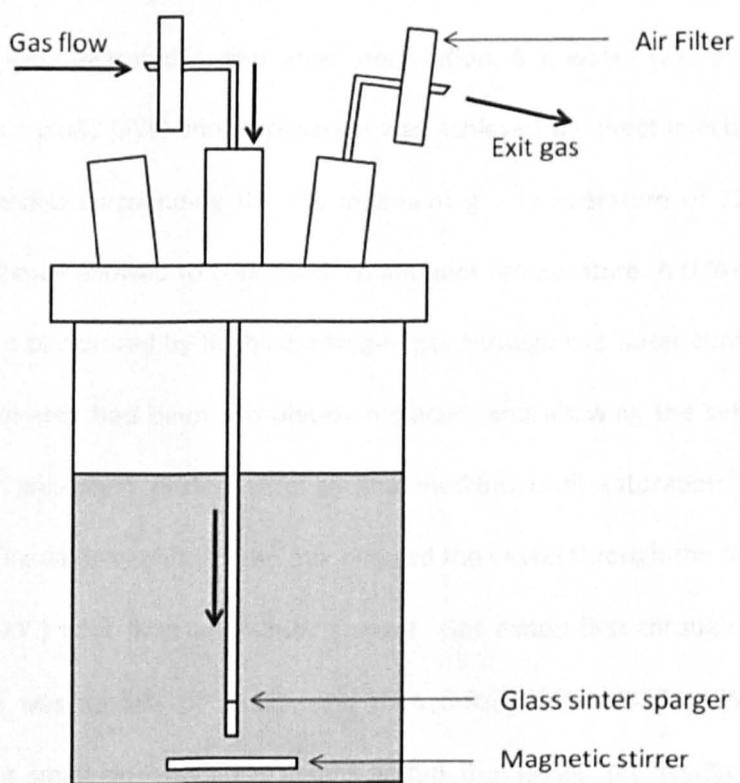
Fermentation vessels were opened, the entire contents decanted into two 50 ml centrifuge tubes and centrifuged at 1,500 RCF for 5 min. As a consequence triplicate mini fermentations were required for each time point assessed. Wort/beer samples from the mini fermentations were then decanted into another centrifuge tube and frozen (-20°C) until analysis. Yeast cells were resuspended in 50 ml sterilised RO water and 3 ml added to 7 ml of 100 % ethanol (Burke *et al.*, 2000). Cells were stored at 4°C or frozen at -20°C, as necessary until required for analysis.

## 2.8 5 L SCALE FERMENTATIONS

### 2.8.1 Control yeast propagation

10 ml YPD media was inoculated with a loop full of yeast stock from a YPD agar slope and incubated for 48 hr at 25°C and 120 rpm, before being transferred to 200 ml YPD (48 hr).

This culture was then used to inoculate 2 L of YPD contained within a propagation vessel (Figure 2.4). The propagation vessel consisted of a reaction vessel (Fisher Scientific, UK) with a magnetic stirrer (Figure 2.4). Oxygen, filtered through a hepa-vent 0.1 µm pore size (Whatman, U.K.), entered through a glass sinter sparger and exited through a heap-vent (direction of gas flow illustrated by arrows on Figure 2.4). Propagation temperature was maintained at 25°C using a flow heater (Grant, U.K.) which heated a water bath containing the propagation vessel. After 72 hours growth, the yeast slurry was then centrifuged at 1,500 RCF for 5 min. The resulting yeast concentrate was resuspended 50:50 with spent YPD media. The number of viable cells per ml was established using methylene blue staining and fermentation media inoculated at  $1.5 \times 10^7$  viable cells per ml.



**Figure 2.4. Propagation vessel. Reaction vessel with air, filtered through a HEPA filter, entering the vessel via a glass sinter sparger (the direction of gas flow through the vessel is indicated the bold arrows). Propagation medium was agitated using a magnetic stirrer.**

## **2.8.2 Dried yeast rehydration**

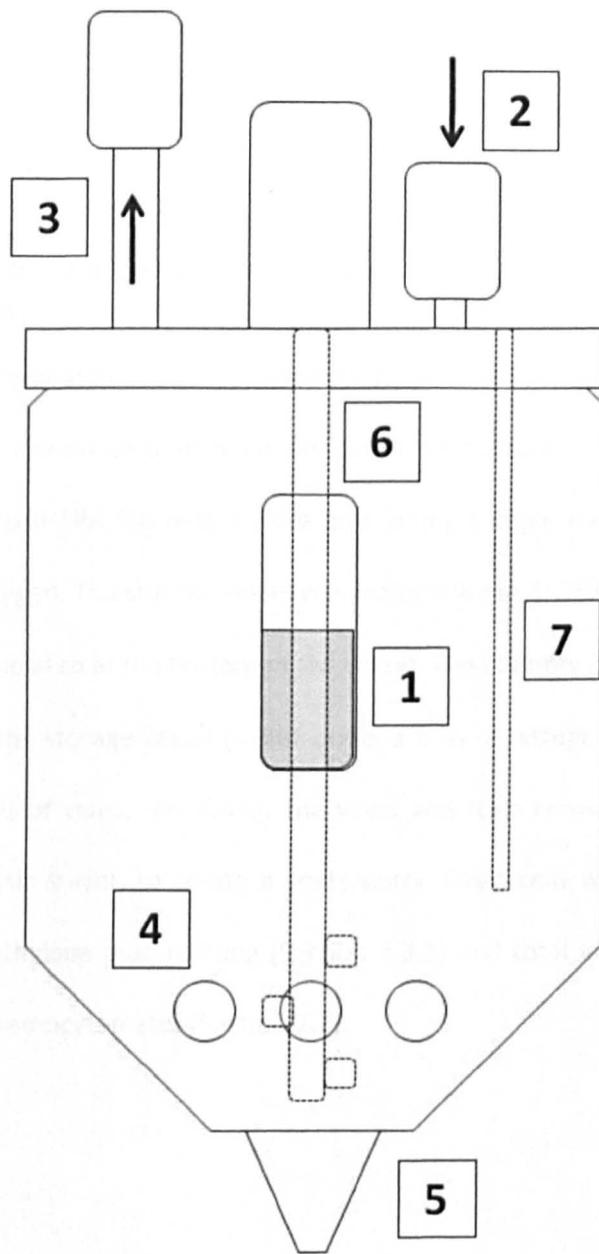
The rehydration parameters were the same as outlined previously in Section 2.7.3, with the exception of the quantity of dried yeast (40 g) and water (400 ml) which were both scaled up as more yeast was required. As stated previously no attemperation was required (Section 2.7.3). The number of viable cells per ml was established using methylene blue staining and fermentation media inoculated at  $1.5 \times 10^7$  viable cells per ml.

## **2.8.3 5 l scale fermentation parameters**

Triplicate 5 l fermentations were completed in 15 l capacity (3 – 10 L working volume) Techfors-S Bioreactors (Infors HT, Switzerland) (Figure 2.5). pH probes (Mettler-Toledo, U.K.) were calibrated at pH 7 and pH 4 and inserted into an Ingold port in each FV. A dissolved oxygen sensor (DO probe) (TruDO, Finesse, Switzerland) was also inserted into an Ingold port and calibrated in-situ after sterilisation. 5 L water was dispensed into the fermentation vessels (FVs) and sterilisation was achieved by direct injection of steam into the double jacket surrounding the FV, maintaining a temperature of 121°C for 15 min. Vessels were then allowed to cool down to ambient temperature. A 0 % calibration of the DO probe was performed by flushing nitrogen gas through the water contained within the vessel until oxygen had been completely replaced, and allowing the sensor's reading to stabilise. Air was then passed through the medium until saturation to allow 100 % calibration. The air/oxygen/nitrogen mix entered the vessel through the NOVASIP-steam in place (Pall, U.K.) inlet filter and sinter sparger. Gas exited first through a condenser, to ensure there was no loss of media, and then through an exit filter (NOVASIP). Whilst maintaining a small positive air pressure within the vessel, the sterilisation water was removed through the bottom sampling point.

Brewery wort (5 l) was transferred to the laboratory aseptically via a sterilised 19 l capacity cornelius vessel (The Cornelius Co., U.S.A). The Cornelius vessel was pressurised using

nitrogen filtered through a hepa-vent 0.1 µM pore size (Whatman, U.K.) and the wort was dispensed into the fermentation vessel through a sampling port in the top plate of the fermentation vessel. Antifoam A (15 % v/v) aqueous emulsion of silicon polymer, Sigma-Aldrich, UK) was autoclaved (121°C and 103 kPa for 15 min) in 5 ml batches and 5 ml was pipetted into the fermentation vessel immediately prior to the addition of wort. Wort was agitated throughout oxygenation and fermentation at 200 rpm. The temperature of the FVs was maintained at 15°C using a chiller unit (FL1703, Julabo, Germany) to circulate temperature controlled water through jackets surrounding each vessel. Once vessels were at the required temperature, a 40 % O<sub>2</sub> and 60 % N<sub>2</sub> gas mix was passed through the sinter sparger for approximately 1 hour (until DO sensor reading stabilised) to allow oxygenation of the wort to 180 % ( $\approx$ 18 ppm). Yeast was inoculated at  $1.5 \times 10^7$  cells per ml. Temperature, pH, DO<sub>2</sub> and stirring values were all recorded using Iris NT (version 5.02.709.0997, Infors, Bottmingen, Switzerland).



**Figure 2.5. Schematic of Infors bioreactor.** 1: Viewing panel. 2: Air inlet, through HEPA filter. 3: Air outlet, first through a condenser and then HEPA filter. 4: Three Ingold ports, one utilized for a pH probe and another for a DO sensor. 5: Sampling port, 8 mm manual valve with tri-clamp connector. 6: Rotor. 7: Temperature probe.

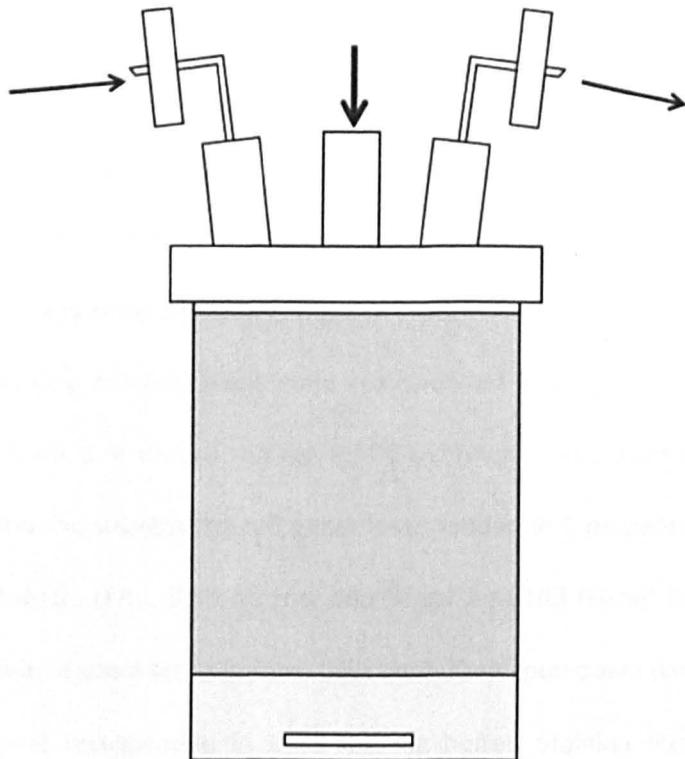
#### 2.8.4 Sampling from fermentation vessels

Samples (50 ml) were removed through an 8 mm manual valve sample port with tri-clamp connector (Figure 2.5) (sterilised with 70 % methylated spirits) every 8 hours (every four at the beginning of fermentation). Collected samples were centrifuged at 1,500 RCF for 5 min and the wort/beer supernatant was decanted into three 15 ml centrifuge tubes and frozen

(-20°C) until analysis. Yeast cell fractions were resuspended in 50 ml sterilised RO water and 3 ml of this suspension pipetted into 7 ml of 100 % ethanol. Cells were stored at 4°C until analysis for DNA content.

#### **2.8.5 The cropping, storage and subsequent repitching of yeast into a new fermentation**

After six days of fermentation, beer was harvested from the fermentation vessel directly into a storage vessel, the set-up of which is illustrated in Figure 2.6. The storage vessel was flushed with nitrogen (HEPA filtered), before and during sample collection, to minimise yeast exposure to oxygen. The storage vessel was maintained at 4°C for 48 hr, during which time the yeast accumulated at the bottom of the vessel. The majority of the separated beer was decanted from the storage vessel (whilst under a flow of nitrogen), leaving a volume, similar to the volume of yeast, remaining. The yeast was then resuspended in the spent wort, using a magnetic stirrer, to create a yeast slurry. Dead cells within the population were stained via methylene blue staining (Section 2.3.1) and total and viable cell counts performed using a haemocytometer (Section 2.2).



**Figure 2.6.** Yeast storage vessel. The small arrows indicate gas flow into and out of the vessel. The large arrow indicates the port through which the sample entered the vessel, which was sealed immediately after vessel was filled.

## 2.9 ANALYSIS OF FERMENTATION SAMPLES

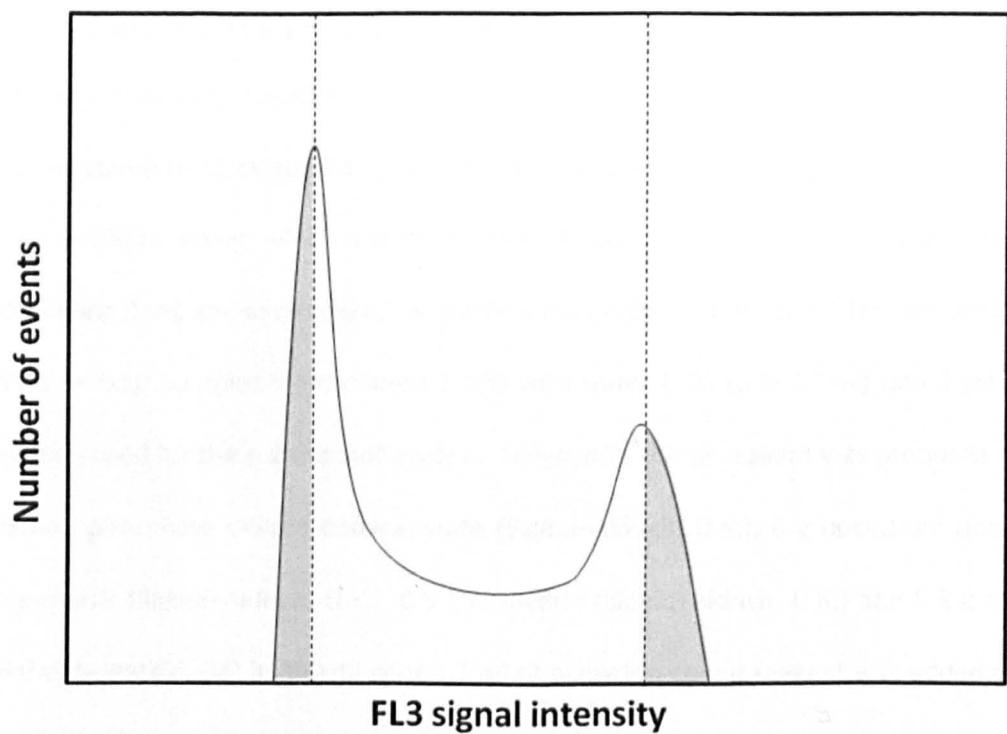
### 2.9.1 Estimation of the DNA content of individual cells

The DNA content of yeast cells was estimated using propidium iodide staining and flow cytometry analysis based on the method of Burke *et al.* (2000). Yeast samples were resuspended in 50 ml sterilised RO water and 3 ml of this suspension was added to 7 ml of 100 % (v/v) ethanol, producing a final concentration of 70 % ethanol. Cell suspensions were stored at 4°C until required at which time 1.5 ml aliquots were placed into a micro-centrifuge tube. Cells were isolated by centrifugation (1,500 RCF for 5 min) and resuspended in 1 ml tris-buffer (50 mM tris/HCl pH 7.5). This was repeated before cells were placed in a sonicating water bath to separate flocs (sonicated for 3 x 10 sec). Cell populations were observed under a light microscope (Olympus BH-2) to ensure complete cell separation. If cells remained joined, sonication was repeated using the same protocol.

Populations comprising discrete cells were collected by centrifugation (1,500 g for 5 min) and the Tris buffer decanted from the tube. 10 ml of 10x RNase stock solution was prepared by dissolving 100 mg of RNase (Sigma–Aldrich, U.K.) in 333 µl of 3 M NaOAc (pH 5) (Sigma–Aldrich, U.K.) and 9.7 ml H<sub>2</sub>O, which was subsequently boiled for 30 min to ensure removal of any residual DNase. The 10x stock was then diluted with Tris buffer to produce a 1x working solution. Cells were resuspended in 1 ml 1x RNase solution and incubated at 37°C for 1 hr before storage at 4°C overnight. Cells were centrifuged (1,500 RCF for 5 min) and the subsequent cell pellet resuspended in 1 ml pepsin solution (50 mg pepsin (Sigma–Aldrich, U.K.), 9.45 ml H<sub>2</sub>O and 550µl 1 M HCl (Fisher Scientific, UK)) and incubated for 5 min at room temperature. Cells were then spun down (centrifuged at 1,500 RCF for 5 min) and resuspended in 1 ml staining buffer. Staining buffer was made by dissolving 50 mg Propidium iodide (Sigma–Aldrich, U.K.), 21.8 g tris (Sigma–Aldrich, U.K.), 10.52 g NaCl (Fisher Scientific, UK) and 14.26 g MgCl<sub>2</sub> (Fisher Scientific, UK) in 800 ml water. The pH was adjusted to 7.5 and the buffer was made up to 1 L with H<sub>2</sub>O. The staining buffer and cells were incubated at room temperature for 1 hour and stored on ice.

Samples were analysed using a Coulter FC 500 flow cytometer with argon laser (488 nm) and FL3 filter (675/30 nm) (Beckman Coulter, U.S.A.). Fluorescence (FL3 channel), forward scatter and side scatter signals were recorded for 100,000 events. Flow cytometry standard (FCS) data files were analyzed using WEASEL v2.5 (The Walter and Eliza Hall Institute of Medical Research, Australia). A scatter plot of forward scatter versus FL3 was used as the basis for the creation of a gate to exclude cell debris (low intensity signal) and cell aggregates (high intensity signal) from the desired data (Massodi *et al.*, 2010). A histogram of FL3 signal intensity (Figure 2.7), using the gated events, was then used to create regions designated “1xDNA” ( $\approx$ 110 signal strength) and “2xDNA” ( $\approx$ 220). The peak reflect method was used to estimate the percentage of cells with 1xDNA and 2xDNA (Givan, 1992). DNA

content was estimated for the triplicate fermentation samples and the standard deviation shown.



**Figure 2.7. Schematic of FL3 histogram and peak reflect method of estimating the DNA content of cells. Dotted lines represent the relative location of peaks expected for 1xDNA and 2xDNA. The total number of cells in each group was estimated by multiplying the number of events in the grey regions (reflecting the peak) by two.**

### 2.9.2 Analysis of alcohol content and specific gravity in wort and beer samples

Beer or wort samples previously obtained from fermentation (Sections 2.7.6 and 2.8.4) were thawed, vortexed and centrifuged at 1,500 RCF for 5 min to remove cell debris and trub. Samples were then analysed with an Alcolyzer-Plus: beer system, which incorporated a DMA 4500 density meter (Anton Paar Ltd., Hertford, U.K.), for combined alcohol content and specific gravity measurement. Alcohol by volume (ABV) measurements were completed, using the Alcolyzer, based on near infrared (NIR) absorption in the 1150 to 1200 nm wavelength range. The density meter measured specific gravity using an oscillating U-tube. Prior to each set of readings, distilled water was used as a control

measurement to ensure the instrument was performing within the manufacturer's specified range and that no calibration was required.

### 2.9.3 Analysis of free amino nitrogen (FAN) in wort and beer samples

FAN was measured based on the ASBC methods of analysis protocol (ASBC, 1992a). A glycine standard stock solution was made by dissolving 107.2 mg glycine (Sigma-Aldrich, U.K.) in 100 ml water, which was then diluted 1:100 with water. This produced a standard containing 2 mg amino nitrogen/L as every 1 mg of glycine contains 0.186 mg of nitrogen. Wort or beer samples were diluted 1:100 with water (100 µl in 10 ml) and 2 ml of this solution used for the subsequent analysis. Ninhydrin colour reagent was produced by 10 g sodium phosphate dibasic dodecahydrate (Sigma-Aldrich, U.K.), 6 g potassium phosphate monobasic (Sigma-Aldrich, U.K.), 0.5 g ninhydrin (Sigma-Aldrich, U.K.) and 0.3 g fructose (Fisher Scientific, UK) in 100 ml water. 1 ml of ninhydrin colour reagent was added to each 2 ml sample in capped test tubes. Tubes were heated for 16 minutes in a boiling water bath, increasing the rate of formation of Rhuemann's purple from ninhydrin and the amine group of amino acids (Rhuemann, 1910). Reactions were then cooled for 20 minutes in a 20°C water bath. 5 ml of a dilution solution (2 g KIO<sub>3</sub> (Sigma-Aldrich, U.K.) dissolved in 600 ml water, added to 400 ml 96 % ethanol (Fisher Scientific, UK)) was added to each sample, mixed thoroughly and the absorbance at 570 nm recorded and compared to that of a water blank. For each set of samples triplicates of glycine standards and blanks were produced. FAN was then calculated using Equation 2.6 (ASBC, 1992a).

**Equation 2.6. Calculation of free amino nitrogen (FAN) in samples of wort and beer as outlined in the ASBC methods of analysis (ASBC, 1992a). The calculation is based on the absorbance of a standard solution of known FAN.**

$$FAN \text{ mg/L} = \frac{\text{net absorbance of test solution}}{\text{net absorbance of glycine standard}} * 200$$

## **2.9.4 Estimation of amino acid content in beer and wort samples**

Amino acids were isolated from samples and derivatized using the EZ:faast amino acid kit (Phenomenex, U.K.). Using the amino acid concentrates supplied with the analysis kit (Phenomenex, U.K.), standard solutions of the targeted amino acids were made at 50, 100 and 200 nmol/ml. 20 µl of each standard, beer or wort sample were combined with 100 µl of internal standard norvaline (20 nmol, Phenomenex, U.K.). This solution was mixed and passed through the EZ:faast solid phase extraction absorbent (contained within a pipette tip), which was subsequently washed with 200 µl propanol (Phenomenex, U.K.). A solution of propanol and sodium hydroxide (200 µl, Phenomenex, U.K.) was then used to remove the absorbent, and the amino acids retained on it, from the pipette tip. 50 µl chloroform (Phenomenex, U.K.) and 100 µl iso-octane (Phenomenex, U.K.) were sequentially added to the solution to derivatize, chemically modify, the amino acids. Derivatization of amino acids was required to produce a compound more suited to GC analysis than amino acids themselves. The amino acids were recovered in the upper organic layer using a Pasteur pipette, dried and the sample redissolved in 100 µl Iso-octane:chloroform (80:20 v/v, Phenomenex, U.K.). Samples were transferred into a GC vial insert, which was placed inside a vial and capped all provided in the analysis kit (Phenomenex, U.K.). Prior to analysis using Gas Chromatography – Mass Spectrometry (GC-MS), samples were stored at -20°C and assigned a random running order. A random running order was used to ensure any systematic variation within the analysis apparatus which may occur, with respect to length of time it was operated or the number of samples that had been processed, were not biased towards particular time points.

For GC-MS analysis, 1 µl of the sample was injected in splitless mode (split close for 10 sec) using an AS3000 auto-sampler (Thermo Fisher Scientific, U.K.). The injector of the trace GC ultra gas chromatograph (Thermo Fisher Scientific, U.K.) was maintained at 250°C, with an initial oven temperature of 90°C which was increased to 320°C at 20°C/min (transfer line

from the oven to mass spectrometer, 300°C). Helium (55 kPa) was used as the carrier gas to elute the amino acids from the ZB-AAA column (10 m x 0.25 mm internal diameter, 0.1 µm film thickness, Phenomenex, U.K.). Preliminary runs were performed in full ion mode, allowing selection of appropriate ions and windows of detection (outlined in Table 2.11). The DSQ II mass spectrometer (Thermo Fisher Scientific, U.K.) was then operated in selected ion mode (outlined in Table 2.11). The ratio of the amino acid to the internal standard was used to normalise individual samples. The concentrations of amino acids in standard solutions (known concentrations of amino acids) were shown to have linear relationships with the peak areas on the chromatograph. The multiplication factor of this linear relationship, which varied depending on the amino acid, was then used to calculate the unknown amino acid concentrations of samples.

**Table 2.11.** The amino acids detected during GCMS analysis of wort and beer samples. The retention time in the column of each amino acid is indicated as well as the ion used for its detection and quantification. The windows of detection and the ions detected during these windows are also outlined.

Amino acid	Abbreviation	Retention time (min)	Ion for quantification	Ion detected in selective ion mode (SIM)	Window of detection (min)
Alanine	ALA	2.15	130	101, 114, 130, 144, 158	0.00-2.42
Sarcosine	SAR	2.27	130		
Glycine	GLY	2.35	144		
Abscisic acid	ABA	2.53	144	116, 130, 144, 158, 172	2.42-3.00
Valine	VAL	2.70	158		
$\beta$ -aminoisobutyric acid	$\beta$ -AIB	2.81	116		
Norvaline	NOR (IS)	2.91	158	116, 130, 156, 172	3.00-3.35
Leucine	LEU	3.06	172		
allo-Isoleucine	AILE	3.10	130		
Isoleucine	ILE	3.15	172		
Threonine	THR	3.49	101	101, 144, 146, 156, 180, 243	3.35-3.74
Serine	SER	3.55	146		
Proline	PRO	3.66	156		
Asparagine	ASN	3.83	155	116, 130, 155	3.74-4.23
Aspartic acid	ASP	4.74	130	101, 116, 130, 146, 172, 244	4.23-5.51
Methionine	MET	4.78	101		
4-Hydroxyproline	4HYP	4.99	172		

**Table 2.11 continued. The amino acids detected during GCMS analysis of wort and beer samples.**  
**The retention time in the column of each amino acid is indicated as well as the ion used for its detection and quantification. The windows of detection and the ions detected during these windows are also outlined.**

Amino acid	Abbreviation	Retention time (min)	Ion for quantification	Ion detected in selective ion mode (SIM)	Window of detection (min)
Glutamic Acid	GLU	5.32	172	101, 116, 130, 146, 172, 244	4.23-5.51
Phenylalanine	PHE	5.34	146		
$\alpha$ -Aminoadipic acid	AAA	5.80	184	84, 101, 114, 156, 184, 244	5.51-7.15
Glutamine	GLN	6.31	84		
Ornithine	ORN	6.98	156	116, 155, 172, 170, 180	7.15-7.93
Lysine	LYS	7.40	116		
Histidine	HIS	7.69	180	107, 130, 206, 244	7.93-9.00
Tyrosine	TYR	8.14	116		
Tryptophan	TRP	8.58	130	146, 155, 248	9.00-11.00
Cystine	C-C	9.74	248		

### 2.9.5 Internal amino acid content of yeast cells

Control yeast (cultivated on YPD medium), Fed-batch yeast and ADY samples of LAL1 were assayed for their relative amounts of amino acid. The extraction of amino acids was based on the method of Martinez-Force and Benitez (Martinez-Force and Benitez, 1995). Cells were harvested by centrifugation, resuspended in 5 ml water and washed 5 times with distilled water. The suspension was placed in a boiling water bath for 15 minutes to lyse cells, centrifuged and the pellet discarded. The supernatant was filtered through a 0.45  $\mu$ m pore size filter (Millipore, U.S.A.) and stored at -20°C until required for analysis. 100  $\mu$ l of

this supernatant was analysed for amino acid concentration using the EZ:faast analysis kit (Phenomenex, U.K.) as described previously (Section 2.9.4).

#### **2.9.6 Analysis of the sugar content of wort and beer by high performance liquid chromatography (HPLC)**

Standard stock solutions of sugars were made in 10 ml volumes to the concentrations outlined in Table 2.12. Stock solutions were mixed to produce 'standard 1', which was sequentially diluted (5 ml: 5 ml) to produce Standards 2, 3 and 4 (Table 2.12). For each analysis subsequently described, water was used as a blank reference. For the analysis of unknown samples, wort or beer was compared to a standard solution by pipetting 1ml of each sample into a 1.5 ml microfuge tube with 100 µl of internal standard (Melizitose 100 mg/ml). The mixture was then passed through a solid phase extraction cartridge (strata-X 33 µm Polymeric Reversed Phase 30mg/1ml cartridge Phenomenex, U.K.), previously conditioned by passing 1 ml methanol through the sorbent bed and equilibrated with 1 ml of water. After this cleanup procedure the samples were aliquoted into glass vials in preparation for HPLC analysis.

Samples were arranged in a random running order and placed in the automatic sampler. A random running order was used to ensure any systematic variation within the analysis apparatus which may occur, with respect to length of time it was operated or the number of samples that had been processed, were not biased towards particular time points.

5 µl of sample was injected onto an amino column (250 mm x 4.6 mm internal diameter, Spherisorb NH<sub>2</sub> with 5 µm particle size, Waters Corporation, U.K.). The sugars were eluted using an 80 % acetonitrile 20 % water mix (which was sonicated to degass the liquid prior to use) at a flow rate 0.7 ml/min. Detection of sugars was performed using a Wyatt Refractive Index Detector (Wyatt Technology Corporation, U.S.A.). Chromatograms were analysed using the AZUR chromatography data system (Datalys, France). The elution order

of the sugars was the same as previously described (Buckee and Long, 1982), and the retention times are outlined in Table 2.13. Peaks were manually assigned, subtracting the baseline signal from the peak height. The ratios of standard to the internal standard (IS, melizitose) were then plotted against the known concentration in the sample, to check for linearity of detection and determine the quantifying factor for unknown samples (which was the gradient of the graph as the response was linear). The ratio of target compound to IS (melizitose) peak areas was then used to calculate the quantity of the unknown sugar in the sample (Equation 2.7).

**Table 2.12. The composition of standard sugar solutions used during the quantification of the sugar content of wort and beer samples by HPLC**

Sugar	Standard Stock solution (mg/ml)	Concentr- ation	Amount in 10 ml Standard 1 (ml)	Standard 1 (mg/ml)	Standard 2 (mg/ml)	Standard 3 (mg/ml)	Standard 4 (mg/ml)
Sucrose	300	X 10	1	30	15	7.5	3.75
Fructose	100	X 10	1	10	5	2.5	1.25
Glucose	300	X 10	1	30	15	7.5	3.75
Maltose	300	X 2	5	150	75	37.5	18.75
Maltotriose	300	X 10	1	30	15	7.5	3.75
Glycerol	60	X 10	1	6	3	1.5	0.75

**Equation 2.7. Calculation to quantify the amount of sugar in an unknown sample of wort or beer based on a gradient value calculated from the standard sugar solutions**

$$\frac{\text{Target compound peak area}}{\text{Internal standard peak area}} * \text{Gradient} = \text{Target compound in sample (mg/mL)}$$

**Table 2.13. Retention time of the sugars in the amino column used for their separation during HPLC analyses.**

Sugar	Expected Time (min)	Retention
Glycerol	1.63	
Fructose	2.42	
Glucose	2.95	
Sucrose	3.97	
Maltose	4.77	
Melizitose (IS)	6.92	
Maltotriose	9.37	

### **2.9.7 Assessment of the vicinal diketone content (VDK) of wort and beer samples**

Diacetyl (Sigma-Aldrich, UK) and 2,3-pentanedione (Sigma-Aldrich, UK) standards of 20, 10, 5, 1, 0.5, 0.25, 0.125 and 0 ppm were prepared in 5 % (v/v) ethanol (Fisher Scientific, UK). A 250 ppm stock solution of the internal standard 1,2-dichloropropane (Sigma-Aldrich, UK) was prepared in absolute ethanol, which was subsequently diluted 1:50 with water to produce a working solution. In a 22 ml headspace flask (Fisher Scientific, UK) 5ml of wort, beer or standard solution was combined with 100µl of internal standard working solution and 3.5 g of ammonium sulphate (Sigma-Aldrich, UK). The mixture was agitated and the vial sealed using an aluminium crimp seal with pre-fitted septum (Fisher Scientific, UK). The

vials and contents were heated for 30 minutes at 70°C, using a GC oven (Thermo-Fisher Scientific, U.K.), and allowed to cool to room temperature.

The samples were equilibrated for 5 minutes at 45°C with agitation (500 rpm), before 1 ml of headspace was sampled using the combiPAL autosampler (CTC Analytics, Switzerland) in automated head space syringe mode with a the syringe temperature at 60°C. The injector of the gas chromatograph (Trace GC Ultra, Thermo Fisher Scientific, U.K.) was maintained at 125°C, whilst the initial oven temperature was 40°C. The oven remained at 40°C for 2 min and was subsequently increased to 130°C at a rate of 10°C per minute. Helium (1 ml/min constant flow) was used as the carrier gas to elute the diacetyl and 1,2-dichloropropane from the ZB-Wax column (Phenomenex, U.K.) which was 30 m in length, had an internal diameter of 0.25 mm and 1 µm film thickness.

The DSQ I mass spectrometer (Thermo-Fisher Scientific, U.K.) was operated in selected ion mode (see Table 2.14 for ions detected) with a dwell time of 0.45 sec. The chromatograms produced from the mass spectrometer were recorded and analysed using Xcalibur software (Thermo-Fisher Scientific, UK). The peaks were manually assigned, subtracting the baseline signal from the peak height. The standard values were then plotted against the known concentration in the sample to check for linearity of detection and to determine the quantifying factor (gradient of the graph). The ratio of target compound to the internal standard (1,2- dichloropropane) peak area was then used to calculate the quantity of the target sugar in the sample.

**Table 2.14. The compounds detected during head-space analysis used to assess the unknown VDK content of wort and beer samples.**

Compound	Approximate retention time (min)	Ion detected
Ethanol	6.11	43
Diacetyl	6.93	86
1,2- dichloropropane (IS)	8.23	63
2,3-pentanedione	8.37	43

**Equation 2.8. The calculation for unknown VDK content of beer and wort samples using chromatographs generated during headspace analysis**

$$\frac{\text{Target compound peak area}}{\text{Internal standard peak area}} * \text{Gradient} = \text{Target compound in sample (ppm/mL)}$$

## 2.10 STATISTICS

Although some specific statistical analysis has already been described, some techniques are common throughout the study. These are described here and it has been indicated in the results sections when they have been used.

Calculation of means and standard deviations of triplicate samples were completed using Excel (Microsoft, U.S.A.). Analyses of statistical differences between samples were completed using Minitab (Version 16, Minitab Inc., U.S.A.). In each instance the null hypothesis was that no significant difference existed between data sets. One-way analysis of variance (ANOVA) was performed using Minitab. If the P value generated by the test was less than 0.05 then the null hypothesis of no significant difference was rejected. Whilst ANOVA can indicate an overall significant difference between data sets, further analysis is required to assess where the difference or differences exist. Using Minitab pair-wise comparison of means was completed using the Tukey *a posteriori* test. When statistical analysis has been completed, the test used has been indicated in the results section.

## CHAPTER 3: REHYDRATION OF ACTIVE DRY BREWING YEAST AND ITS EFFECT ON CELL VIABILITY

Data from this Chapter has been used in the preparation of a manuscript entitled *Rehydration of active dry brewing yeast and its effect on cell viability*, which has been accepted for publication in the Journal of the Institute of Brewing and Distilling.

### 3.1 INTRODUCTION

A common observation concerning the condition of active dried yeast (ADY) is that the viability is poor (between 60-80 %) in comparison to that exhibited by propagated yeast (approaching 100 %). The low viability can be compensated for by the application of a higher pitching rate, and it has been demonstrated that viability will subsequently recover during fermentations and serial repitching to more favourable levels (Powell and Fischborn, 2010). Over pitching using a population with poor viability does, however, increase the number of dead cells in the fermentation. The presence of dead cells has been suggested to result in abnormal flocculation, haze formation and less stable foam structure, characteristics which have been observed during fermentations utilising dried yeasts (Finn and Stewart, 2002). Therefore, improvements to this parameter are important in order to increase the chances of this technology being widely adopted by the brewing industry.

Although there are stresses involved in the propagation of ADY (discussed in Chapter 1), these are not thought to result in cell death. However, the effects of drying and subsequent rehydration on cell populations are believed to be responsible for the loss in viability. It is therefore proposed that optimisation of both processes may lead to increased cell viability.

The process by which cells are dried can influence the viability of a yeast culture dramatically. Fluidised bed drying is the most common means of producing ADY for direct use in industrial fermentations, although other forms of drying such as freeze-drying and spray drying also exist. Freeze-drying consists of the removal of water by sublimination of a

frozen culture under vacuum (Kawamura *et al.*, 1995). Viability of yeast preserved in this manner is sometimes as low as 0.1 % (Lodato *et al.*, 1999) and consequently this method is not suitable for yeast storage. Spray-drying utilises a stream of hot air to rapidly dry droplets of a slurry solution, producing a powder (Morgan *et al.*, 2006). Whilst industrial scale spray-drying is common for some bacterial species (Millqvist-Fureby *et al.*, 2000; Corcoran *et al.*, 2004), it is not widely used for yeast as it also produces low viability cultures (Elizondo and Labuza, 1974). Drying using a fluidised bed system (Bayrock and Ingledew, 1997a; Grabowski *et al.*, 1997) is less stressful to yeast cells than both freeze and spray drying and is furthermore capable of producing large quantities of biomass.

Whilst the mode of dehydration can influence the capacity of a yeast cell to recover from desiccation, the process of rehydration is equally significant. Rehydration of the yeast is the domain of the brewer, instead of the ADY manufacturer, therefore optimisation and communication of the mechanism of rehydration is important for the success of the technology. Previous studies have investigated the effect of temperature (Simonin *et al.*, 2008), media composition and rehydration protocols (Attfield *et al.*, 2000; Soubeyrand *et al.*, 2006; Vaudano *et al.*, 2009) on the recovery of ADY. However, these studies have focused on the analysis of wine and baking yeast strains, which are typically more tolerant to the stresses imposed by dehydration and rehydration than brewing yeast (Tobias Fischborn, personal communication). Optimisation of the rehydration procedure for brewing yeast strains has only been the subject of limited investigation (Gosselin and Fels, 1998; Fischborn *et al.*, 2004). In this study the effect of rehydration time and temperature on the viability of yeast cultures after rehydration was assessed, as well as methods to estimate live cells in rehydrated ADY cultures.

## **3.2 RESULTS**

Optimisation of the rehydration procedure and accurate measurement of cell viability ensures the pitching yeast is of maximal viability, reducing any deleterious effect that high concentrations of dead cells may have on fermentation. Three industrially produced ADY strains were rehydrated at various temperatures and assessed using four different methods of viability assessment. The data generated were used to assess the effect of rehydration time (Sections 3.2.1 and 3.3.1) and temperature (Section 0) on yeast viability, as well as the most appropriate method of assessing viability in ADY (Section 3.2.3 and 0).

### **3.2.1 The effect of rehydration time on estimated cell viability**

Typical protocol requires yeast to be rehydrated for up to an hour before utilisation. Because of operational constraints this practice is often not adhered to when ADY is employed on an industrial scale. To determine the effect of rehydration time on the viability of the subsequent culture, rehydration was conducted according to the protocol outlined in Chapter 2. Samples for viability testing were recovered in triplicate before rehydration (Sample Point A), after initial mixing (Sample Point B) and subsequently at 15 min intervals (Sample Points C1-C4). The viability of each culture was assessed using slide culture, bright field and fluorescent stains.

Analysis of samples at time point A revealed that although some variation in viability was observed according to the method employed, viability was exclusively lower (25-50 %, 40-55 % and 20-55 % for strains LAL1, LAL2 and LAL4 respectively) than at subsequent sample points for each of the strains analysed (Figures 3.1-3.6). At sample point C4, when rehydration was deemed to be complete, the viability was observed to range from approximately 65-75 % for strain LAL1 (Figures 3.1-3.2) to 75-85 % for strains LAL2 and LAL4.

### **3.2.2 The effect of rehydration temperature on cell viability**

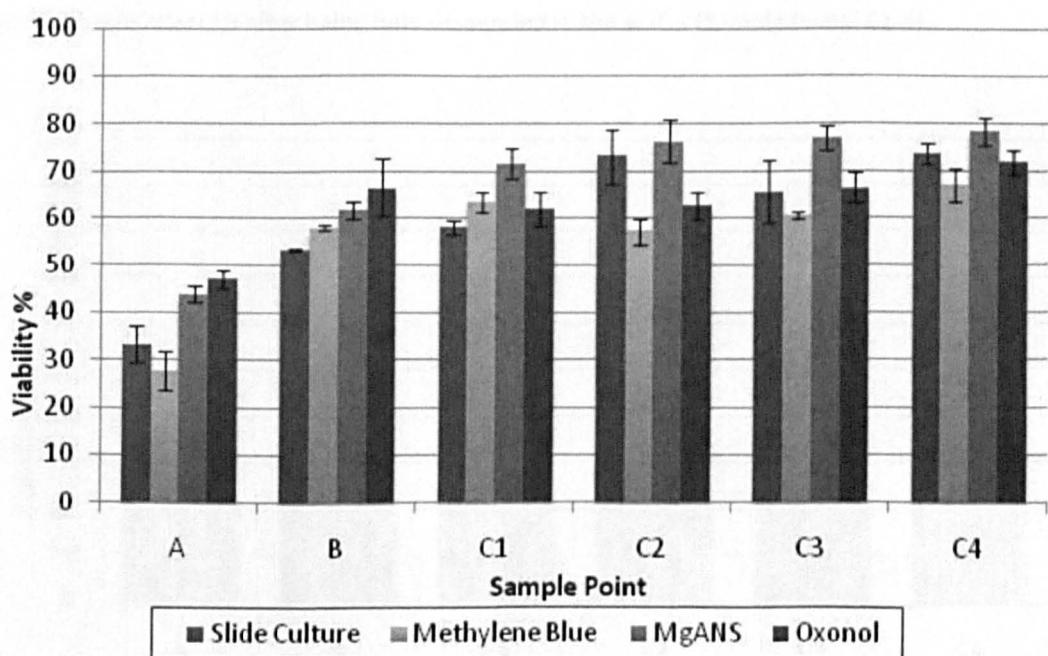
Laroche and Gervais (2003) suggested that survival during rehydration is determined by the osmotic pressure, the temperature of the yeast cell and surrounding medium. These factors are key in determining the state of the membrane, with rehydration across an unstable membrane a significant cause of cell death. Phase transitions, the source of instability, may be avoided depending on the temperature of rehydration. In order to determine the effect of rehydration temperature on cell viability, each yeast strain was rehydrated at both 25°C and 30°C.

The final viabilities of ADY cultures (Table 3.1) were analysed using a multi-factorial ANOVA to determine the significance of the temperature of rehydration and method of assessment on the viability estimation of each strain. Analysis was made based on a nested design, in which assessment type was nested within temperature of rehydration.

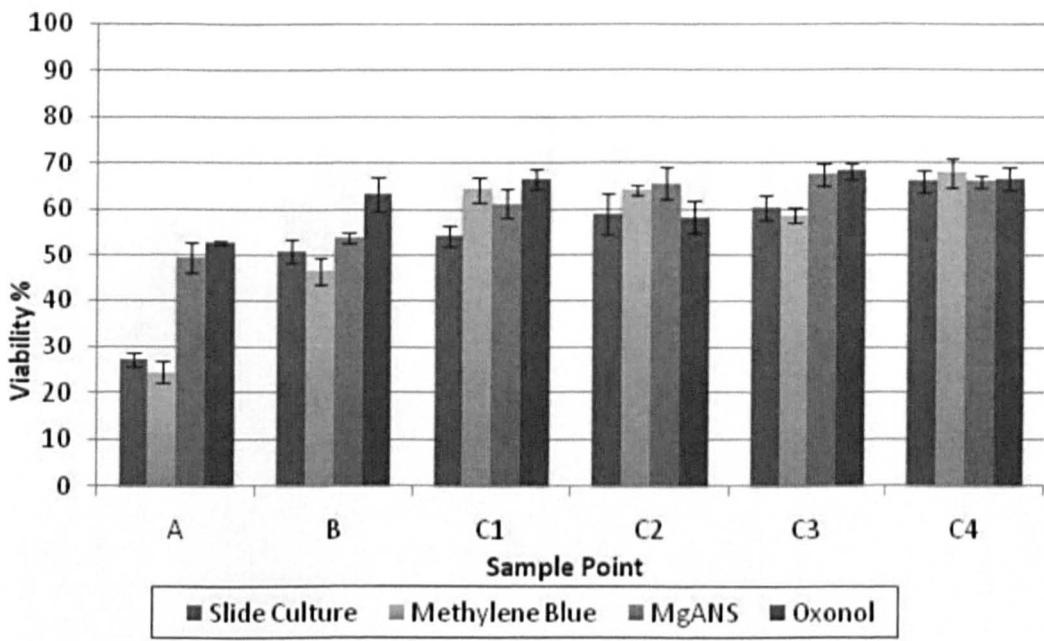
For the lager strain LAL1 it can be seen that cell survival was dependent on the temperature (statistically significant at P<0.05) of rehydration, with a greater viability at time point C4 (complete rehydration) when 25°C was applied compared to rehydration at 30°C (Figures 3.1 and 3.2). Although some variation was observed according to the method by which viability was estimated, at 25°C a viability of approximately 73 % could be achieved compared to that of 67 % at 30°C (Table 3.1). This pattern of results was not observed for the other strains analysed, with the ale strain LAL4 exhibiting a higher viability when rehydrated at 30°C (approximately 80 %) than at 25°C (approximately 72 %) although this was not statistically significant. Interestingly, the viability of ale strain LAL2 did not appear to be as temperature dependent, with similar viabilities observed at both 25°C and 30°C (approximately 75 % and 78 % respectively).

### 3.2.3 Methods for assessing ADY Viability

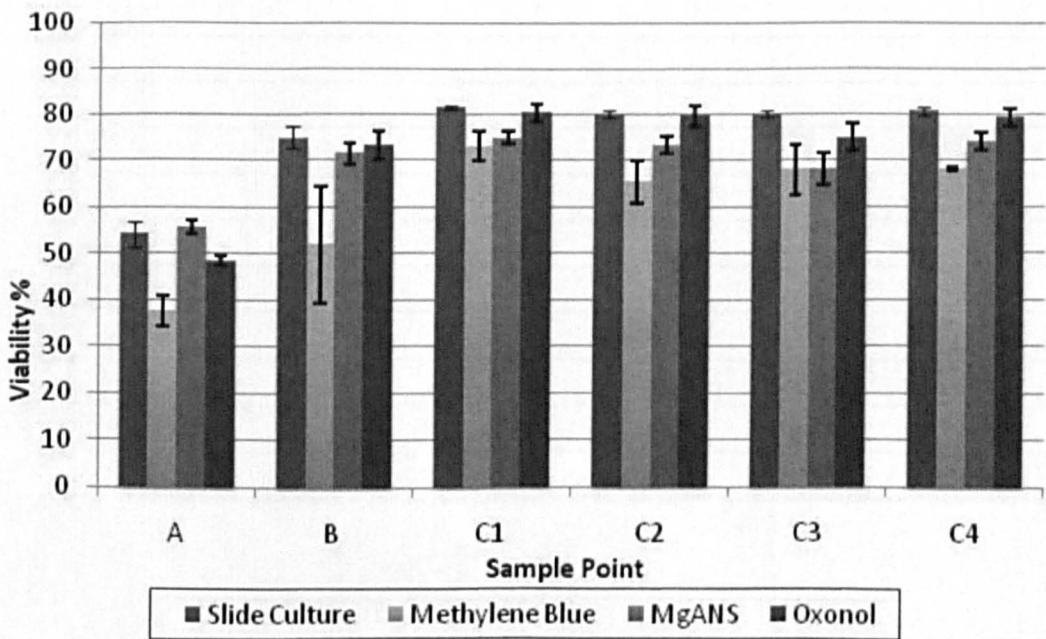
Analysis of samples at time point A revealed that a large variation in viability was obtained when different methods were applied (Figures 3.1-3.6). This variation may reflect the period of most change within the yeast cells, and population as a whole, which displays greater variation with respect to viability assessment. At sample point C4, when rehydration was deemed to be complete, the discrepancies between the percentage viability when different assessment techniques were applied was considerably reduced. Irrespective of the means by which the viability of the yeast slurry was determined no specific trends were observed and no individual technique gave consistently high or low estimates.



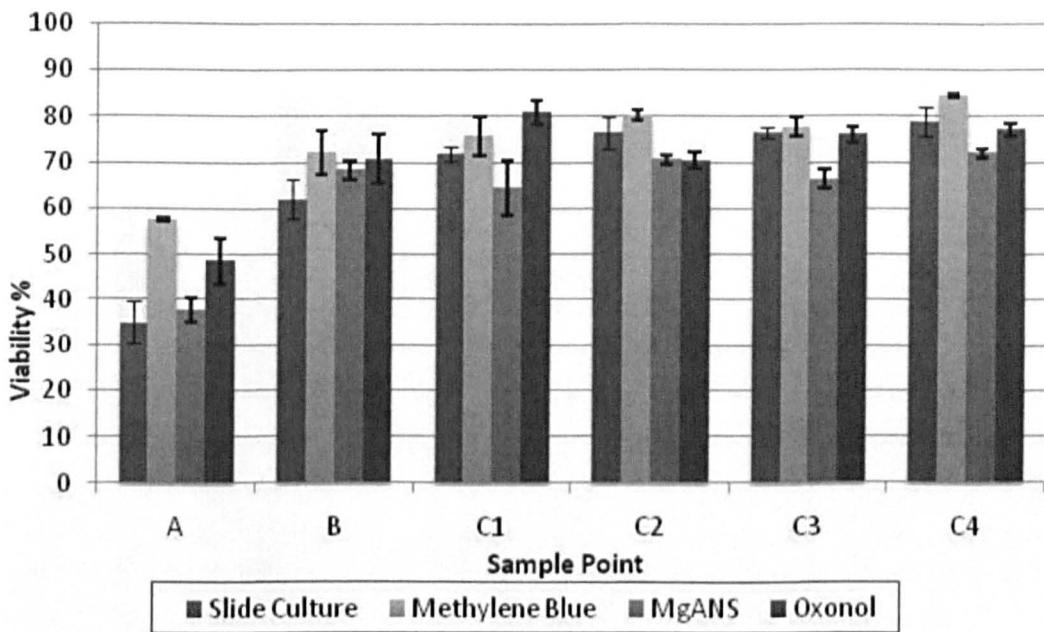
**Figure 3.1** Viability during rehydration of LAL1 at 25°C. Mean viabilities of ADY populations (triplicate samples with standard error shown). Yeast were assessed immediately after removal from the package (Sample Point A), after 15 min settled on the surface of water (Sample Point B) and at 15 min intervals after being fully suspended in the water (Sample Points C1-4).



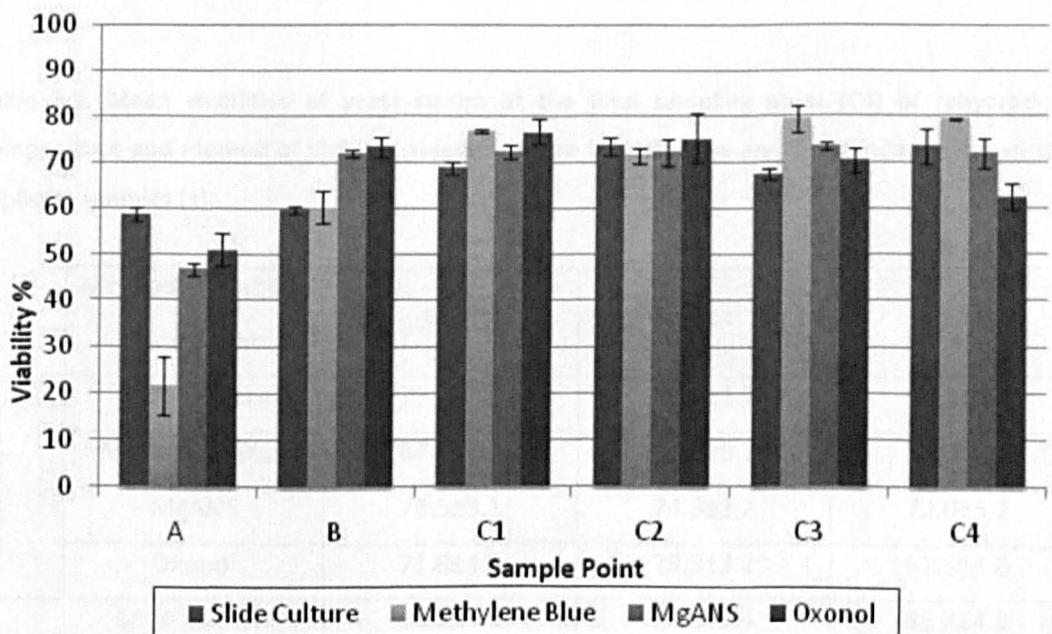
**Figure 3.2 Viability during rehydration of LAL1 at 30°C.** Mean viabilities of ADY populations (triplicate samples with standard error shown). Yeast were assessed immediately after removal from the package (Sample Point A), after 15 min settled on the surface of water (Sample Point B) and at 15 min intervals after being fully suspended in the water (Sample Points C1-4).



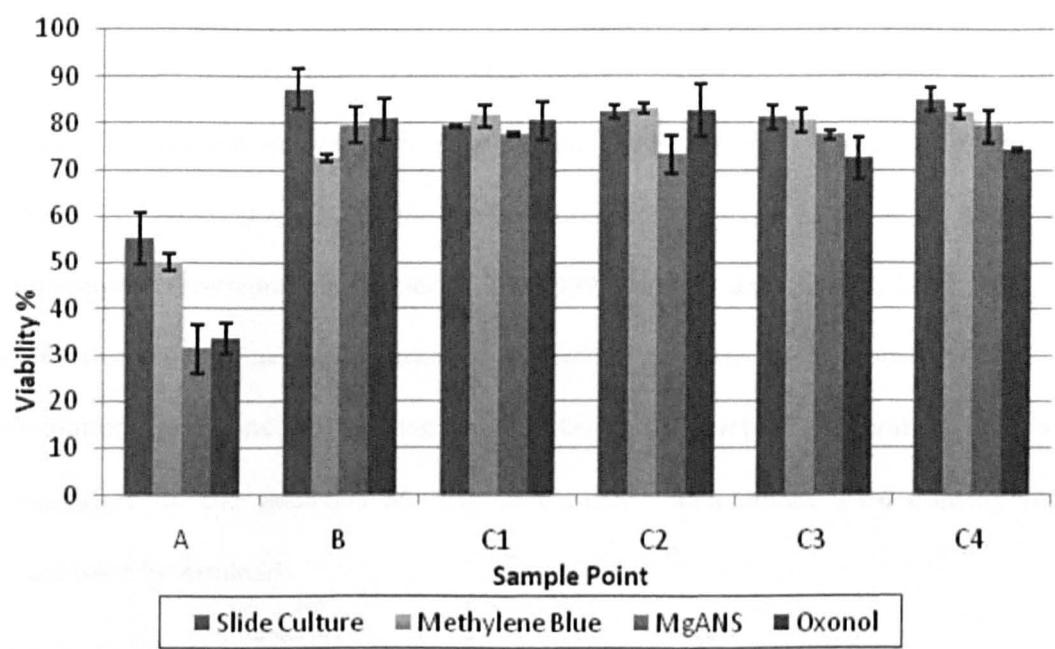
**Figure 3.3 Viability during rehydration of LAL2 at 25°C.** Mean viabilities of ADY populations (triplicate samples with standard error shown). Yeast were assessed immediately after removal from the package (Sample Point A), after 15 min settled on the surface of water (Sample Point B) and at 15 min intervals after being fully suspended in the water (Sample Points C1-4).



**Figure 3.4** Viability during rehydration of LAL2 at 30°C. Mean viabilities of ADY populations (triplicate samples with standard error shown). Yeast were assessed immediately after removal from the package (Sample Point A), after 15 min settled on the surface of water (Sample Point B) and at 15 min intervals after being fully suspended in the water (Sample Points C1-4).



**Figure 3.5** Viability during rehydration of LAL4 at 25°C. Mean viabilities of ADY populations (triplicate samples with standard error shown). Yeast were assessed immediately after removal from the package (Sample Point A), after 15 min settled on the surface of water (Sample Point B) and at 15 min intervals after being fully suspended in the water (Sample Points C1-4).



**Figure 3.6 Viability during rehydration of LAL4 at 30°C.** Mean viabilities of ADY populations (triplicate samples with standard error shown). Yeast were assessed immediately after removal from the package (Sample Point A), after 15 min settled on the surface of water (Sample Point B) and at 15 min intervals after being fully suspended in the water (Sample Points C1-4).

**Table 3.1. Mean viabilities of yeast strains at the final sampling point (C4) of rehydration.** Temperature and method of viability assessment are indicated, as are the standard error of the triplicate samples ( $\pm$ ).

	Method of Viability Assessment	LAL1	LAL2	LAL4
Rehydrated at 25°C	Slide Culture	73.7±3.9	80.8±1.7	73.5±6.9
	Methylene Blue	67.0±6.1	68.2±0.7	79.4±0.5
	MgANS	78.5±5.1	74.3±3.2	72.0±5.7
	Oxonol	71.8±4.4	79.3±3.2	62.5±4.8
Rehydrated at 30°C	Slide Culture	66.1±4.4	78.9±5.4	85.2±4.2
	Methylene Blue	68.0±5.6	84.7±0.9	82.6±2.7
	MgANS	66.1±2.2	72.2±1.6	79.4±6.1
	Oxonol	66.8±4.2	77.2±2.2	74.4±0.7

### **3.3 DISCUSSION**

The viabilities of industrial ADY cultures are believed to be dependent on multiple factors including the strain employed (Luna-Solano *et al.*, 2000), the method of drying (Grabowski *et al.*, 1997; Lodato *et al.*, 1999), the temperature of drying (Luna-Solano *et al.*, 2000), the temperature of rehydration (Poirier *et al.*, 1999; Laroche and Gervais, 2003), and the rehydration media (Rodriguez-Porrata *et al.*, 2008). In order to determine the effect of rehydration conditions on brewing yeast cells, the impact of rehydration time and temperature on the viabilities of three industrially manufactured dried brewing yeast strains were determined.

#### **3.3.1 The effect of rehydration time on estimated cell viability**

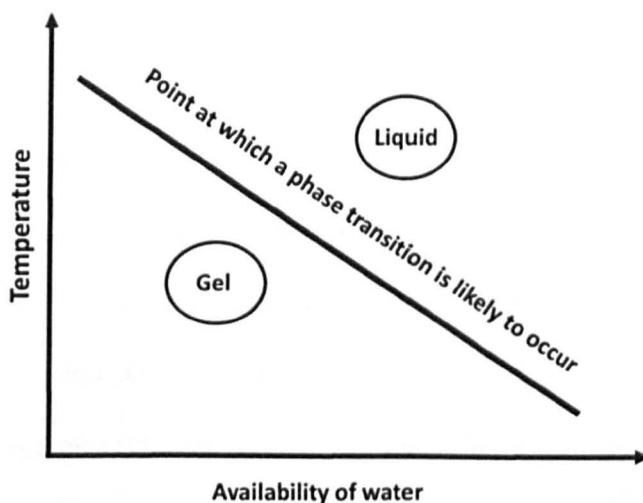
At sample point A each viability test indicated significantly lower viabilities compared to later time points. This suggests that the use of viability tests to assess ADY quality before rehydration has been completed may provide unreliable data and a more accurate estimation of cell viability can only be obtained once a culture has been fully rehydrated. However, it is also important to consider that the lower apparent viability at Sample Point A may indicate that there are differences in the physiological and metabolic state of yeast populations at this stage of the process. Viability analysis by means of slide culture, as well as the different staining methods, demonstrated an increase in the estimated number of live cells throughout rehydration (Figures 3.1-3.6). Consequently, although it is evident that cells are able to recover their capacity to replicate when fully rehydrated, these data suggest that yeast cells at an early stage of rehydration may not be able to respond to the change in environmental conditions required for viability assessment, perhaps reflecting the fragile condition of the membrane at this point. This hypothesis is supported by previous data indicating that direct pitching of yeast into wort can be detrimental and that pre-incubation is important to ensure fermentation performance (Tobias Fischborn and Chris Powell, personal communication). While our data indicates that a shorter incubation

period than previously suggested by the manufacturer (20-60 min) may be possible for the strains analysed here, it is noted that the current measure of yeast condition (viability) does not guarantee fermentation performance (Kobayashi *et al.*, 2007). Although viability did not appear to change significantly during the latter stages of rehydration, it is possible that this period is required to remove products of the dehydration stress response, such as trehalose which can cause inhibition of yeast metabolic pathways (Gancedo and Flores, 2004; Sebollela *et al.*, 2004) (Chapter 1.). Consequently, although the precise benefits of allowing yeast to slowly acclimatise to the environment have not been directly assessed here, it is suggested that this period may play a significant role in determining how ADY performs in industrial scale fermentations.

Furthermore, while the data presented here indicates that ADY populations in their dry form and during the initial stages of rehydration may demonstrate different physiological properties to fully rehydrated or wet yeast slurries, it is suggested that they should not immediately be considered to be non-viable, as the apparent viability increases during rehydration. Whilst drying results in cells in a dormant state which have non-viable characteristics, viable characteristics can be recovered during rehydration. This highlights the difficulties in assessing viability as an absolute phenotype. Practically, this is of significance given that an accurate assessment of the viable state of yeast cultures is important to ensure that the correct pitching rate is achieved. It is proposed that measurements of ADY viability should be treated with caution if taken at an early point during the rehydration process and that a more accurate estimation can only be achieved once the yeast has been fully rehydrated.

### **3.3.2 The effect of rehydration temperature on cell viability**

The temperature of rehydration is believed to be particularly important in avoiding structural damage as a result of phase transition events (characterised by a loss of fluidity) within the plasma membrane (Simonin *et al.*, 2008). As the availability of water is reduced the temperature at which a fluid membrane exists is increased, meaning a phase transition during rehydration is likely to occur (Figure 3.7). Such events may be avoided by rehydration at a temperature above the phase transition temperature of the cell membrane(Poirier *et al.*, 1999), which may lead to improved viability in certain yeast strains. However, thermal tolerance in yeast is also known to be strain dependent and determined by factors such as the presence of protectants (De Virgilio *et al.*, 1993) and synthesis of heat shock proteins (Trott and Morano, 2003). Therefore the benefit of increased rehydration temperatures in terms of membrane stability may be reduced in some yeast strains due to poor heat resistance. Lager yeast are known to have an optimum growth temperature which is considerably lower than those of ale yeast, the former being unable to grow at temperatures of above 34°C (Walsh and Martin, 1977). This suggests that the optimum temperatures for not only yeast function, but also rehydration, may be lower for lager strains than for ale yeast. This hypothesis is supported by our observation that higher viabilities were obtained for LAL1 (lager strain) at 25°C, while strains LAL2 and LAL4 (ale strains) were able to be rehydrated with good results at the higher temperature of 30°C. As a consequence, it is proposed that the rehydration temperatures selected for ADY should be aligned with the optimum functional temperature of each yeast strain and that deviation from these temperatures may adversely affect viability, a key factor in achieving a successful fermentation. Furthermore, it is anticipated that correct preparation of ADY may prevent excessive cell death (some cell death must be anticipated when using ADY) and that as a result the abnormal characteristics reported to be associated with using yeast in this form may be alleviated.



**Figure 3.7.** The relationship between the availability of water and temperature on the state of the cell membrane. The membrane can exhibit gel or liquid properties and is largely determined by the temperature and water availability, although several other factors can play a role. The point at which a change in phase occurs is termed a phase transition and is an important consideration when rehydration is occurring.

### 3.3.3 Methods for assessing ADY Viability

The basic criterion for a yeast cell to be considered viable is that it has the ability to reproduce. Although techniques based on cultivation are able to directly assess the capacity of cells to divide, such methods are typically time consuming. Methods based on cell staining are frequently adopted within industry as a means of estimating brewing yeast viability. Staining protocols are typically used to provide an indication of viability based on either membrane exclusion, the staining of cellular compounds, or by the ability of cells to convert or degrade the stain during cellular metabolism. Consequently, although such stains do not directly determine the capacity of cells to divide, they assess specific aspects of the cell which are critical to function and as such may be used as a measure of brewing yeast viability (Van Zandycke *et al.*, 2003b).

Despite its widespread use within the brewing industry for viability assessment, methylene blue is known to be inaccurate for the assessment of cultures with a viability of less than 90 % (Jones, 1987; O'Connor-Cox *et al.*, 1997; Smart *et al.*, 1999) and may not represent the most appropriate test for use with ADY, while other stains such as Oxonol and MgANS may

be more suitable. Oxonol is a potentiometric fluorescent stain which is excluded from viable yeast, but enters cells when the trans-membrane potential is lost (Lloyd and Dinsdale, 2000). On entry, the dye binds to intracellular lipids and proteins, and fluoresces indicating that the cell is non-viable (Epps *et al.*, 1994). 8-Anilino-1-naphthalenesulfonic acid hemimagnesium salt hydrate (MgANS) is excluded from viable cells by the cell membrane, but can enter dead cells where it binds to cytoplasmic proteins, forming a highly fluorescent complex (McCaig, 1990). With the differing modes of action for each of these methods it was anticipated that the results obtained may provide a more accurate reflection of the viable state of ADY cells. Analysis of samples at time point A revealed that a large variation in viability was obtained when different methods were applied. As suggested previously, this indicates that the use of viability tests to assess ADY quality before rehydration has been completed yields unreliable data, and a more accurate estimation of cell viability can be obtained once a culture has been fully rehydrated. However, even at later time points, such as C4, no individual technique gave consistently high or low estimates. Consequently no single viability technique can be recommended for analysis of rehydrated ADY cultures. This was perhaps surprising given that the mode of action of each of the methods employed differed, however the current data suggests that standard viability techniques employed in breweries are adequate for analysis of ADY as long as the yeast population is completely rehydrated, as discussed earlier.

### **3.4 CONCLUSIONS**

ADY cultures have been reported to exhibit lower cell viabilities than wet yeast populations of the same strain, leading to altered fermentation performance. Consequently it is important to ensure that ADY viability is maximised prior to inoculating a fermentation vessel. It is clear from this study that the method of rehydration may play a particularly important role in maintaining population health and yeast slurry viability. Specifically, incomplete rehydration, or rehydration at a sub-optimal temperature is likely to result in

impaired viability. While the current study has focused on the rehydration of yeast under laboratory conditions, these results may indicate that directly pitching ADY into wort (particularly cooler, lager type worts) could potentially result in viability loss and negatively influence fermentation performance. Furthermore, it is suggested that the optimum temperature of rehydration should be determined for each individual ADY strain and that these guidelines should be applied in the brewery in order to help ensure yeast viability and final product quality.

## CHAPTER 4: IMPACT OF DEHYDRATION AND REHYDRATION ON BREWING

### YEAST DNA INTEGRITY

Data from this Chapter formed the basis of a paper entitled *Dried Yeast: Impact of Dehydration and Rehydration on Brewing Yeast DNA Integrity*, which has been published in the Journal of the American Society of Brewing Chemists (2010 Volume 68 Number 3 pages 132-138).

#### 4.1 INTRODUCTION

The reduced viability exhibited when yeast cells are dehydrated and subsequently rehydrated, as demonstrated in Chapter 3, has been widely reported (Finn and Stewart, 2002; Fischborn *et al.*, 2004; Cyr *et al.*, 2007). However, reports regarding the damage thought to result in cell death have mainly focussed on the plasma membrane (Attfield *et al.*, 2000; Simonin *et al.*, 2007b), whilst other cellular damage which might be caused by the dehydration and subsequent rehydration of yeast has largely been ignored. Extensive DNA damage has been linked to yeast cell death in laboratory strains (Del Carratore *et al.*, 2002) and is one possible alternative for the cause of cell death seen in ADY.

Before investigating the effect of drying and rehydration on ADY DNA, it was necessary to consider how brewing yeast genome instability might be assessed. The nucleus houses the majority of DNA contained within the brewing yeast cell, accounting for between 80 and 85 % of the total DNA (Hammond, 2003). This DNA is stored on 16 distinct chromosomes each of which, in brewing yeast, may be present in multiple but not necessarily identical copies. . Weihenstephan 34/70, a common lager brewing yeast strain, contains 36 chromosomes with a total size of approximately 26.1 Mb (Nakao *et al.*, 2009). The nucleus, however, is not the only location of DNA within the cell; mitochondria contain DNA between 70 Kb (Nakao *et al.*, 2009) and 85 Kb (Foury *et al.*, 1998) in size (depending on the strain), typically present in 10-40 copies (Hammond, 2003). It is well documented that the brewing yeast

genome is prone to instability resulting in modifications in ploidy, chromosome length, chromosomal rearrangements and mitochondrial DNA integrity (Pedersen, 1994; Sato *et al.*, 1994; Chi and Arneborg, 1999; Sato *et al.*, 2001; Gibson *et al.*, 2008; James *et al.*, 2008). From an evolutionary perspective, genome instability as a consequence of stresses encountered during fermentation, provides the means for the adaptation of strains to industrial environments (James *et al.*, 2008). This is because genetic damage often manifests itself as phenotypic changes exhibited by the yeast (Bidard *et al.*, 1995; Bell *et al.*, 1997), although this is not always the case with a degree of robustness intrinsic to the cell (Wagner, 2000). This robustness is due to duplication, or redundancy, found within the genome (Goffeau *et al.*, 1996), but also compensatory actions of unrelated genes (Wagner, 2000). Nevertheless, if genetic damage occurs during ADY production and utilisation, even without an immediate phenotypic effect, this damage may manifest itself during its first use or when repitched. Although such variability may occasionally prove beneficial, it is generally accepted that genetic instability can cause process problems during fermentation including changes in flocculation capacity (Bidard *et al.*, 1995) and maltotriose utilization (Bell *et al.*, 1997).

The plethora of stresses which yeast are exposed to during dehydration and rehydration were discussed in Chapter 1 and some of these stresses are of particular relevance when considering the potential for damage to DNA. Water plays a fundamental role in the structure of DNA (Watson and Crick, 1953b), stabilising the double helix (Tao and Lindsay, 1989), therefore osmotic stress and dehydration of a cell could have a detrimental effect. *In vitro*, the desiccation of DNA in bacteria leads to a loss of supercoiling, aggregation and loss of resolution during agarose gel electrophoresis (Shirkey *et al.*, 2003). Although an equivalent study has not been performed with yeast, analysis of bacterial DNA has indicated that the structure can readily refold into an active conformation on rehydration, preventing permanent damage from occurring (França *et al.*, 2007). Therefore, the majority

of DNA damage that may occur is likely to be attributable to stresses associated with the loss of water, production of ADY, and rehydration, rather than dehydration itself. A decrease in cellular volume accompanies dehydration and the subsequent cytoplasmic crowding increases the chance of molecular interactions, some of which may also result in DNA damage (França *et al.*, 2007). Perhaps of greater significance are the high levels of reactive oxygen species (ROS) detected in yeast cells after dehydration, which can display more than a 10-fold increase (Pereira *et al.*, 2003). Oxidative stress has been shown to induce chromosomal damage in yeast in the form of strand breaks (Ramotar *et al.*, 1991). Due to the production of reactive oxygen species (ROS) within the mitochondria, mtDNA has been reported to exhibit a particularly high occurrence of DNA damage (O'Rourke *et al.*, 2002; Doudican *et al.*, 2005). Indeed, in certain eukaryotic cells it has been shown that reactive oxygen species damage mitochondrial DNA more than nuclear DNA (Yakes and VanHouten, 1997), with oxidative stress commonly causing mitochondrial damage in brewing yeast, manifesting in the form of respiratory mutants (Gibson *et al.*, 2008). Another potential source of DNA damage in ADY is heat stress. Although the heat stress experienced by ADY during production could be considered to be mild (approximately 35°C), heat shock can result in major structural changes to the nucleolus (Liu *et al.*, 1996), whilst it may also lead to the formation of further ROS (Bruskov *et al.*, 2002).

Given that DNA is known to be sensitive to stresses associated with desiccation, and that genetic stability is an important characteristic of brewing yeast, this investigation aims to assess the genetic integrity of commercial ADY products.

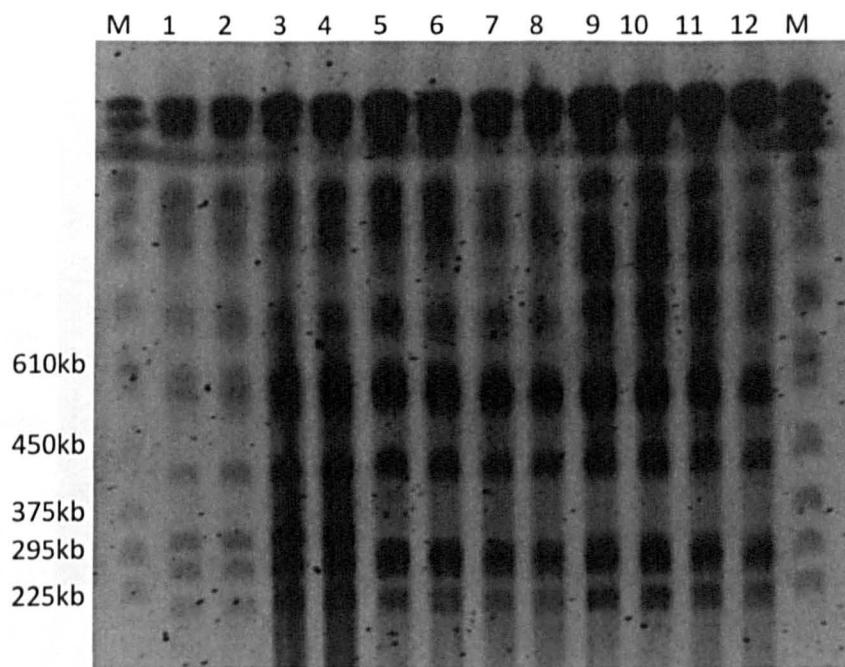
## **4.2 RESULTS**

The potential for DNA damage to ADY products is clear (discussed in Section 4.1), however, the size of the yeast genome means any potential damage may be difficult to identify. Since the early work of scientists analyzing the structure of DNA (Watson and Crick, 1953a) there have been many developments in the techniques designed to probe its sequence within organisms. Many of these techniques are directly applicable to yeast due to its status as a model organism (Drubin, 1989; Botstein *et al.*, 1997; Zeyl, 2000; Oliver, 2001; Game, 2002; De Freitas *et al.*, 2003; Lagali *et al.*, 2003; Morgan *et al.*, 2009). These techniques range from measuring the entire DNA content of individual cells in a population (Givan *et al.*, 1988; Haase and Reed, 2002) to sequencing the entire genome of isolates (Goffeau *et al.*, 1996; Nakao *et al.*, 2009). The former is useful for analysis of the yeast cell cycle, but is less likely to detect DNA damage which may occur during ADY preparation. Sequencing, however, represents a costly and time consuming endeavour, despite recent advances (Shendure and Ji, 2008). However, there are several targeted techniques which allow the probing of specific areas of genome with known increased susceptibility to damage. Identifying these “hotspots” in nuclear DNA can be achieved using PFGE-CHEF analysis and PCR using primers targeting inter-delta sequences. Restriction fragment length polymorphisms (Piskur *et al.*, 1998), mitochondrial copy number (Stuart *et al.*, 2006) and the presence of respiratory mutants (Goldring *et al.*, 1970) can be similarly used to assess mtDNA stability.

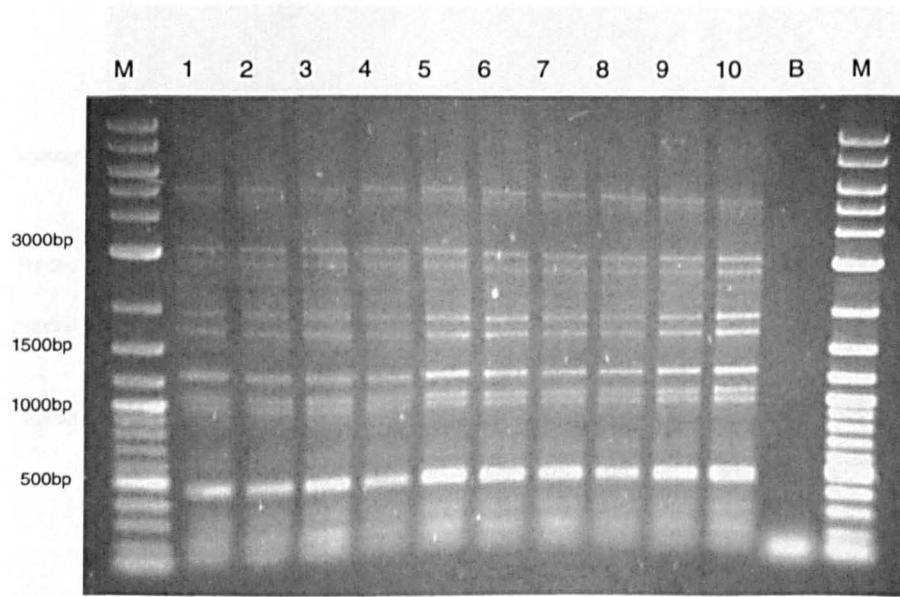
### **4.2.1 Nuclear DNA Stability**

Large scale chromosomal rearrangements were assessed by comparing the karyotypes derived from dried and control (laboratory grown, non-dried) brewing yeast using PFGE-CHEF analysis. It was observed that for each sample, an identical fingerprint was produced in terms of the size and number of chromosomes obtained (Figure 4.1), indicating that major structural damage did not occur during dehydration and rehydration of ADY.

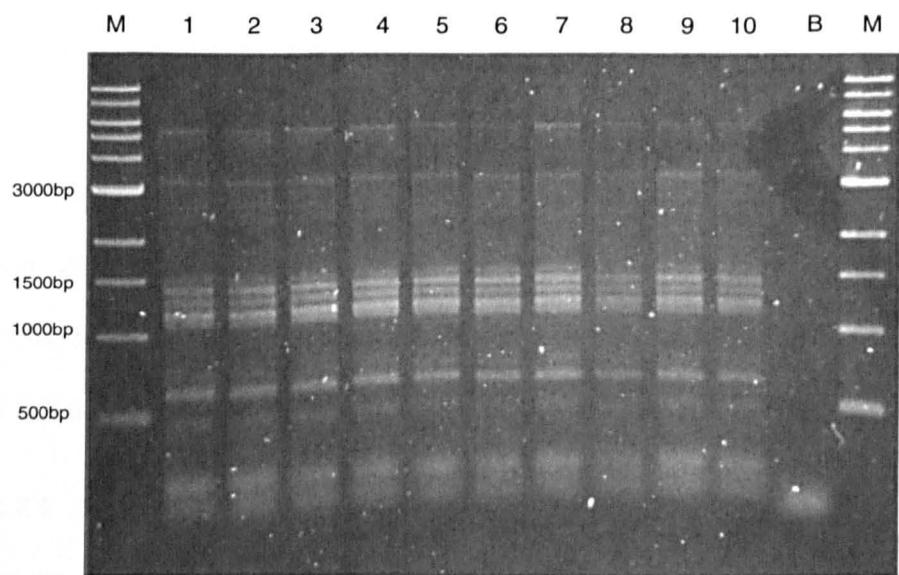
Whilst large-scale chromosomal rearrangements were not observed to occur in the ADY strains investigated here, it should be noted that karyotyping does not permit more minor changes to the DNA to be detected. Therefore, an investigation into sequence changes in the DNA was conducted using PCR analysis with primers designed to amplify inter-delta sequences (Legras and Karst, 2003). Inter-delta sequences are long terminal repeats (LTRs) that flank retrotransposons Ty1 and Ty2 but can be found elsewhere in the genome of yeast. Transposons can be a major source of genetic change due to Ty-driven translocation (Rachidi *et al.*, 1999). Analysis of inter-delta regions has previously been used for yeast strain differentiation and identification (Ness *et al.*, 1993; de Barros Lopes *et al.*, 1996; Legras and Karst, 2003), and can be applied to detect genetic variants. The application of this analysis to LAL1, LAL2 and LAL4 strains yielded strain dependent reproducible and unique profiles for each strain. To determine the impact of dehydration and rehydration on these profiles, ADY and control populations of each strain were cultivated on solid medium and 5 representative colonies in each case were recovered for analysis. No variation in the profiles obtained for ADY and control populations of LAL1 (Figure 4.2), LAL2 (Figure 4.3) and LAL4 (Figure 4.4) could be detected indicating that Ty element movement had not occurred.



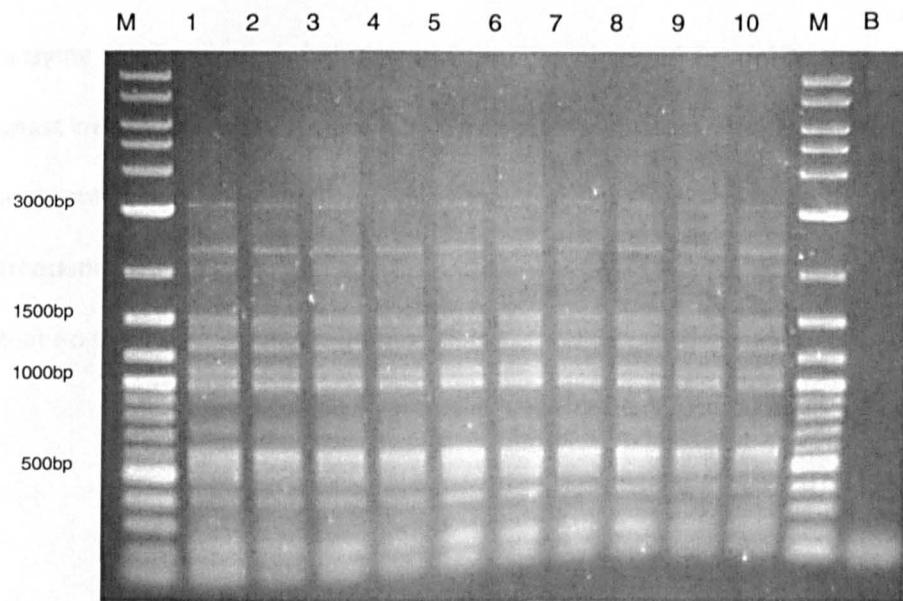
**Figure 4.1.** Karyotyping. M: Chromosomes Isolated from *Saccharomyces cerevisiae* strain YPH80; 1 and 2:LAL1 Control; 3 and 4: LAL1 Dried; 5 and 6: LAL2 Control; 7 and 8: LAL2 Dried; 9 and 10: LAL4 Control; 11 and 12: LAL4 Dried.



**Figure 4.2.** Lager yeast LAL1. Analysis of inter-delta regions by PCR fingerprinting. M: 2-Log DNA Ladder , Lanes 1-5: LAL1 Control (colony isolates); Lanes 6-10: LAL1 Dried (colony isolates), B: Blank.



**Figure 4.3.** Ale yeast LAL2. Analysis of inter-delta regions by PCR fingerprinting. M: 2-Log DNA Ladder, Lanes 1-5: LAL2 Control (colony isolates); Lanes 6-10 LAL2 Dried (colony isolates), B: Blank.



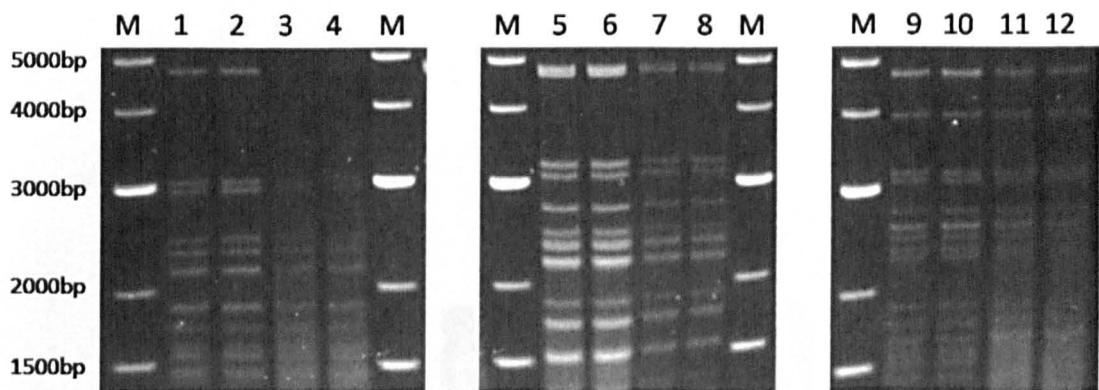
**Figure 4.4.** Ale yeast LAL4. Analysis of inter-delta regions by PCR fingerprinting. M: 2-Log DNA Ladder, Lanes 1-5: LAL4 Control (colony isolates); Lanes 6-10: LAL4 Dried (colony isolates), B: Blank.

## **4.2.2 Mitochondrial DNA Stability**

Although mitochondrial DNA accounts for a lower percentage of total DNA than nuclear DNA, the DNA is more susceptible to damage (Yakes and VanHouten, 1997) with the petite mutant commonly occurring during fermentation and storage (Morrison and Suggett, 1983; Good, 1993; Jenkins *et al.*, 2009). Therefore several aspects of mitochondrial DNA were probed including DNA sequence conformity (Section 4.2.2.1), copy number (Section 4.2.2.2) and relative resistance to mutagen challenge (Section 4.2.2.3).

### **4.2.2.1 Mitochondrial DNA sequence conformity**

Analysis of restriction sites within mtDNA can be used to detect variation within the mitochondrial genome as these sites can be transposed or deleted when mutations or sequence changes occur. Consequently, the size and number of DNA fragments spliced during RFLP has been shown to differ in petite cells when compared to respiratory competent individuals (Castreron *et al.*, 2002). Analysis of mtDNA using the restriction enzyme *HinfI* indicated that the RFLP profiles obtained from ADY and non-dried control yeast were comparable (Figure 4.5). To assess the relative concentration of petites for ADY and control non-dried cultures, TTC overlay plates were inoculated and enumerated. Petite frequency was negligible in all samples assessed (less than 1 %). It is therefore proposed that no significant mtDNA sequence changes had occurred during the production of ADY.

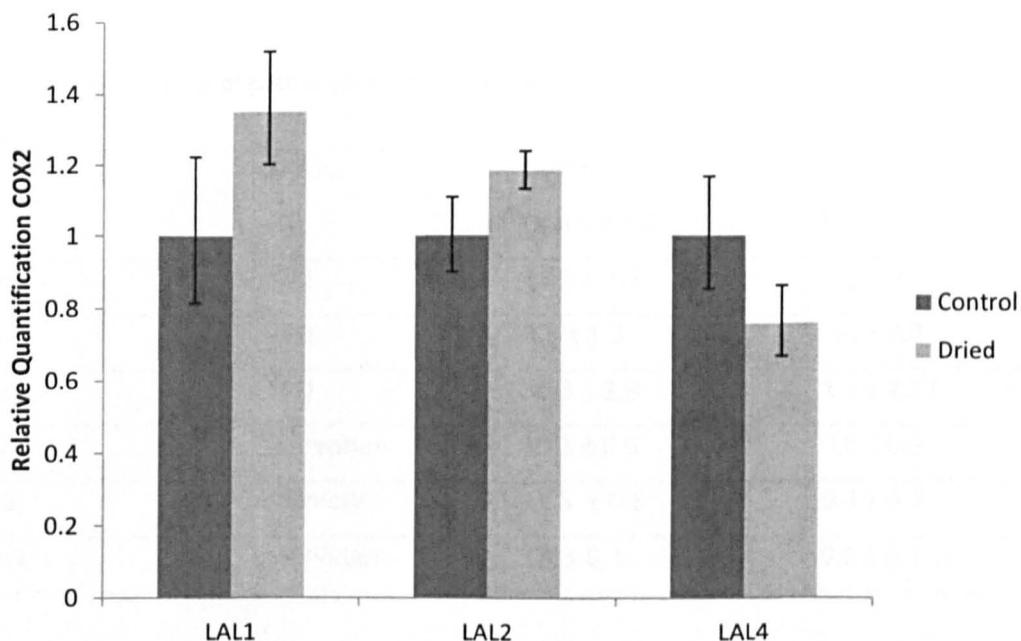


**Figure 4.5. RFLP analysis of mtDNA digested with HinfI.** M: 2-Log DNA Ladder, 1 and 2: LAL1 Control; 3 and 4: LAL1 Dried; 5 and 6: LAL2 Control; 7 and 8: LAL2 Dried; 9 and 10: LAL4 Control; 11 and 12: LAL4 Dried

#### 4.2.2.2 Mitochondrial DNA copy number assessment

Mitochondrial DNA damage can remain hidden due to the number of mitochondria and/or mitochondrial DNA copy number present in a cell. Given that ADY production incorporates desiccation stress, and that mitochondrial copy number is known to vary according to the physiological condition of cells (Visser *et al.*, 1995; Sia *et al.*, 2003), the effect of drying on mtDNA copy number was assessed. To this end, the relative proportions of mtDNA were assessed using two housekeeping genes, *ACT1* (located on chromosome IV), and *COX2* (located in the mitochondria) were examined. By comparing these genes by qPCR it was possible to assess the relative quantity of mitochondria with complete *COX2* genes between ADY and non-dried control samples. The relative quantification (RQ) value of the non-dried control sample was assigned to be a value of one, and the ADY sample was calculated in relation to this. Strains LAL1 and LAL2 were observed to exhibit a higher RQ value (higher number of mtDNA copies) in ADY than in control samples, however strain LAL4 exhibited a higher RQ value in the control sample (Figure 4.6). Analysis of the variation within the samples suggested that the differences observed were not significant.

Consequently, these data provide further evidence to suggest that DNA damage does not occur during ADY production and utilization.



**Figure 4.6. Relative quantification of COX2 with reference to ACT1. Minimum and maximum Relative Quantification (RQ) values indicated.**

#### 4.2.2.3 Relative resistance to mutagen challenge

To support the hypothesis that mtDNA copy number and sequence was not affected by dehydration and rehydration, the propensity of ADY and control cultures to form petites was assessed. Petite formation is not solely a function of mtDNA integrity but can also be influenced by chromosomal DNA integrity. Indeed *GSH1* which is a gene located on chromosomal DNA is required to maintain the non-petite phenotype and damage to this gene will lead to petite formation (Kistler *et al.*, 1990). The propensity of ADY and control samples to form petite mutants was assessed using the mutagen ethidium bromide. It can be seen that mutagenesis using ethidium bromide yielded more petites in non-dried cultures than in rehydrated ADY cultures irrespective of the strain examined (Table 4.1). Starved LAL1 and LAL2 yeast also exhibited a higher petite frequency (13.1 % and 11.3 %)

than corresponding ADY populations (0.0 % and 0.4 %) following exposure to ethidium bromide. This trend was also apparent for LAL4 albeit to a lesser extent, with control yeast exhibiting 1.4 % petites compared to 0.1 % for ADY (Table 4.1).

**Table 4.1. Percentage of petites present after incubation with 100 µM ethidium bromide**

Sample	Media	Control (± standard error)	ADY (± standard error)
LAL1	YPD	15.5 ± 2.9	0.2 ± 0.3
LAL2	YPD	9.5 ± 1.5	0.0 ± 0.0
LAL4	YPD	30.3 ± 3.8	1.5 ± 2.17
LAL1	Starvation	13.1 ± 0.6	0.0 ± 0.0
LAL2	Starvation	11.3 ± 0.8	0.4 ± 0.2
LAL4	Starvation	1.4 ± 0.3	0.1 ± 0.1

#### 4.3 DISCUSSION

It is well documented that the brewing yeast genome is prone to instability resulting in modifications in ploidy, chromosome length, chromosomal rearrangements and mitochondrial DNA integrity (Pedersen, 1994; Sato *et al.*, 1994; Chi and Arneborg, 1999; Sato *et al.*, 2001; Gibson *et al.*, 2008; James *et al.*, 2008). From an evolutionary perspective, genome instability as a consequence of stresses encountered during fermentation provides the means for the adaptation of strains to industrial environments (James *et al.*, 2008). Although such variability may occasionally prove beneficial, it is generally accepted that instability causes process problems during fermentation. Based on the premise that yeast genetic instability is not typically beneficial to the brewer, the purpose of this investigation was to assess the impact of dehydration and rehydration on the genome integrity of ale and lager ADY.

Previous reports have demonstrated that freeze dried yeast cells can exhibit chromosomal breaks (Lopes *et al.*, 1999). However, on a commercial scale ADY is typically produced

using fluidised bed drying, a technique which differs significantly from other methods of drying. By karyotyping and analysing of inter-delta regions of the genome, it was demonstrated that when ADY produced using fluidised bed drying was rehydrated its genetic profiles were comparable to control yeast. This data suggests that the fluidised bed drying process is less stressful to DNA than freeze drying. In support of this hypothesis, the latter technique (comprising the removal of water by sublimation from a frozen culture under vacuum (Kawamura *et al.*, 1995)), often results in a viability as low as 0.1 % (Lodato *et al.*, 1999), whereas for fluidized bed dried cultures the typical viabilities fall within the range of around 60-80 % (Gosselin and Fels, 1998; Finn and Stewart, 2002; van den Berg and Van Landschoot, 2003). These results are in keeping with a previous study which found the genetic profile of a dried lager yeast to be comparable to that of control yeast and maintain this through a series of five fermentations (Powell and Fischborn, 2010). This study, in combination with the work highlighted here incorporating ale strains, indicates that genetic damage to nuclear DNA does not occur as a result of fluidised bed drying.

Yeast mitochondrial DNA is known to be particularly susceptible to damage, resulting in the formation of petite mutants (Goldring *et al.*, 1970; Nagley and Linnane, 1970; Jenkins *et al.*, 2009). Typically brewing yeast populations contain around one percent petites (Silhankova *et al.*, 1970). Finn and Stewart demonstrated that petite occurrence for ADY was comparable to that typically observed in brewing cropped yeast (Finn and Stewart, 2002). In contrast, freeze dried yeast cultures typically comprise high levels of petites (Russell and Stewart, 1981). Several stresses have been linked to the formation of petites during brewing yeast fermentation and yeast handling and some of these are also associated with the fluidized bed ADY production process, such as oxidative stress (O'Rourke *et al.*, 2002; Doudican *et al.*, 2005) and starvation (Wallis *et al.*, 1972; Wallis and Whittaker, 1974).

To investigate the impact of fluidized bed dehydration and rehydration on yeast propensity to form petites, mtDNA was assessed for gross damage leading to the formation of respiratory deficient mutants, mtDNA copy number, sequence rearrangements and resistance to mutagen challenge. Petite frequency was negligible in all samples assessed (less than 1 %). The formation of a petite requires the damage (or total deletion) of all copies (typically between 20 to 50 within a single cell) of mitochondrial genome within the cell (Wimasalena, Nicholls and Smart, unpublished data). The absence of petites therefore does not necessarily equate to the absence of mtDNA damage within the cell. Therefore damage affecting some but not all of the mtDNA may be termed 'sub-petite' damage. Although methods for analyzing sub-petite damage have not previously been established, it is proposed that this phenotype may be detected by analysis of mtDNA sequence conformity, mtDNA copy number assessment, and the relative resistance to a mtDNA mutagen.

The data presented here suggests that the potential for mtDNA damage was reduced in ADY when compared to non-dried yeast. While the precise rationale for this difference between cultures has yet to be determined, Schneider-Berlin *et al.* (2005) demonstrated that petite induction through exposure to ethidium bromide was slower in non-dividing cells than in dividing individuals. Cells within an ADY population are typically in a non-dividing state (data not shown), whereas those present in laboratory grown control populations are often more asynchronous with respect to the cell cycle, with some budding cells evident. To test the hypothesis that the differences observed between ADY and control yeast sensitivity to ethidium bromide challenge were related to cell population synchronicity, starvation was used to synchronise control populations. Similar trends were recorded, suggesting that cell division does not fully account for the differences observed.

Therefore, the relative resistance of ADY to ethidium bromide challenge remains unexplained. A variety of factors may contribute to the phenotype observed including the protective effect of previously induced stress responses during production. Osmotic, oxidative, thermal and starvation stresses are all present during the production of ADY (Perez-Torrado *et al.*, 2005). Whilst each stress elicits its own response, they often share common features which have been termed the general environmental stress response (Ruis and Schuller, 1995). Under this response, exposure to one stress factor can provide protection against other stresses, termed cross protection (Lewis *et al.*, 1995; Park *et al.*, 1997). Currently the mode of action for the mutagenic effect of ethidium bromide on yeast mitochondria is unclear (Schneider-Berlin *et al.*, 2005), although the plasma membrane has been implicated in the capacity to restrict its assimilation into the cell (Brunner *et al.*, 1982; Coote *et al.*, 1994). It is proposed that ADY and control yeast populations may therefore differ with respect to their capacity to assimilate the mutagen, although the mechanism by which ethidium bromide uptake occurs remains the subject of further investigation.

#### 4.4 CONCLUSIONS

Analysis of colonies and populations derived from ADY yeast prepared by fluidized bed drying indicated that no evidence for chromosomal and mtDNA genome damage could be found. In addition, the tolerance to ethidium bromide mutagen challenge appeared to differ, with ADY populations demonstrating a reduced sensitivity to petite formation when compared to control populations. The reasons for this difference are not known. It is recognized that due to the nature of molecular based techniques, which require analysis of populations or a selection of representative isolates derived from the population, DNA damage occurring at an extremely low rate may not be detected. However, at the basic genetic level there is no evidence to suggest that ADY prepared using fluidized bed drying should exhibit impaired fermentation performance as a result of DNA damage during drying.

## CHAPTER 5: PROPERTIES OF THE PLASMA MEMBRANE SUBSEQUENT TO YEAST

### REHYDRATION

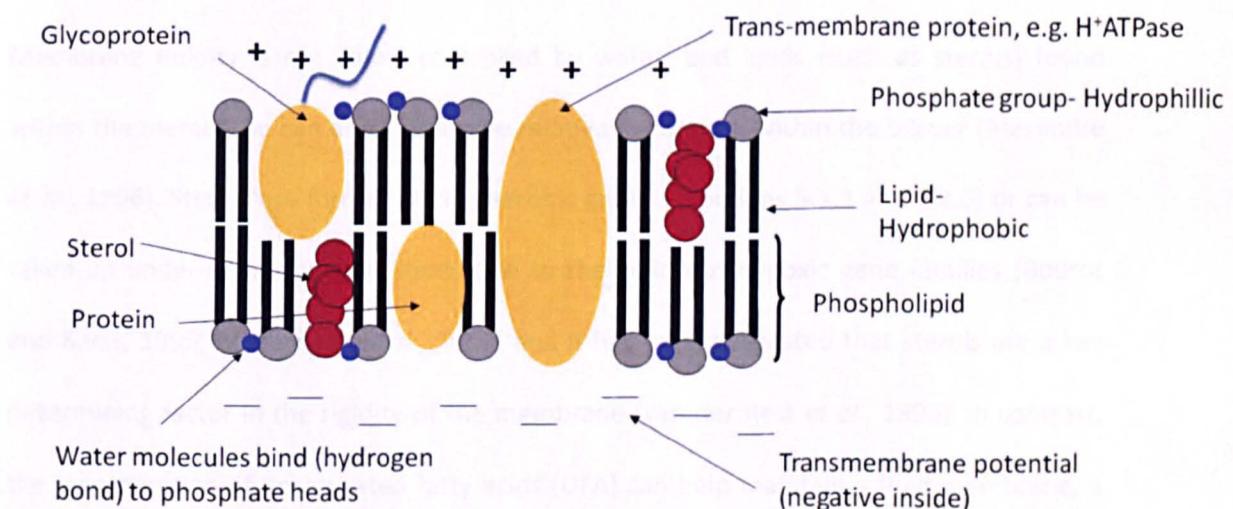
#### 5.1 INTRODUCTION

The cell envelope comprises the cell wall, plasma membrane and periplasmic space.

The cell wall is porous and molecules smaller than 600 Da are able to readily permeate its structure (Scherrer *et al.*, 1974). In contrast the plasma membrane does not promote the free movement of molecules and this structure acts as a selective barrier to the cell.

#### 5.1.1 The composition and structure of the plasma membrane

The basic structure of the plasma membrane consists of a phospholipid bilayer with interspersed protein structures (Figure 5.1).



**Figure 5.1. Schematic of the structure of the yeast plasma membrane. The bulk of the structure consists of phospholipids arranged in a bilayer. Transport proteins span the entire width of the bilayer, whilst others may penetrate only partially. In viable cells a transmembrane potential is maintained with a negative potential inside the cell.**

Of the phospholipids present, the majority are phosphatidylglycerol, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine (Blagovic *et al.*, 2005). The interactions

between these phospholipids and the structure they form are key to the organelle's function. Singer and Nicholson (1972) proposed the fluid mosaic model to describe the freedom of movement demonstrated by the phospholipid bilayer, although membranes are often described as exhibiting a liquid-crystal state (Leslie *et al.*, 1995; Laroche and Gervais, 2003; Simonin *et al.*, 2008). This description reflects the frequent movement within each layer, but the limited movement between the two layers which constitute the bilayer. Water plays a key role in maintaining this basic structure by surrounding the phospholipids and creating the hydrophilic and hydrophobic regions required to maintain the bilayer structure (Chapman, 1994). Water also separates phosphate head groups on the outer and inner surface of the lipid bilayer and therefore assists in maintaining an ordered and fluid membrane (Chapman, 1994). Due to its role within the membrane, it is likely that the loss of water could be important in relation to dried yeast, as alterations in fluidity have been suggested to promote mortality under osmotic stress (Simonin *et al.*, 2007b).

Membrane fluidity is not solely controlled by water, and lipids (such as sterols) found within the membrane can also determine relative movement within the bilayer (Alexandre *et al.*, 1996). Sterols are formed during aerobic growth (Sections 5.1.2 and 5.2.6) or can be taken-up under anaerobic conditions due to the action of hypoxic gene families (Bourot and Karst, 1995; Alimardani *et al.*, 2004) and it has been suggested that sterols are a key determining factor in the rigidity of the membrane (van der Rest *et al.*, 1995). In contrast, the incorporation of unsaturated fatty acids (UFA) can help maintain a fluid membrane, a regulation sometimes required in response to stress, for example osmotic (Rodriguez-Vargas *et al.*, 2007). Fluidity is maintained due to the lower melting point of UFA compared to saturated fatty acids (Rodriguez-Vargas *et al.*, 2007).

Although the individual components of the membrane contribute to fluidity, this property is not uniform throughout (Karnovsky *et al.*, 1982), with the presence of "lipid rafts"

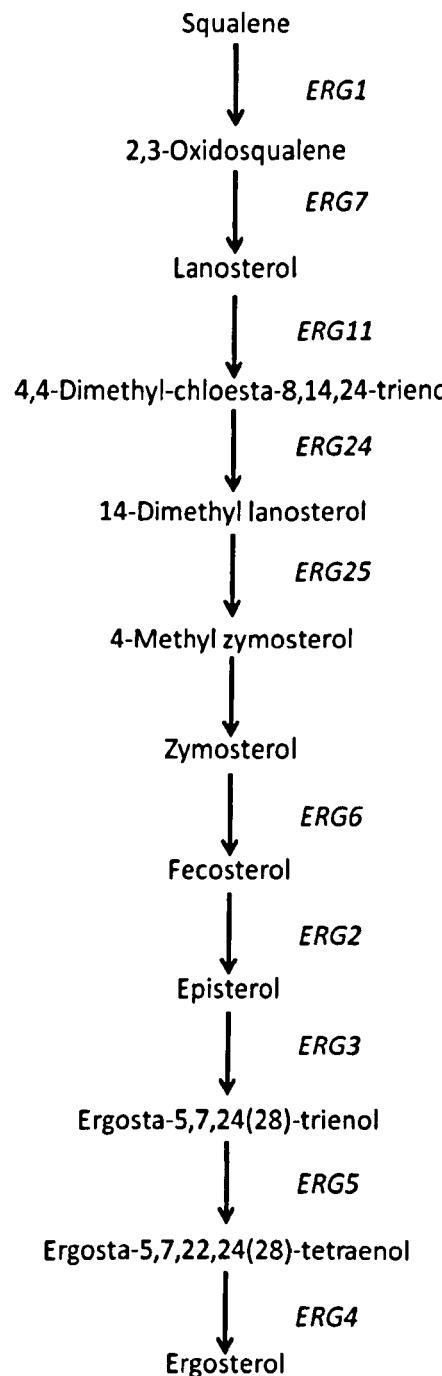
throughout the structure (Bagnat *et al.*, 2000). These rafts are characterised by areas of distinct and differing lipid content, resulting in modified fluidities, which are presumably important for certain cellular functions (Bagnat *et al.*, 2000). These compositional and fluidity changes are important for membrane function, indeed certain membrane bound enzymes can become inactivated when lipids are removed (Dufour and Goffeau, 1980).

### 5.1.2 Ergosterol biosynthesis and accumulation in *S. cerevisiae*

Ergosterol, a key component of the plasma membrane, has several roles in yeast. Its presence is required in relatively small amounts to enable growth, and this has been termed its “sparking” function (Rodriguez and Parks, 1983; Lorenz *et al.*, 1989). Ergosterol also has an important structural role in the plasma membrane (discussed in Section 5.1.1.) and like other sterols plays a key role in maintaining membrane fluidity (Lees *et al.*, 1979; Lees *et al.*, 1989) and membrane permeability (Bard *et al.*, 1978; Kleinhans *et al.*, 1979).

Sterols can be synthesised in yeast cells from acetyl-CoA formed during the glycolysis of carbon substrates. The condensation of acetyl-CoA and acetoacetyl-CoA forms  $\beta$ -hydroxy- $\beta$ -methyl glutaryl coenzyme A (HMG CoA) (Rudney, 1957). HMG CoA is then converted to mevalonic acid (Ferguson *et al.*, 1986), a precursor in the mevalonate pathway, which can ultimately lead to the biosynthesis of squalene. In the presence of oxygen squalene can be converted to ergosterol via the pathway outlined in Figure 5.2. Many of the sterols generated at the beginning of fermentation, when catabolite derepression occurs, are esterified and stored (Quain and Haslam, 1979). During the anaerobic conditions of fermentation sterol esters are hydrolysed to free esters and utilised within the cell .As anaerobic conditions persists (Quain and Haslam, 1979), squalene accumulates in the cell (Blagovic *et al.*, 2005) due to the requirement of oxygen for its conversion to 2,3 oxidosqualene in the ergosterol pathway (Jahnke and Klein, 1983). The genes *ERG9* (encodes the enzyme squalene synthase), *ERG1* (squalene epoxidase) and *ERG7* (lanosterol

synthase) are required to produce the first sterol in the pathway (lanosterol) and are thus viewed as essential for aerobic viability to ensure some sterol synthesis in yeast (Lees *et al.*, 1995). *ERG11* and *ERG24* are also essential for cell growth, but genes encoding later steps such as *ERG2* and *ERG3* are not essential (Lees *et al.*, 1995).



**Figure 5.2. The late steps of the ergosterol synthesis pathway adapted from Palermo *et al.* (1997). The genes responsible for the various steps are indicated next to the relevant arrows.**

### **5.1.3 The changes in the plasma membrane due to the dehydration and subsequent rehydration of yeast cells.**

During dehydration it has been proposed that water molecules are removed from the membrane and the phospholipid head groups are forced together. The resultant increase in packing density of the phospholipid head groups, has been purported to lead to an increase in van der waals interactions among hydrocarbon chains (Laroche and Gervais, 2003; França *et al.*, 2007). As a consequence the membrane undergoes a phase transition, entering a gel phase. In contrast rehydration has been proposed to reverse this process, returning the membrane to a liquid-crystalline state (Laroche *et al.*, 2001; Laroche *et al.*, 2005). It has been suggested that biological membranes which contain a heterogeneous mixture of lipids, could result in gel and liquid-crystalline phases co-existing (Laroche and Gervais, 2003). This in turn could lead to packing defects at boundaries between the differing domains, potentially resulting in cytoplasmic leakage and cell death (Laroche and Gervais, 2003). Retention of yeast cell viability for rehydrated ADY appears to depend on the temperature at which rehydration occurs. It has been proposed that this may relate to the rate at which water permeates the plasma membrane during phase transition (Laroche and Gervais, 2003). Plasmolysis, which is defined as the cell membrane shrinking, but not detaching, from the cell wall can also occur during dehydration and rehydration leading to membrane rupture. If the cell shrinks, due to loss of water, the membrane can have a tendency to fuse in areas, and therefore become leaky (Hoekstra *et al.*, 2001; Guyot *et al.*, 2006).

Trehalose enriched membranes appear to tolerate a wider range of phase transition temperatures (Leslie *et al.*, 1994) and it has been proposed that trehalose stabilises the membrane during transition. This is one reason why trehalose accumulation during the production of dried brewing yeast is encouraged (Powell and Fischborn, In press).

In this Chapter an investigation into the impact of dehydration and rehydration, at optimal and sub-optimal temperatures, on the fluidity of the plasma membrane was conducted.

## 5.2 RESULTS

### 5.2.1 Selection and verification of yeast mutants displaying altered fluidity characteristics

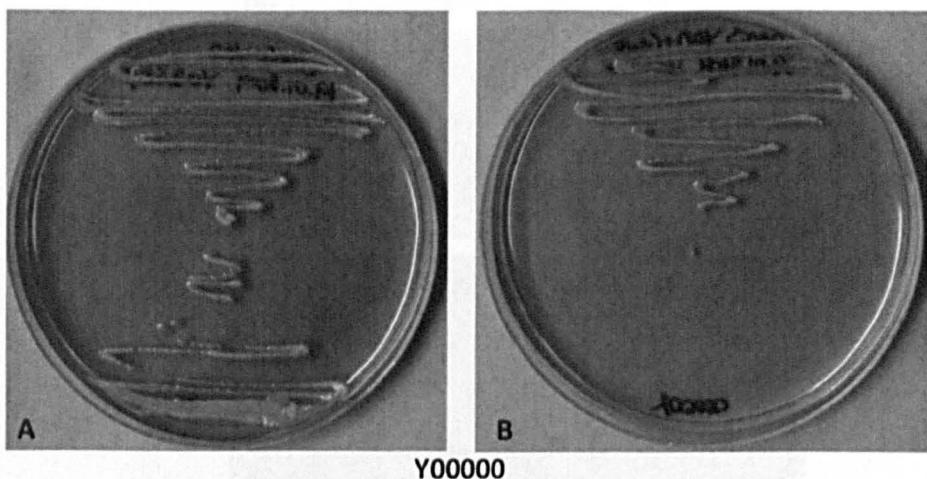
In order to investigate the impact of dehydration and rehydration on membrane fluidity, control strains with demonstrable fluidity differences were required to confirm that analytical approaches used were functioning correctly. Candidate genes, the deletion of which has been proposed to alter membrane fluidity, were identified through a series of searches in the *Saccharomyces* Genome Database ([www.yeastgenome.org](http://www.yeastgenome.org)) and associated publications (Sharma, 2006; Abe and Hiraki, 2009). *ERG2* (YMR202w) and *ERG3* (YLR056w) two genes found in the synthetic pathway for ergosterol (Figure 5.2), were deemed suitable for investigation. *ERG2* encodes a non-essential C-8 sterol isomerase (Ashman *et al.*, 1991) and its deletion causes the accumulation of ergosta-5,8,22-trienol, fecosterol and ergosta-8-enol (Munn *et al.*, 1999). *ERG3* encodes a non-essential C-5 sterol desaturase (Arthington *et al.*, 1991) the deletion of which causes the accumulation of Ergosta-7-enol, Ergosta-7,22-dienol, Fecosterol and Episterol (Heese-Peck *et al.*, 2002). Despite these key differences in phenotype it should be noted that variations in the quantities of each sterol accumulated sterols accumulated, instead of the normal ergosterol, had been noted to differ with respect to strain and growth conditions (Munn *et al.*, 1999). Previous studies using mutant *S. cerevisiae* strains containing non-functioning *erg* genes demonstrated differences in fluidity (Sharma, 2006; Abe and Hiraki, 2009) a property of significance when considering dried yeast mortality.

Mutant yeast strains with these candidate genes deleted were procured from EUROSCARF (Frankfurt, Germany). Deletion mutants from this source have the entire gene of interest

deleted and a kanamycin resistance cassette (KanMX4) inserted. This insertion, which produces resistance to the antibiotic G418 in *S. cerevisiae*, was confirmed with the inoculation of the yeast onto YPD agar containing the antibiotic and observation of growth. Figure 5.3 demonstrates the growth of both the wild type (Y00000/BY4741) and the mutant (Y02667) strains on YPD alone and the growth of just the mutant strain when inoculated on YPD and G418. The effect of insertion of the KanMX4 cassette into the mutant Y00788 is shown in Figure 5.4. However, this figure simply demonstrates the presence of the insertion and not the specific location, which was verified using PCR analysis. The *ERG3/YLR056w* open reading frame is 1098 bp in length. Primers were used to amplify a 1580 bp region of the genome in the wild type strain and a 2116 bp region in the mutant strain. The DNA amplicons produced (Section 2.7.1) were identified to be of the anticipated sizes, verifying the deletion of the gene and insertion of the kanMX4 cassette (1634 bp in length) (Figure 5.5).

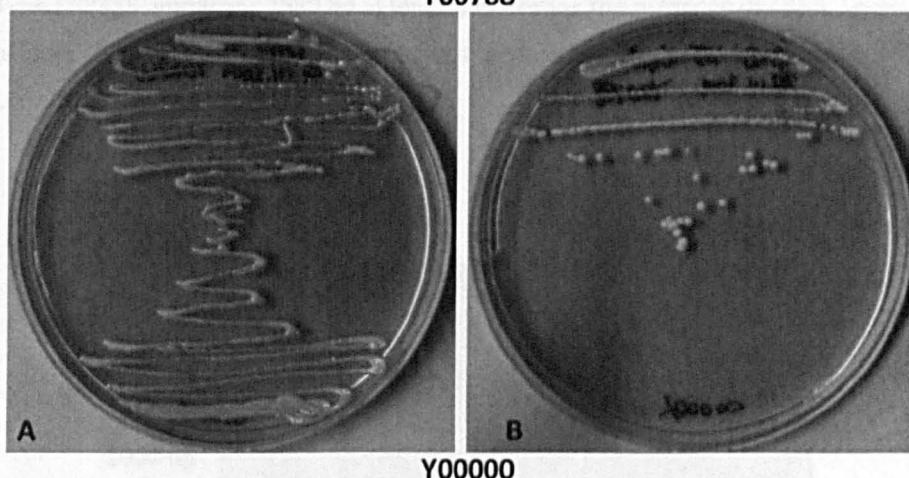
The *ERG2/YMR202w* open reading frame is 669 bp in length. Primers were used to amplify a 1203 bp region of the genome in the wild type strain and a 2134 bp region of the mutant strain (Y00788) to verify the deletion of the target gene and insertion of the cassette (Figure 5.6).

**Y02667**

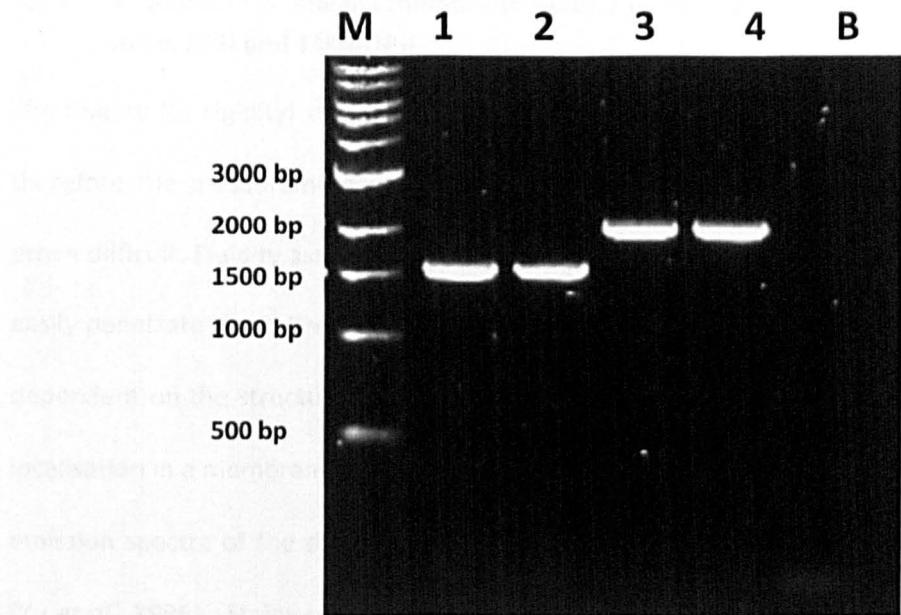


**Figure 5.3. Confirmation of G418 resistance in EUROSCARF strains. Picture A shows growth of Y00000 and Y02667 on YPD plates, whilst B shows growth of Y02667 and the lack of growth of the wild type Y00000 on YPD plates containing G418.**

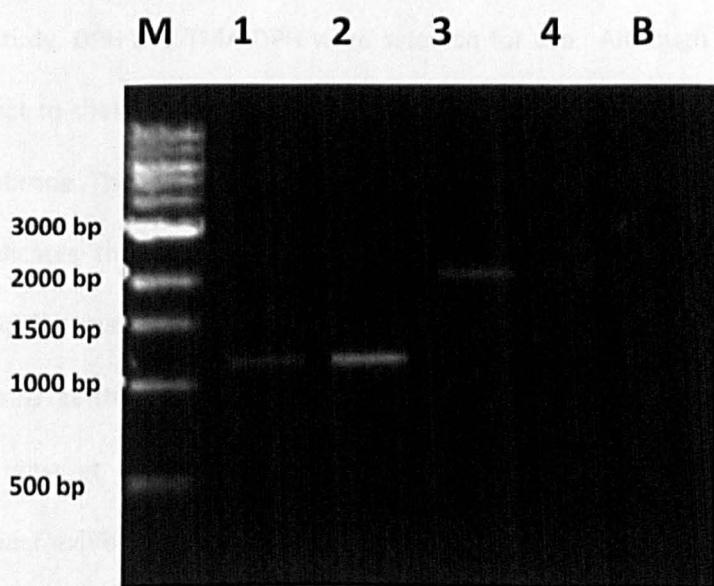
**Y00788**



**Figure 5.4. Confirmation of G418 resistance in EUROSCARF strains. Picture A shows growth of Y00000 and Y00788 on YPD plates, whilst B shows growth of Y00788 and the lack of growth of the wild type Y00000 on YPD plates containing G418.**



**Figure 5.5.** Confirmation of the deletion in the EUROSCARF strain Y02667 using PCR analysis with primers designed to flank the gene of interest. M: 1kb ladder (New England Biolabs), Lanes 1 and 2: Wild Type (Y0000) amplicons 1580 bp in length, indicating the presence of ERG3. Lanes 3 and 4: Y02667 amplicons 2116 bp in length, indicating the deletion of ERG3 and insertion of kanMX4 cassette. B:Blank



**Figure 5.6.** Confirmation of the deletion in the EUROSCARF strain Y00788 using PCR analysis with primers designed to flank the gene of interest. M: 1kb ladder (New England Biolabs), Lanes 1 and 2: Wild Type (Y0000) amplicons 1203 bp in length, indicating the presence of ERG2. Lanes 3 and 4: Y00788 amplicons 2134 bp in length, indicating the deletion of ERG2 and insertion of kanMX4 cassette. B: Blank.

## **5.2.2 Assessment of plasma membrane fluidity using depolarisation of the fluorescent stains DPH and TMA-DPH**

The fluidity (or rigidity) of a membrane is determined by many factors (Section 5.1) and therefore the measurement of this property using compositional differences alone can prove difficult. Fluidity assessment has therefore tended to focus on the use of dyes which easily penetrate the cell wall, bind to the membrane and elicit emission spectra which are dependent on the structure to which the dye has localised. For the dye merocyanine 540, localisation in a membrane is determined by the density of lipids (McEvoy *et al.*, 1988). The emission spectra of the dye laurdan is determined by the state of the plasma membrane (Yu *et al.*, 1996). Stains such as 1,6-Diphenyl-1,3,5-hexatriene (DPH) (Laroche *et al.*, 2001; Rodriguez-Vargas *et al.*, 2002; Simonin *et al.*, 2007b; Simonin *et al.*, 2008) and its analogue N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium p-toluenesulfonate (TMA-DPH) (Sharma, 2006; Abe and Hiraki, 2009) have anisotropic properties which have been used to indicate fluidity based on the dye's movement within the membrane.

In the current study, DPH and TMA-DPH were selected for use. Although they are similar dyes with respect to their fluorescence emission, they differ in terms of their localisation within the membrane. The hydrophobic stain DPH is thought to localise near the lipid tails, therefore it indicates the fluidity of the inner membrane (Kaiser and London, 1998; Repáková, 2004; Sharma, 2006). In contrast, TMA-DPH carries a positive charge which ensures it remains at the water lipid interphase, thus it reflects the outer membrane fluidity (Prendergast *et al.*, 1981). DPH has been observed to also aggregate in the cytoplasm of yeast exhibiting non-functioning ergosterol biosynthetic genes, rather than localising in the plasma membrane (Abe and Hiraki, 2009). The reasons for this change in localisation and indeed the target of the actual staining are unknown (Abe and Hiraki, 2009). To investigate the specificity of each stain, the cellular localisation of DPH (Figure 5.7) and TMA-DPH (Figure 5.8) were observed microscopically. It can be seen that DPH

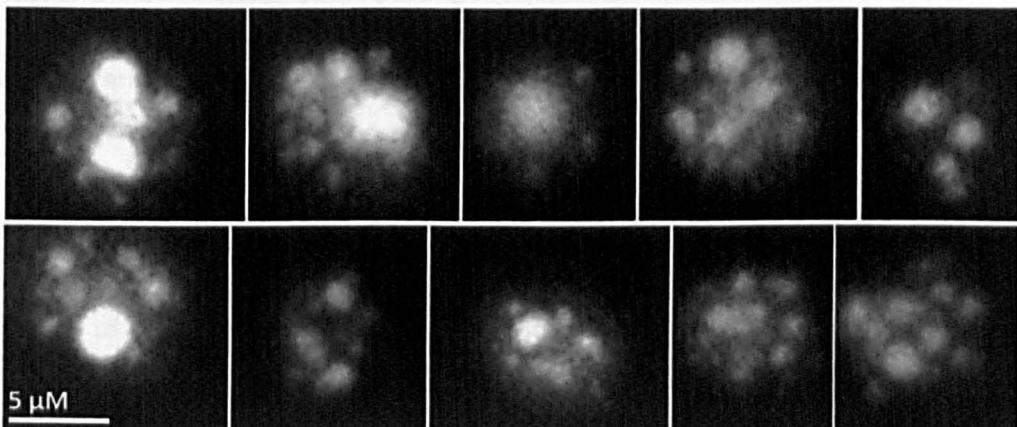
becomes localised to intracellular components (unknown) and not the plasma membrane, whilst TMA-DPH appears to be confined to the plasma membrane alone. As a consequence, DPH was disregarded for use in the current study and TMA-DPH was selected for further analysis. Upon localising in a membrane TMA-DPH becomes strongly fluorescent. The rod shaped fluorophore becomes orientated parallel to the lipid chains of biological membranes. The absorption and emission oscillators both lie along the long axis of the fluorophore and thus only rotations perpendicular to the long axis will result in depolarisation (Lakowicz and Prendergast, 1978b). There is a significant change in anisotropy exhibited during the phase transition from gel to liquid-crystal (Lentz *et al.*, 1976).

The majority of studies on lipid bilayers (Lakowicz and Prendergast, 1978a; Prendergast *et al.*, 1981) or intact cell membranes (Laroche *et al.*, 2001; Sharma, 2006; Abe and Hiraki, 2009) tend to utilise spectrofluorimeters to assess the fluorescence anisotropy (directionally dependent fluorescence intensity) of dyes such as DPH. This requires the measurement of the whole population of cells, and as a consequence will not indicate the occurrence of any potential sub-populations within the sample. It has previously been suggested that sub-populations of dead yeast cells may demonstrate a pronounced difference in the fluidity of their membrane when compared to viable cells (Learmonth and Gratton, 2002). As non-viable cells represent a significant proportion of cells in dried yeast cultures it is clear that a method which would enable the detection of these cells would be advantageous. Arndt-Jovin *et al.* (1976) demonstrated that DPH anisotropy measurements of cell populations are similar whether measured statically (spectrofluorimeter) or dynamically (flow system). In the current study flow cytometry was selected for analysis to enable each cell, and therefore heterogeneity within the sample, to be evaluated separately. The polarisation property of the dye is normally derived using Equation 5.1. However, the limitations of the flow cytometry method used in the current study

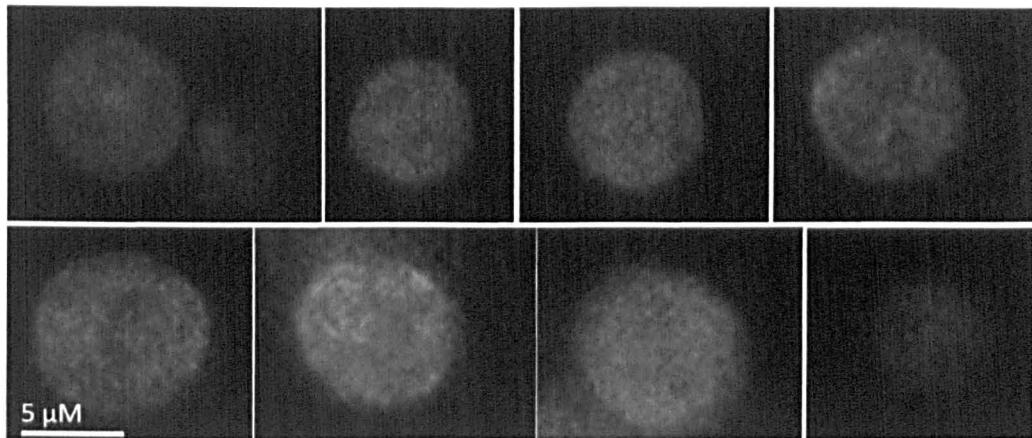
necessitated a ratio of vertically polarised light to horizontally polarised light to be recorded. Although this does not provide absolute values, it does allow comparison between the samples examined here. An increase in the ratio, as with an increase in the polarisation value, indicates a more rigid structure.

**Equation 5.1 Calculation for fluorescence polarisation value  $P$ , With  $I_{\parallel}$  and  $I_{\perp}$  as the fluorescence intensity parallel and perpendicular to the incident laser light polarisation.**

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$



**Figure 5.7.** Yeast cells stained using the fluorescent dye DPH (using the same protocol as used for fluidity assessment) and viewed at a magnification of  $\times 1000$ . The stain is not localised to the plasma membrane, but instead appears to have concentrated in internal organelles.



**Figure 5.8.** Yeast cells stained using the fluorescent dye TMA-DPH (using the same protocol as used for fluidity assessment and viewed at a magnification of x1000. The stain appears to be localised to the plasma membrane, with no obvious staining of internal organelles evident, suggesting it is suitable for specific probing of the plasma membrane.

### 5.2.3 Verification of the fluidity assessment protocol using mutants exhibiting membrane differences

To ensure the dye (TMA-DPH) and instrument (flow cytometer) were capable of determining fluidity differences, control samples were required to test the differentiation capacity of the method. To address this, mutants with impaired fluidity were deemed an appropriate means of achieving controls where differences were more likely to be consistently expressed. Disruption of ergosterol synthesis promotes a reduction in the rigidity of the membrane which has been previously detected by reduced polarisation in the presence of TMA-DPH (Sharma, 2006; Abe and Hiraki, 2009). It has been suggested that these differences may be due to a lack of packing of the lipid acyl chains , whilst the ratio of sterols to phospholipids may also be altered in ergosterol mutants (Abe and Hiraki, 2009). Two yeast strains with disruptions in the ergosterol pathway (Section 5.2.1), were used to ascertain the validity of the fluidity detection system.

Cells were grown at 25°C and stained with TMA-DPH for 1 hour prior to analysis as described in Section 2.7.2. The fluorescence was measured in two planes with the use of polarising filters; the ratio of these intensities provided an indication as to the degree of

fluorescence anisotropy exhibited by the bound dye. The ratio values recorded for the ergosterol mutants were lower than that for the wild type, suggesting they have a more fluid (less rigid) membrane (Table 5.1). This is in agreement with previous work (Sharma, 2006; Abe and Hiraki, 2009).

**Table 5.1.** The ratio of horizontally to vertically polarised light emitted from single yeast cells stained with TMA-DPH. Yeast strains containing single deletions in the ergosterol synthesis pathway were compared to the wild type strain. \*Indicates that these values were significantly different to the values for the control (wild type) strain (significance determined using an analysis of variance)

EUROSCARF strain number	Deleted gene	Ratio of vertical to horizontal polarised light (mean of three replicates of 100,000 events $\pm$ S.D.)
Y00000	Wild type	$0.3335 \pm 0.0020$
Y02667	<i>erg3</i>	$0.3128 \pm 0.0019^*$
Y00788	<i>erg2</i>	$0.3079 \pm 0.0055^*$

#### 5.2.4 The identification of sub-populations contained within rehydrated dried yeast cultures

The cultures analysed in Table 5.1 were homogeneous in terms of their viability and therefore directly comparable. The primary concern with applying the same assay to ADY and the corresponding control sample was that heterogeneity would exist within each population. Indeed one particular source of heterogeneity concerns cell death and its influence on fluidity (Learmonth and Gratton, 2002). ADY populations exhibit a lower viability than control populations, laboratory grown cells, and could therefore be expected to exhibit differences in fluidity as a function of viability. Whilst this topic is interesting, the objective of the analysis was simply to consider differences in fluidity in live cells. To

circumvent the issue of heterogeneity a gating process was used. Gating permits grouping of populations which exhibit significant differences and can be used to exclude or include these populations from future processing (Givan, 1992). The scatter of light by cells is often used to distinguish between different cell types (Olson *et al.*, 1989; Deere *et al.*, 1998; Givan, 2004) and also viability (Givan, 1992). There are two types of scattered light which are commonly recorded by flow cytometer instruments. Forward scatter is a measure of the spread of light which passes directly through cells and can be used as an indication of the size of a cell, with larger cells scattering the light to a greater extent (Givan, 1992). It can also be used as an indication of cell viability as non viable cells often scatter less light. In non-viable cells the plasma membrane may become leaky allowing the carrier fluid, which the cells are suspended in, to diffuse into the cell resulting in a refractive index inside the cell similar to that outside and reducing the degree of scattering (Shapiro, 2003). Side scatter is a measure of the amount of light scattered at 90° to the direction of the incident light. This can give an indication to the degree of cell granularity, which may be reduced in apoptotic cell death as organelles are broken down. In this study, side scatter (PMT1 Lin) and forward scatter (FS Lin) were plotted against each other in an attempt to identify non-viable and viable cells.

Initial gates (Region A) were performed to remove cells (events) which had either horizontally or vertically polarised light intensities beyond the limit of detection. Such events equated to up to 10 % of the sample. A second gate (Region B) was performed using dot plots of scattered light (forward and side scatter), isolating the main body of events, excluding larger events (possibly containing two cells) or smaller events (cell debris).

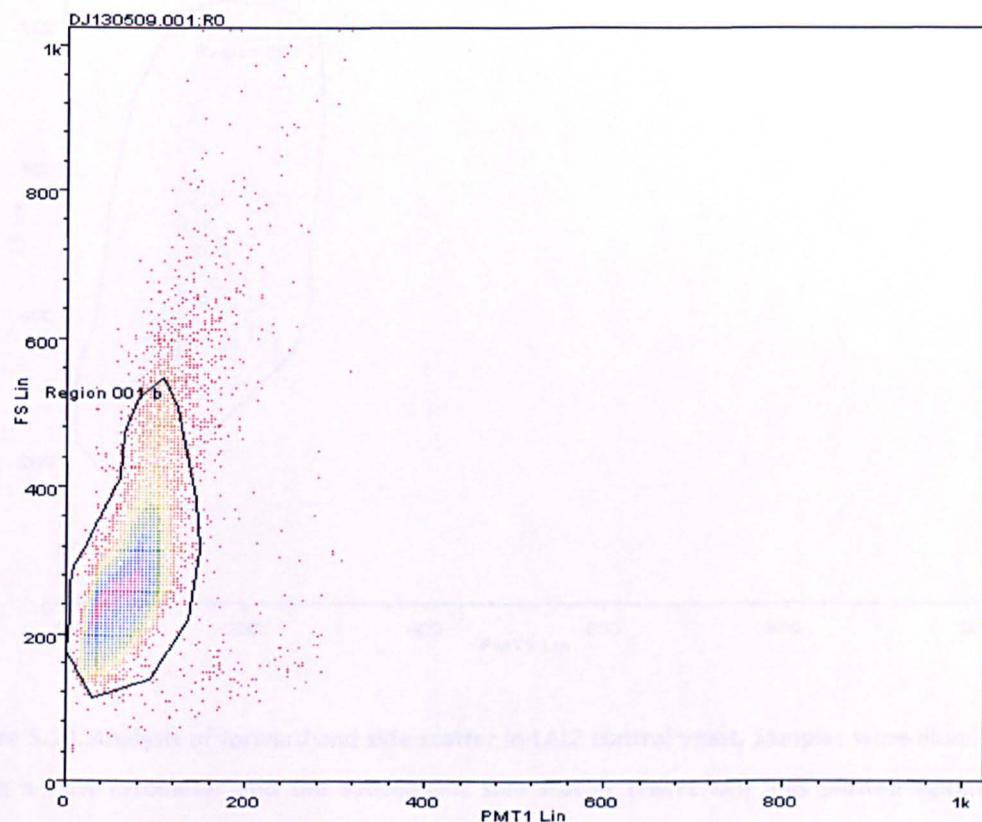
The analysis of control samples of each of the three strains (LAL1, LAL2 and LAL4) yielded similar scatter profiles. The dot plot generated (from forward and side scattered light) shows a single group for each population (Figures 5.9-5.11). However, within each

population there was a relatively large degree of variation with respect to forward scatter, indeed this variation was greater in control populations than in rehydrated yeast populations. One reason for this may be differences in cell size. Cell size is thought to vary with the replicative age of laboratory yeast cells (Hartwell and Unger, 1977), a trait also noted in brewing yeast (Barker and Smart, 1996).

Dried yeast (rehydrated at 30°C) showed less variation, with respect to forward scatter, compared to control yeast (Figures 5.12-5.14), suggesting that the population contained a greater number of small cells. For two of the strains (LAL1 and LAL2) variation in side scatter showed an increase which, combined with variation in forward scatter, indicates the presence of sub-populations (Figures 5.12 and 5.13). However, the proximity of the groups and the lack of a definite separation makes the gates created (indicated in Figures 5.12 and 5.13) subjective. The relative proportions seen in the two sub-populations are similar to those which might be predicted if they contained viable and non-viable cells, but there is some variation to the viability as determined by methylene blue staining. When yeast was rehydrated at 15°C there was a reduction in viability, as might be expected. Analysis of the dot plots generated based on scattered light (Figures 5.15 and 5.16) indicated sub-populations which could be defined, although some overlap was observed. Again, the proportions of events contained within each gate are similar to those which might be expected from viable and non-viable populations.

Whilst LAL4 control yeast demonstrated similar population trends to those observed with the previous control samples (Figure 5.11), the rehydrated yeast responded differently with respect to scattered light. When LAL4 dried yeast was rehydrated at both 30°C (Figure 5.14) and 15°C (Figure 5.17) there was a lack of identifiable sub-populations. This result was interesting because these cultures exhibited viabilities of 75 % and 71 % respectively. In this

instance, fluidity measurements were therefore calculated for both the overall population and the identified sub-populations for LAL1 and LAL2 (Section 5.2.3).



**Figure 5.9. Analysis of forward and side scatter in LAL1 control yeast.** Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.

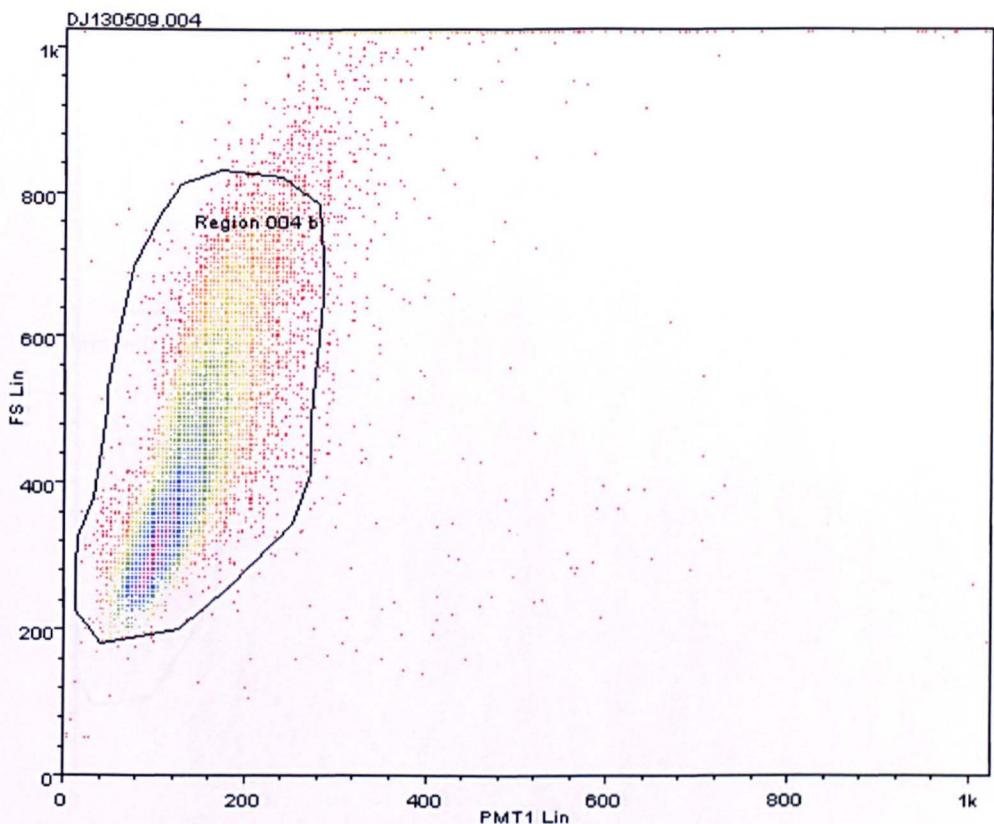
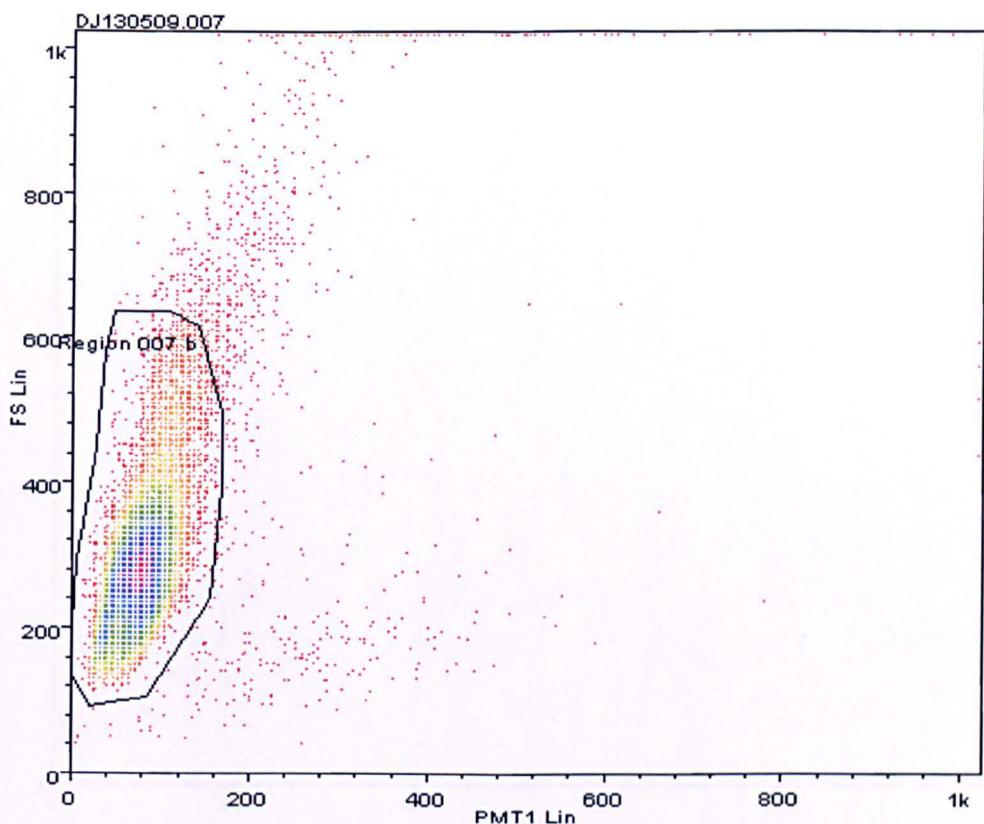
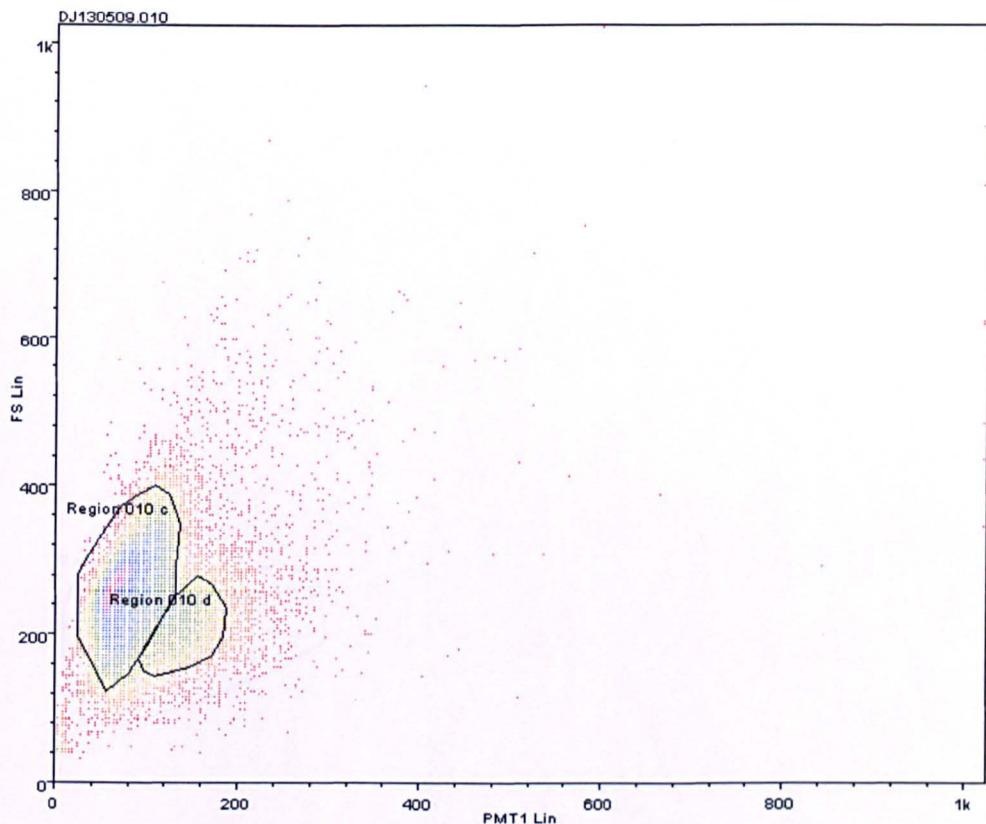


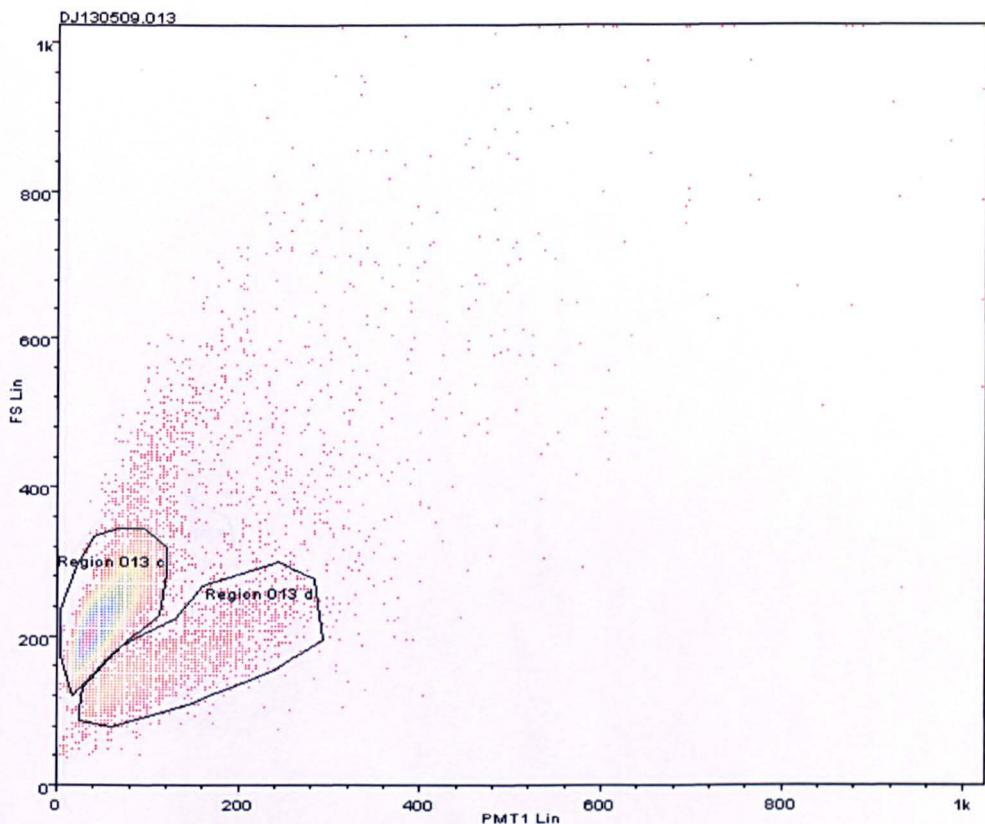
Figure 5.10. Analysis of forward and side scatter in LAL2 control yeast. Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.



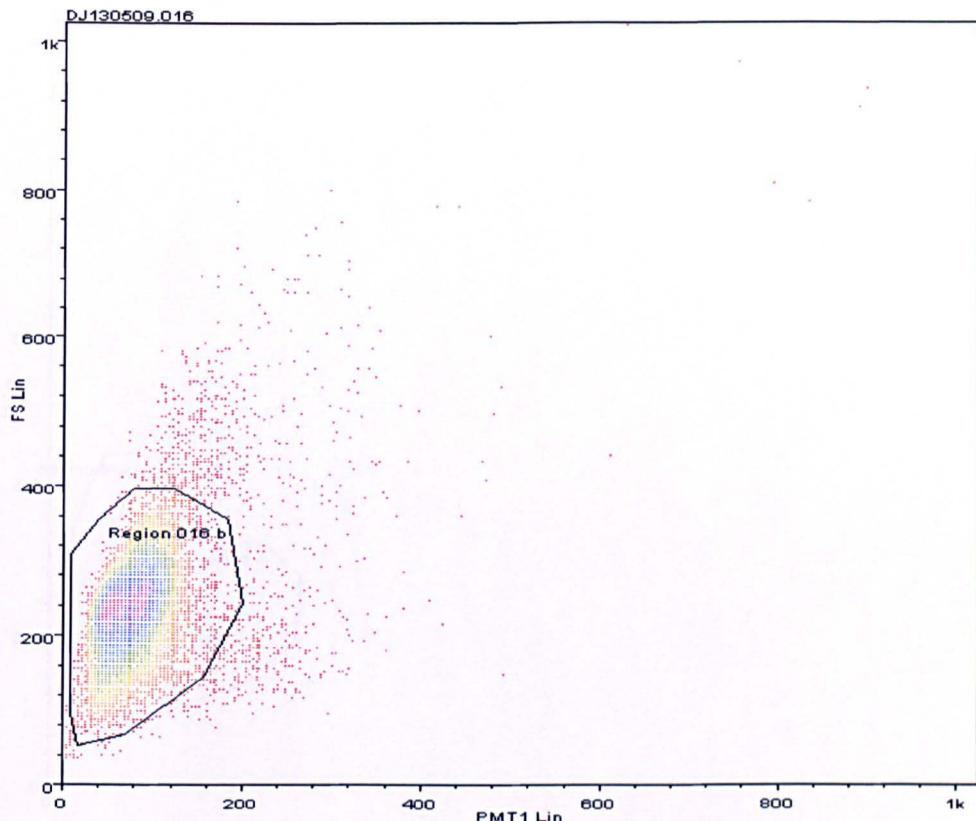
**Figure 5.11. Analysis of forward and side scatter in LAL4 control yeast cells.** Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.



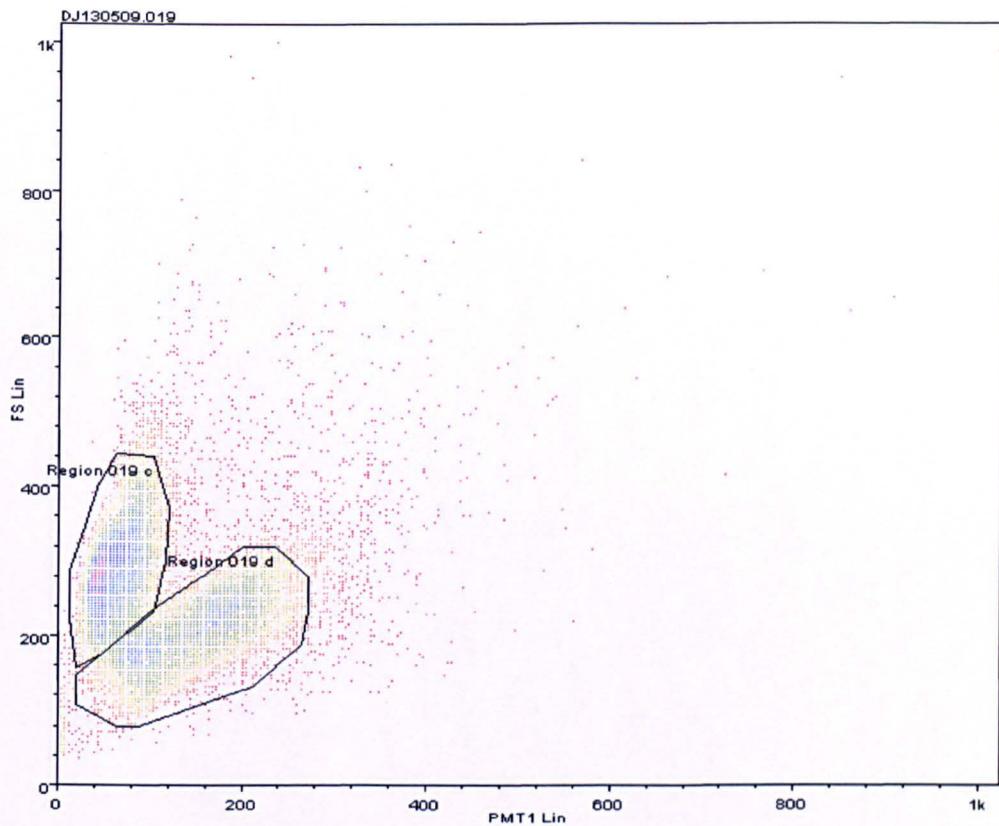
**Figure 5.12. Analysis of forward and side scatter in LAL1 dried yeast cells (rehydrated at 30°C).** Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.



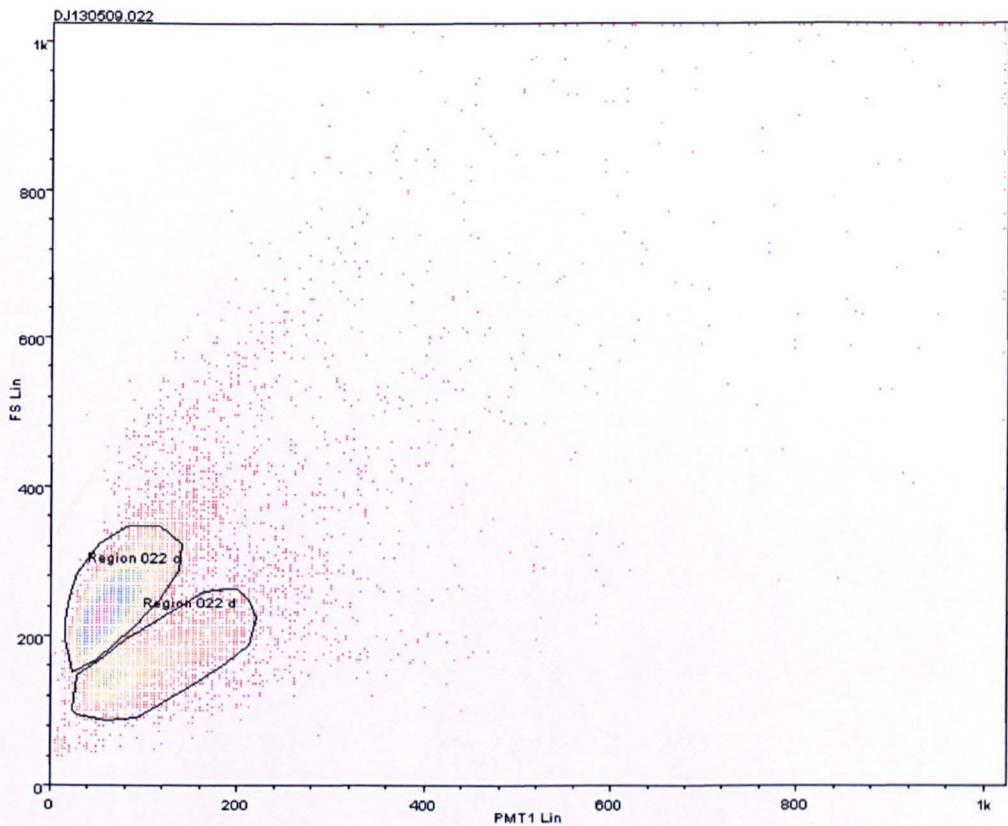
**Figure 5.13. Analysis of forward and side scatter in LAL2 dried yeast cells (rehydrated at 30°C).** Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.



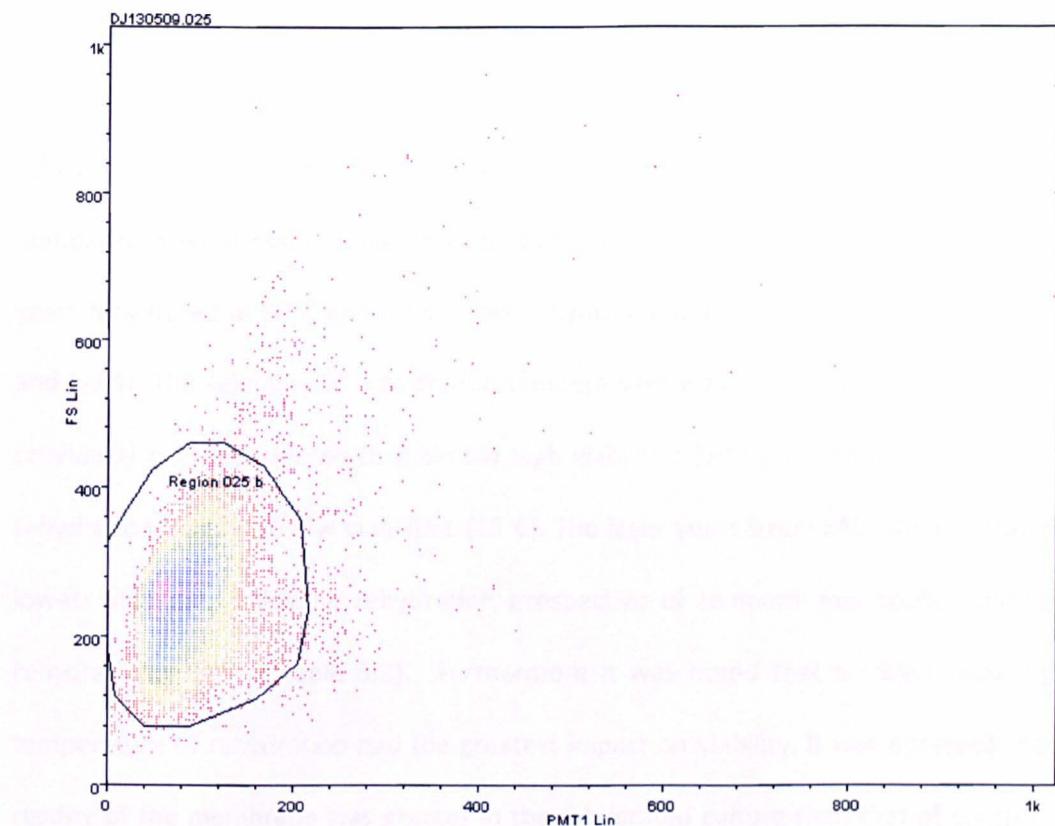
**Figure 5.14. Analysis of forward and side scatter in LAL4 dried yeast cells (rehydrated at 30°C).** Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.



**Figure 5.15.** Analysis of forward and side scatter in LAL1 dried yeast cells (rehydrated at 15°C). Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.



**Figure 5.16. Analysis of forward and side scatter in LAL2 dried yeast cells (rehydrated at 15°C).** Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.



**Figure 5.17.** Analysis of forward and side scatter in LAL4 dried yeast cells (rehydrated at 15°C). Samples were illuminated using a flow cytometer and the subsequent side scatter (PMT1 Lin) was plotted against the forward scatter (FS Lin) using WEASEL analysis software. The coloured regions indicate the density of events, purple where density is greatest, then green and yellow, with red signifying a single event. The polygon labelled “Region 001 b” is a user defined region, based on perceived grouping of cells. This is one example of triplicate sample runs and dot plots which were similar in appearance.

samples were rehydrated at 15°C and 30°C, and the resulting cell morphology was determined by light microscopy (Figure 5.18). The lack of an apparent difference which will be discussed later, has been due to the increased temperature causing the yeast to undergo a morphological change, the yeast cells becoming more spherical when the yeast are grown at 30°C than that of the control yeast at 15°C. Figure 5.19 shows a greater overall reduction of 15% compared to yeast rehydrated at 30°C. The increased radius, exhibited by cells rehydrated at 30°C, will be an important consideration when calculating the volume of each cell. The volume of each cell was calculated by the equation of the volume of a sphere based on the surface area of the cell, calculated by the equation of the surface area of a sphere.

### **5.2.5 Assessment of the relative fluidity of control and rehydrated dried yeast**

Fluidity was assessed using the ratio of vertically polarised light to horizontally polarised light. An increase in the ratio indicates a more rigid property (less fluid membrane). Comparisons were made between yeast that had not been previously dehydrated, dried yeast rehydrated at 30°C and dried yeast rehydrated at 15°C for three strains (LAL1, LAL2 and LAL4). The selection of rehydration temperatures was based on conditions that have previously been considered to maintain high viabilities (30°C) and challenge yeast during rehydration leading to low viabilities (15°C). The lager yeast strain LAL1 demonstrated the lowest viabilities following rehydration irrespective of temperatures applied during the rehydration process (Table 5.2). Furthermore it was noted that for LAL1 reducing the temperature of rehydration had the greatest impact on viability. It was observed that the rigidity of the membrane was greater in the rehydrated culture than that of control cells. The rigidity was also greater when cells were rehydrated at 15°C compared to 30°C. The relative rigidity of the sub-populations (identified in Section 5.2.4) showed little difference compared to control cells when cells were rehydrated at 30°C, but a difference was apparent in cells rehydrated at 15°C (Figure 5.18). The sub-population (B) which it is suggested consisted of non-viable cells, exhibited a greater rigidity compared to viable cells (suggested to be sub-population A), which would be in agreement with the previous work of Learmonth and Gratton (2002). The lack of an apparent difference when cells were rehydrated at 30°C may have been due to the less distinct sub-populations obscuring the results. The membrane rigidity exhibited by LAL2 dried viable yeast (sub-population A) when rehydrated at 30°C was lower than that of the control yeast (Figure 5.19). Rigidity was also greater in cells rehydrated at 15°C compared to those rehydrated at 30°C. The increased rigidity exhibited by cells thought to be non-viable was more apparent for strain LAL2 than that observed for LAL1. As no sub-populations were identified in LAL4, assessment of rigidity was based on the entire population (Figure 5.20). Whilst rigidity was

higher in cells rehydrated at 30°C, and greater still in cells rehydrated at 15°C, this may simply have been a reflection of the greater number of non-viable cells contained in each sample.

**Table 5.2.** The ratio of horizontally polarised light to vertically polarised light emitted from single yeast cells (LAL1) stained with TMA-DPH. YPD grown control samples were compared to ADY rehydrated at 30°C and 15°C. Gate reference is in relation to the regions creating using dot plots of forward and side scatter of light.

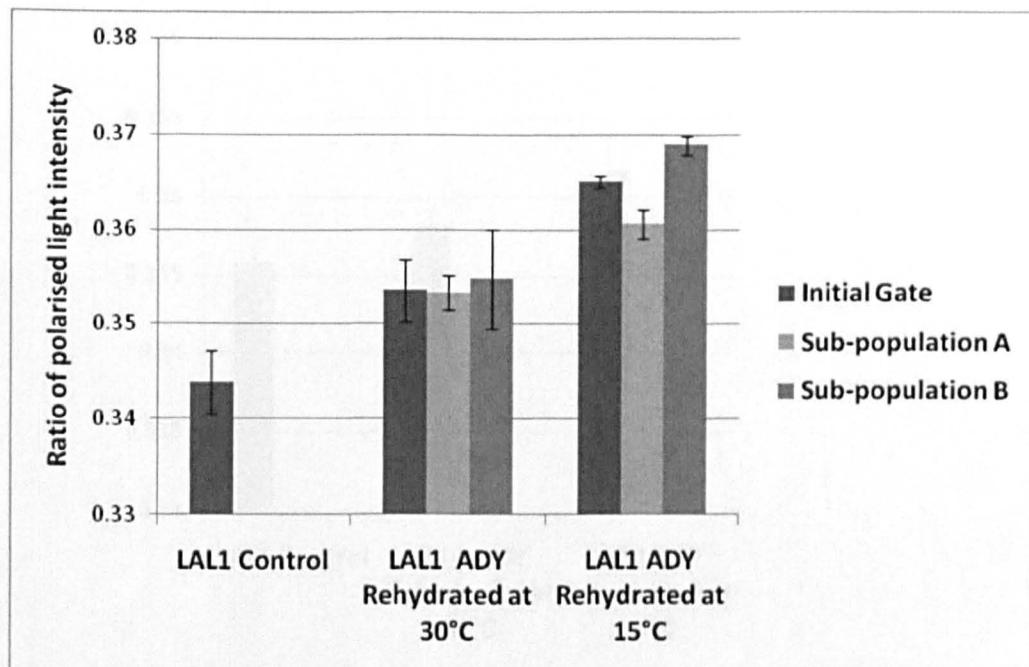
Sample	Viability determined by methylene blue staining	Gate Reference	Percentage of the total population contained within the gate	Ratio of vertical to horizontal polarised light (mean of three replicates of 100,000 events ±S.D.)
LAL1 Control	100 %	Whole Population	91 %	0.3438 ±0.0033
LAL1 ADY Rehydrated at 30°C	67 %	Whole Population	90 %	0.3536 ±0.0027
		Sub-Population A	59 %	0.3534 ±0.0018
		Sub-population B	17 %	0.3548 ±0.0052
LAL1 ADY Rehydrated at 15°C	54 %	Whole Population	93 %	0.3651 ±0.0006
		Sub-Population A	41 %	0.3606 ±0.0014
		Sub-Population B	43 %	0.3689 ±0.0010

**Table 5.3 The ratio of horizontally polarised light to vertically polarised light emitted from single yeast cells (LAL2) stained with TMA-DPH. YPD grown control samples were compared to ADY rehydrated at 30°C and 15°C. Gate reference is in relation to the regions creating using dot plots of forward and side scatter of light.**

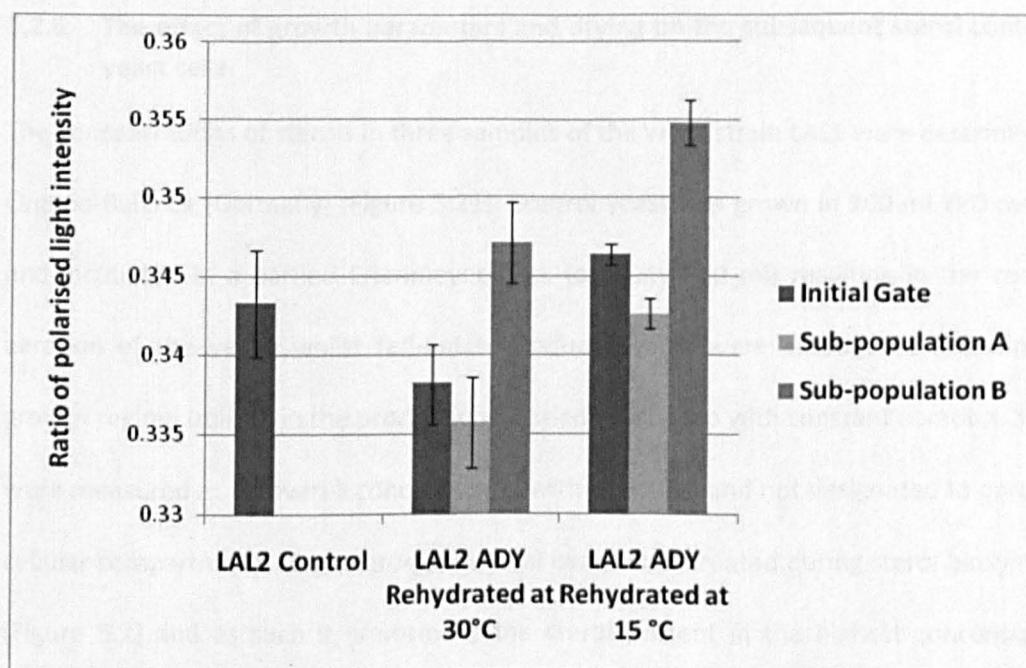
Sample	Viability determined by methylene blue staining	Gate Reference	Percentage of the total population contained within the gate	Ratio of vertical to horizontal polarised light (mean of three replicates of 100,000 events ±S.D.)
LAL2 Control	100 %	Whole Population	94 %	0.3433 ±0.0034
LAL2 ADY Rehydrated at 30°C	81 %	Whole Population	90 %	0.3382 ±0.0025
		Sub-Population A	67 %	0.3357 ±0.0029
		Sub-Population B	18 %	0.3471 ±0.0025
LAL2 ADY Rehydrated at 15°C	77 %	Whole Population	91 %	0.3464 ±0.0006
		Sub-Population A	61 %	0.3426 ±0.0010
		Sub-Population B	25 %	0.3548 ±0.0014

**Table 5.4 The ratio of horizontally polarised light to vertically polarised light emitted from single yeast cells (LAL4) stained with TMA-DPH. YPD grown control samples were compared to ADY rehydrated at 30°C and 15°C. Gate reference is in relation to the regions creating using dot plots of forward and side scatter of light.**

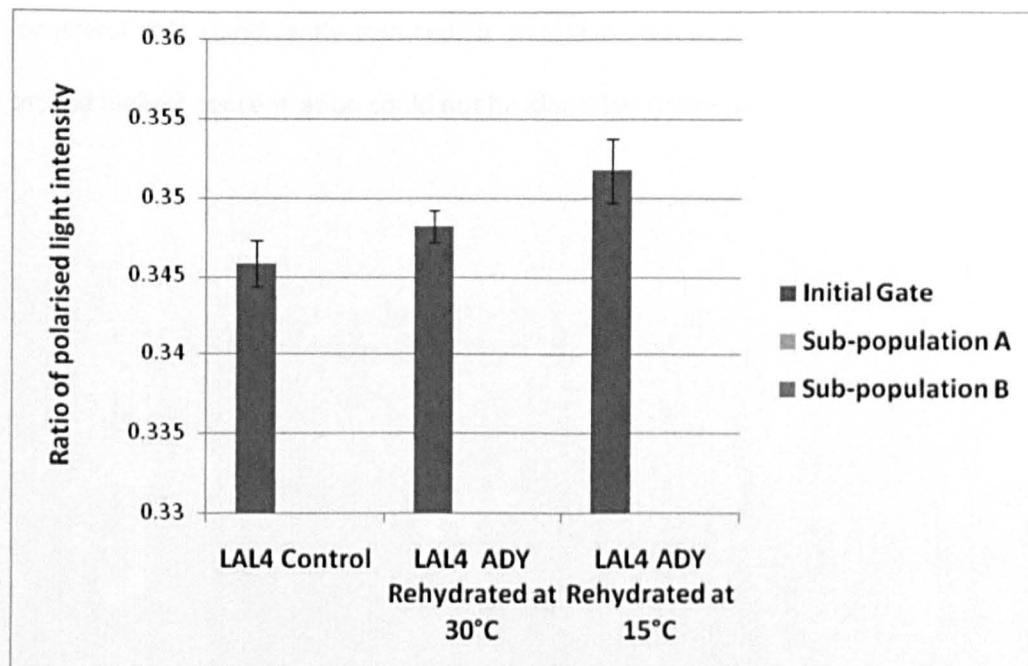
Sample	Viability determined by methylene blue staining	Gate Reference	Percentage of the total population contained within the gate	Ratio of vertical to horizontal polarised light (mean of three replicates of 100,000 events ±S.D.)
LAL4 Control	100 %	Whole Population	93 %	0.3458 ±0.0015
LAL4 ADY Rehydrated at 30°C	75 %	Whole Population	93 %	0.3482 ±0.0010
LAL4 ADY Rehydrated at 15°C	71 %	Whole Population	95 %	0.3517 ±0.0020



**Figure 5.18.** The ratio of vertically polarised light to horizontally polarised light emitted when LAL1 yeast cells are stained with TMA-DPA. Three types of yeast are displayed and sub populations defined by the grouping of cells based on their scatter properties.



**Figure 5.19.** The ratio of vertically polarised light to horizontally polarised light emitted when LAL2 yeast cells are stained with TMA-DPA. Three types of yeast are displayed and sub populations defined by the grouping of cells based on their scatter properties.

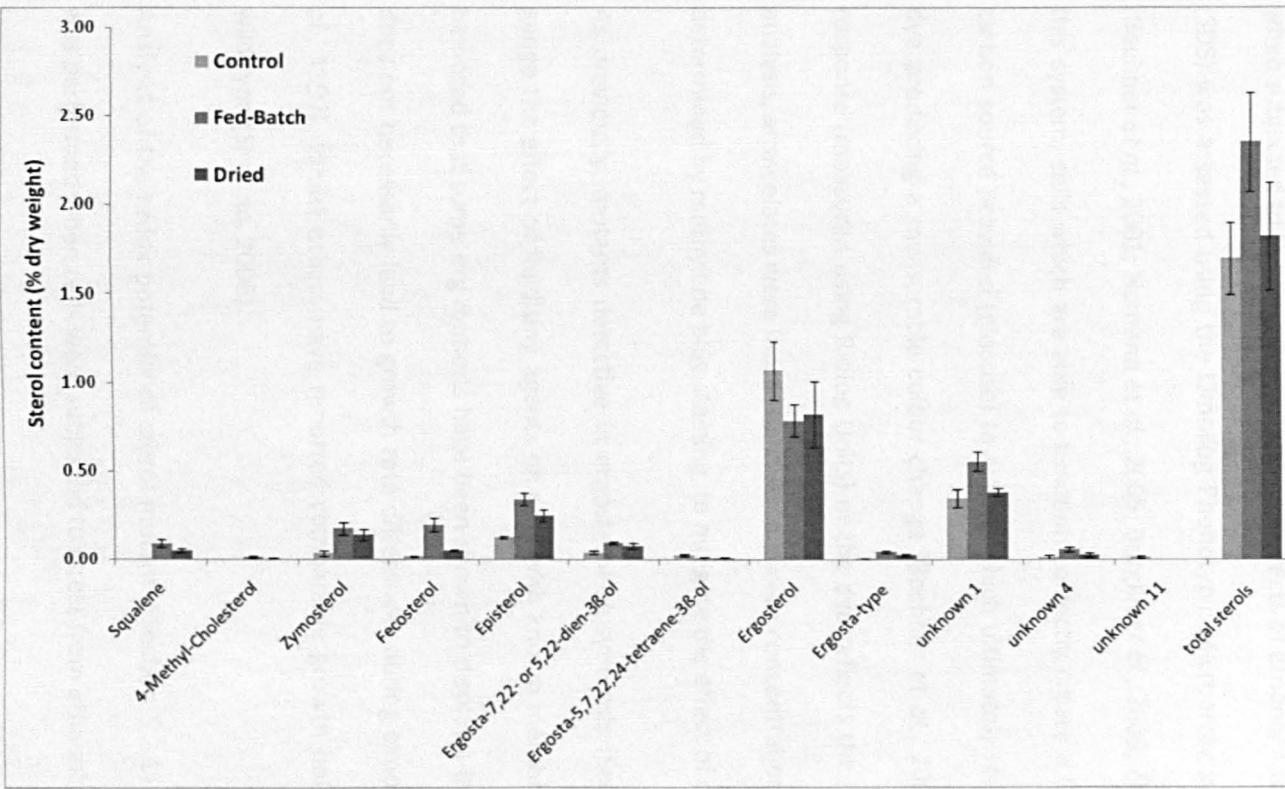


**Figure 5.20.** The ratio of vertically polarised light to horizontally polarised light emitted when LAL4 yeast cells are stained with TMA-DPA. Three types of yeast are displayed and sub populations defined by the grouping of cells based on their scatter properties.

### 5.2.6 The effect of growth parameters and drying on the subsequent sterol content in yeast cells

The concentrations of sterols in three samples of the yeast strain LAL1 were determined by Organo-Balance (Germany) (Figure 5.21). Control yeast was grown in 100 ml YPD medium and incubated in a baffled Erlenmeyer flask (capacity 250 ml) resulting in the constant aeration of the yeast, whilst fed-batch produced yeast were propagated following the growth regime utilised in the production of dried yeast, also with constant aeration. Sterols were measured as an overall concentration within the cell and not designated to particular cellular compartments. Ergosterol is the final compound created during sterol biosynthesis (Figure 5.2) and as such it is normally the sterol present in the highest concentrations within yeast cells, which was the case in all samples here (Figure 5.21). Differences were apparent in the levels of intermediate compounds, which were lower in control yeast compared to fed-batch and dried yeast. The effect of drying on sterol concentration, assessed by comparing fed-batch and dried yeast, appeared to have little effect, although

fecosterol was significantly reduced. It should be noted that the sterol present in the second highest concentration could not be identified (Unknown 1).



**Figure 5.21.** The levels of sterols identified in whole cell populations of three samples of the yeast LAL1. Control cells were grown following a laboratory propagation regime using YPD medium. Fed-batch yeast cells were grown by Lallemand Inc. (Montreal, Canada) following the propagation regime utilised in the production of dried yeast. A proportion of the fed-batch yeast was subsequently dried using a fluidised-bed drier producing dried yeast samples. Cells were sent to Organo-Balance (Germany) for the determination of their respective sterol contents. Sterols were extracted, prior to separation and identification using GC-MS. Unknowns were thought to be sterols, but could not be identified using GC-MS.

### **5.2.7 The affect of membrane perturbing agents on cell function**

When yeast cells are exposed to ethanol the plasma membrane becomes more fluid (Jones and Greenfield, 1987). Other chemicals, such as sodium dodecyl sulphate (SDS), are also known to have similar fluidising effects. The ability of cells to maintain redox potential when subjected to the membrane fluidising effects of ethanol and sodium dodecyl sulphate (SDS) was assessed using the Omnilog Phenotypic Microarray system (Biolog Inc., U.S.A.) (Bochner *et al.*, 2001; Homann *et al.*, 2005; Borglin *et al.*, 2009; Zhang and Biswas, 2009). In this system, cells which are able to function correctly create a flow of electrons from the carbon source provided (glucose) to NADH, which ultimately reduces a tetrazolium based dye producing a measurable colour change (Bochner *et al.*, 2001). The final total colour response (measured using Biolog Units) of the dye reflects the redox potential. For each analysis, array plates were inoculated with a known concentration of viable cells, previously determined by methylene blue staining, to mitigate the effect of cell death on the assay.

As previously, mutants defective in ergosterol biosynthesis (Section 5.2.1) were used to gauge the effect of fluidising agents on cells with known membrane differences. It should be noted that some erg mutants have been shown to display a lag in growth, although this does not necessarily lead to growth rate differences during exponential phase (Palermo *et al.*, 1997). Whilst others have reported comparable growth between sterol mutants and wild type (Sharma, 2006).

Analysis of the redox potential of sterol mutants (Section 5.2.1) and their wild type strain was performed when cells were subjected to stress from ethanol and SDS respectively.

In this study the wild type strain appeared to have a higher redox potential than the mutants, but this difference was not significant (significance determined using an analysis of variance at P<0.05). When grown in the presence of ethanol the redox potential of the wild type was higher than that of the mutants even when the lowest concentration of

ethanol (3 %) was applied (Figure 5.22). This suggests that the wild type cells retained viability in the ethanol environment while mutant strains could not. It is likely that the rigidity of the plasma membrane, influenced by ergosterol, enabled wild type cells to counteract the fluidising effect of ethanol. A similar effect was also observed when 0.04 % SDS was applied (Figure 5.23).

When the test strains (LAL1, LAL2 and LAL4) were analysed LAL1 control yeast demonstrated a higher redox potential than dried yeast in the presence of ethanol, with the difference magnified with increasing ethanol concentrations (Figure 5.24). Both the control and dried cultures of LAL2 demonstrated similar responses to ethanol stress. LAL4 dried yeast demonstrated a lower redox potential than control yeast, however, the difference was not as great as that demonstrated by LAL1. In response to the fluidising effects of SDS, control and rehydrated yeast demonstrated similar responses in LAL2 and LAL4 strains respectively (Figure 5.25). In contrast LAL1 control yeast again demonstrated a higher response than its dried equivalent (Figure 5.25).

It is important to note that this analysis of stress response was conducted under aerobic conditions. Although it is recognised that brewing fermentations are conducted anaerobically, the preliminary stages are aerobic. Consequently, as the environment which ADY first encounters both during rehydration and at inoculation into wort is aerobic in nature, this particular environment was modelled here.

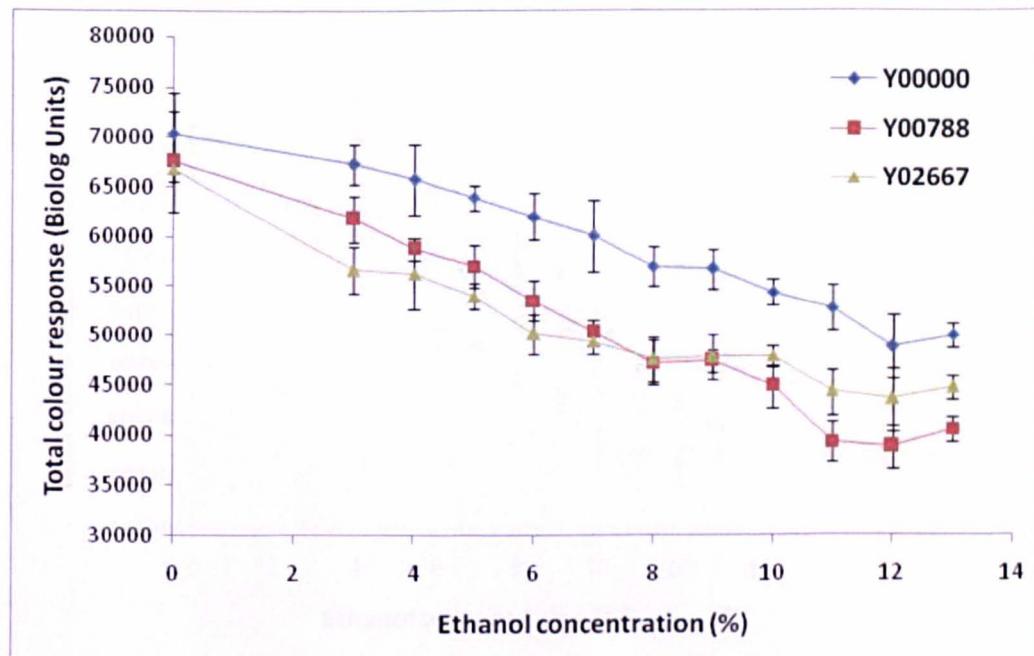


Figure 5.22. The redox potential of yeast cells when challenged with ethanol. The redox potential was assessed using the total colour response (Biolog units) generated by the reduction of a tetrazolium dye after three days incubation. The standard deviation of three replicates is indicated.

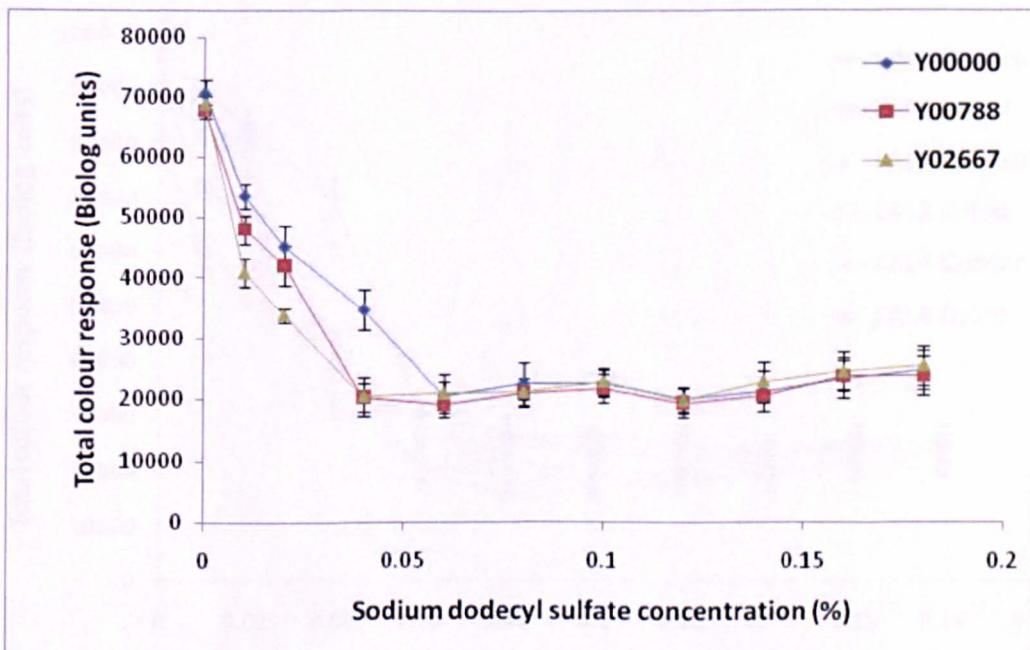


Figure 5.23. The redox potential of yeast cells when challenged with SDS. The redox potential was assessed using the total colour response (Biolog units) generated by the reduction of a tetrazolium dye after three days incubation. The standard deviation of three replicates is indicated.

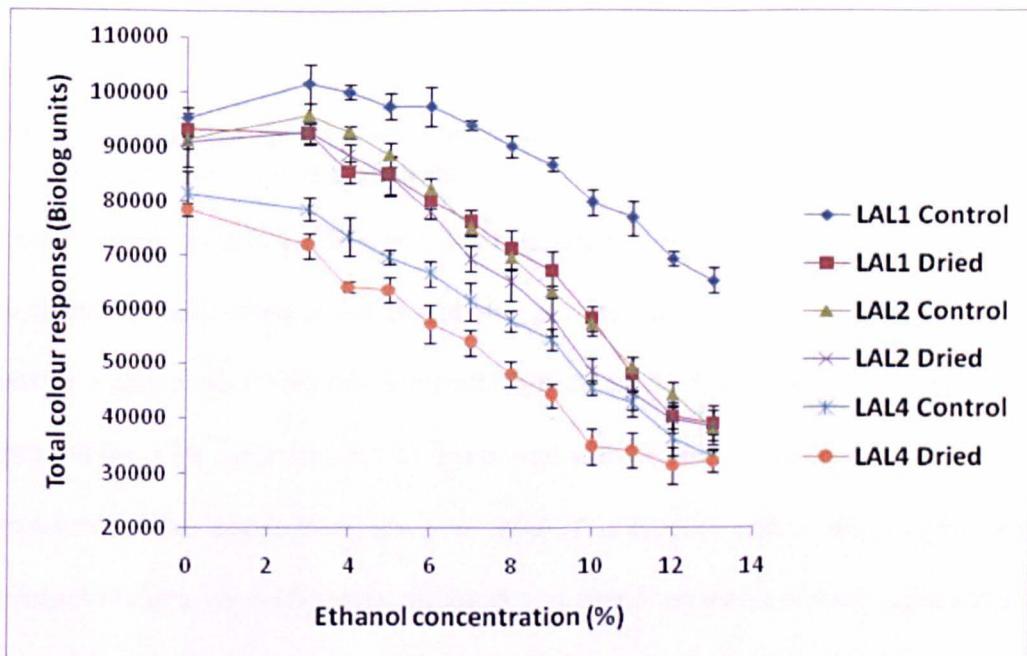


Figure 5.24. The redox potential of dried and control yeast cells when challenged with ethanol. The redox potential was assessed using the total colour response (Biolog units) generated by the reduction of a tetrazolium dye after three days incubation. The standard deviation of three replicates is indicated.

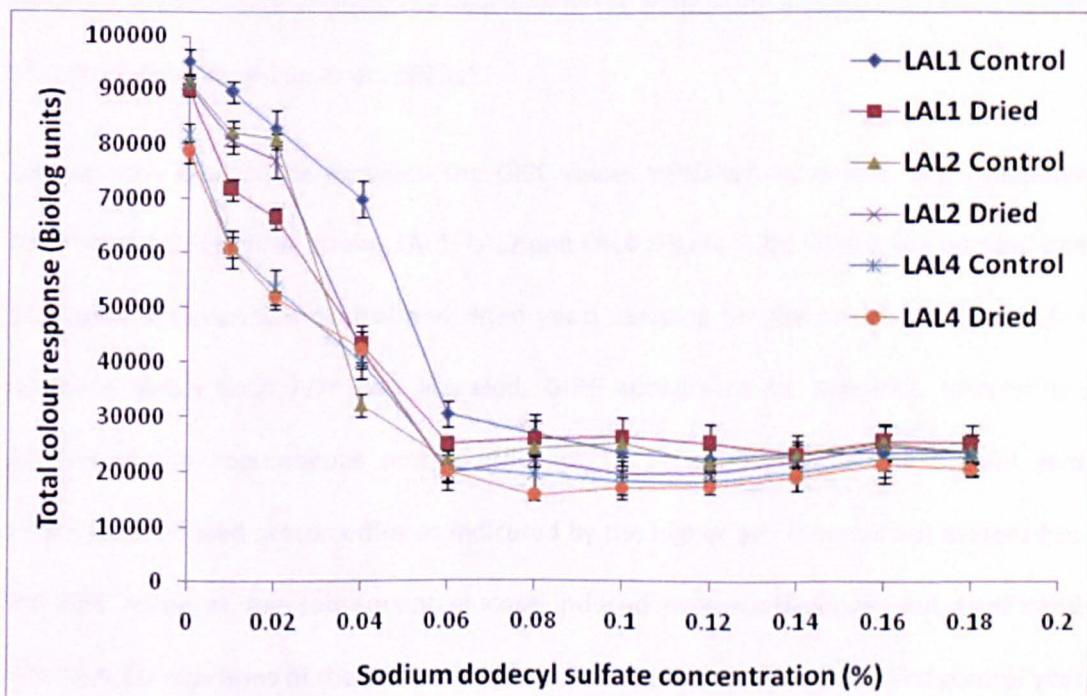


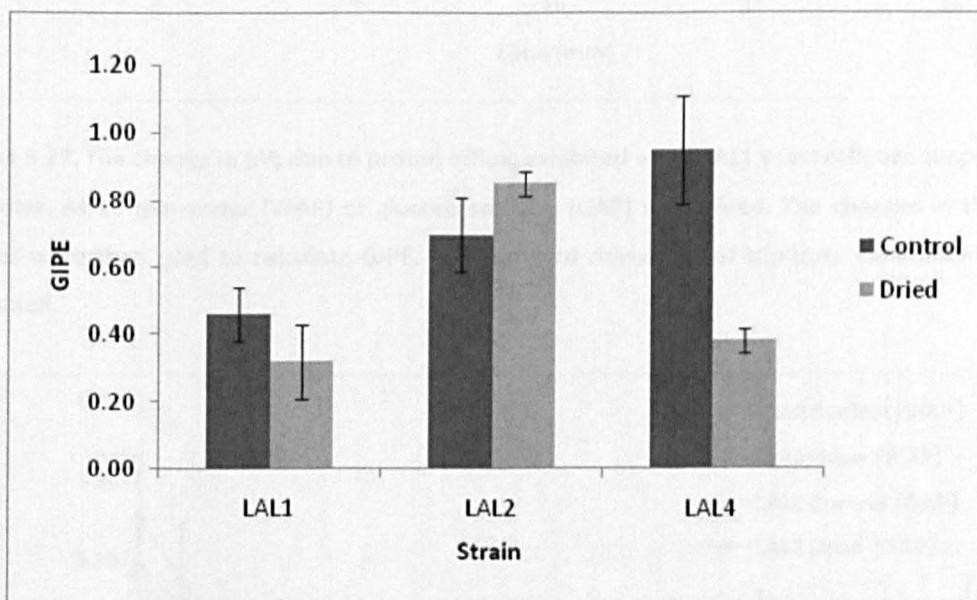
Figure 5.25. The redox potential of yeast cells when challenged with SDS. The redox potential was assessed using the total colour response (Biolog units) generated by the reduction of a tetrazolium dye after three days incubation. The standard deviation of three replicates is indicated.

### **5.2.8 The affect of drying and rehydration on yeast populations' proton efflux in response to a glucose substrate**

Glucose induced proton efflux (GIPE) was calculated using the pH change observed when yeast cells are suspended in water and also glucose solution. Once suspended in water, yeast populations normally cause a reduction in the pH of the surrounding medium due to proton efflux. This reduction can be measured with respect to time, producing a value termed the water acidification power or WAP. This type of proton efflux is thought to represent the utilisation of reserve carbohydrates contained within the cell (Opekarova and Sigler, 1982). When glucose is added, the proton efflux is increased and can be measured to indicate the glucose acidification power or GAP. The difference between these values (the GIPE value) provides an indication of H<sup>+</sup>-ATPase enzyme activity due to the uptake of glucose and also represents a determination of plasma membrane functionality. Its value, however, is dependent on both the leakiness of the membrane and activity of the enzyme H<sup>+</sup>-ATPase (Van Zandycke *et al.*, 2003a).

Comparisons were made between the GIPE values exhibited by control and rehydrated dried yeast for the three strains LAL1, LAL2 and LAL4 (Figure 5.26). There was no significant difference between the control and dried yeast samples for the strains LAL1 and LAL2. However, when LAL4 ADY was assessed, GIPE appears to be impaired. Interestingly, analysis of the spontaneous proton efflux of LAL1 (WAP) indicated that dried yeast displayed a reduced proton efflux as indicated by the higher pH. This was not evident from the GIPE value as the subsequent glucose induced proton efflux was not significantly different. Comparisons of the WAP values for LAL2 showed that the dried and control yeast populations were comparable until the addition of water (10 min). At this point the dried yeast sample maintained a lower pH whereas the control yeast yielded a slight increase. The difference between the populations was mirrored when glucose was added for the

assessment of glucose acidification power (GAP). This resulted in two GIPE values which were not significantly different (assessed using Student's T-Test), despite the underlying differences of the values used in their calculations. LAL4 was the only strain to exhibit significantly different GIPE values between control and dried yeast populations. This difference arose due to a small change in WAP by the control yeast compared to a much greater WAP for the dried yeast population. The final pH value achieved by both yeast cultures upon the addition of glucose was comparable.



**Figure 5.26. The Glucose induced proton efflux (GIPE) of control grown yeast and rehydrated dried yeast for the three strains LAL1, LAL2 and LAL4. The standard deviations of triplicate experiments are indicated.**

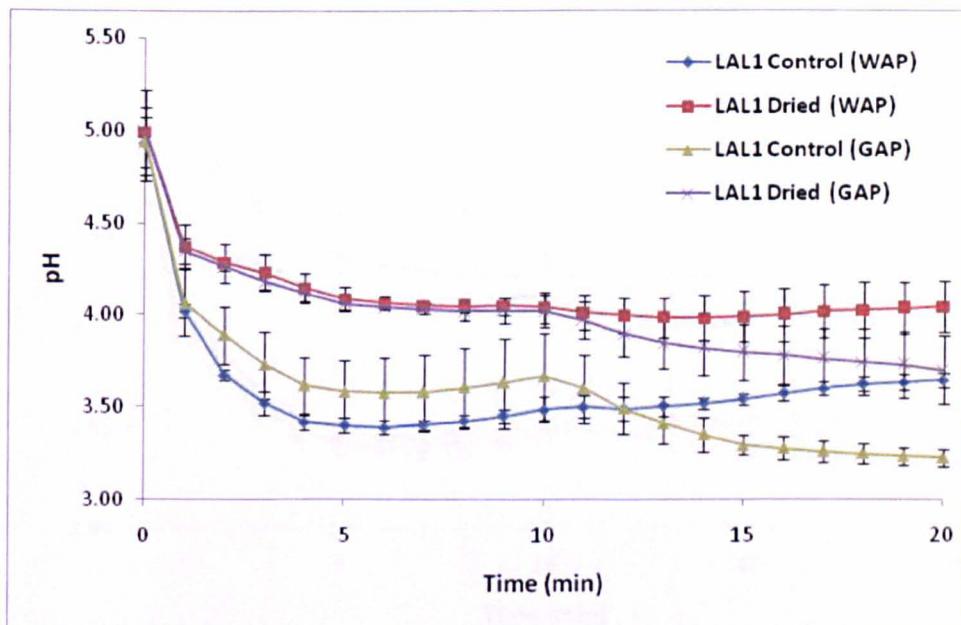


Figure 5.27. The change in pH, due to proton efflux, exhibited when LAL1 yeast cells are suspended in water. At 10 min water (WAP) or glucose solution (GAP) was added. The changes in the pH values were then used to calculate GIPE. The standard deviations of triplicate experiments are indicated.

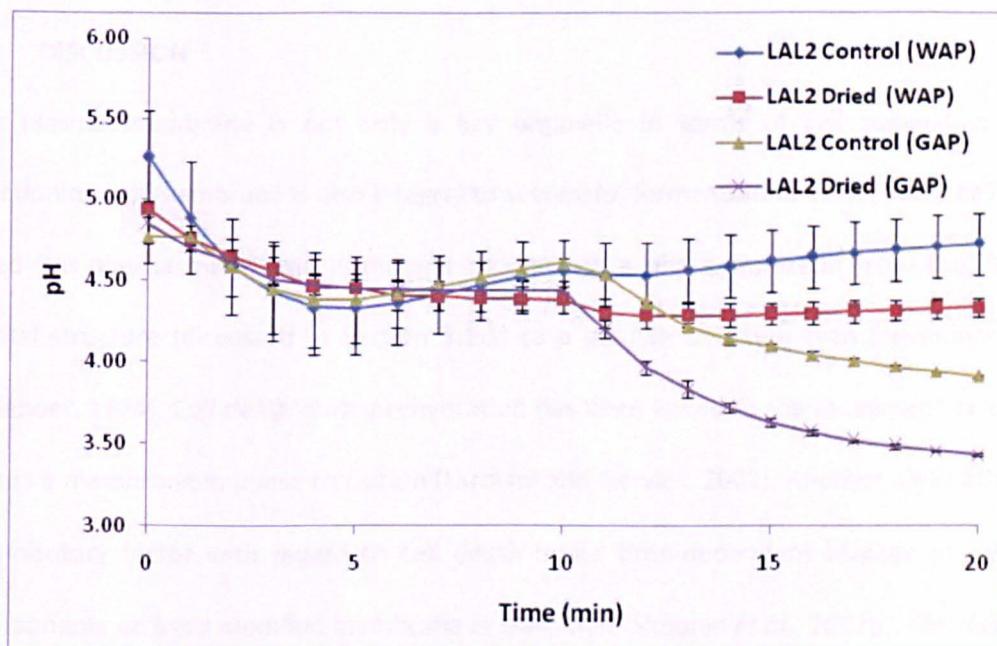


Figure 5.28. The change in pH, due to proton efflux, exhibited when LAL2 yeast cells are suspended in water. At 10 min water (WAP) or glucose solution (GAP) was added. The changes in the pH values were then used to calculate GIPE. The standard deviations of triplicate experiments are indicated.

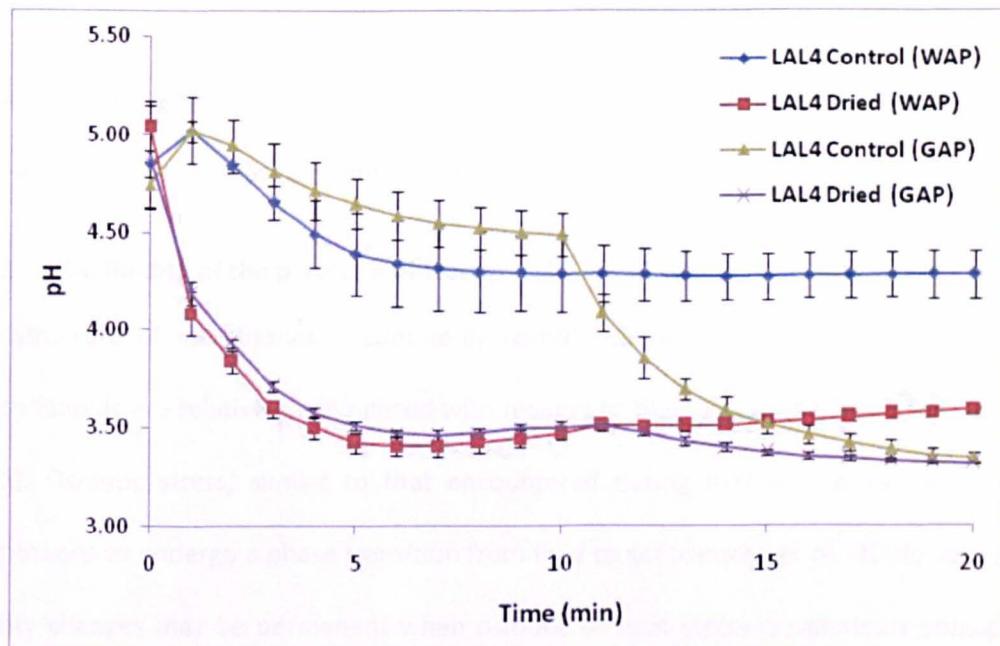


Figure 5.29. The change in pH, due to proton efflux, exhibited when LAL4 yeast cells are suspended in water. At 10 min water (WAP) or glucose solution (GAP) was added. The changes in the pH values were then used to calculate GIPE. The standard deviations of triplicate experiments are indicated.

### 5.3 DISCUSSION

The plasma membrane is not only a key organelle in terms of cell survival; a fully functioning cell membrane is also integral to successful fermentation. When yeast cells are dried the plasma membrane is thought to undergo a phase transition from the liquid-crystal structure (discussed in Section 5.1.1) to a gel like structure (van Steveninck and Leudeboer, 1974). Cell death during rehydration has been linked to the movement of water across a membrane in phase transition (Laroche and Gervais, 2003). Another alternative or contributory factor with regard to cell death is the time-dependent leakage of cellular components across a modified membrane in transition (Simonin *et al.*, 2007b). Morris *et al.* (1986) demonstrated that during osmotic stress cell death could occur due to the shrinkage of the cell occurring without complete plasmolysis (separation of the plasma membrane and cell wall), which led to membrane rupture. Although agreement on the mechanism of necrosis has not been reached, what is clear is the plasma membrane is significantly

perturbed during dehydration and rehydration. It is therefore pertinent to assess the membrane properties of cells which have been dehydrated and rehydrated to ensure that they are fit-for-purpose in subsequent fermentations.

### **5.3.1 The fluidity of the plasma membrane**

The structure of membranes is commonly considered to be in a fluid state, in which phospholipids are relatively unhindered with respect to their sideways movement (Section 5.1.1). Osmotic stress, similar to that encountered during ADY production, can cause membranes to undergo a phase transition from fluid to gel (Laroche *et al.*, 2001). Although fluidity changes may be permanent when osmotic or heat stress is significant enough to cause death (Learnmonth and Gratton, 2002), when death does not occur, changes are often transient (Simonin *et al.*, 2007a). Therefore, although changes in fluidity are likely to occur during dehydration and rehydration, the impact to subsequent fermentation performance may be minimal if the changes are transient.

The stresses encountered by yeast during dehydration and rehydration are significant enough to cause the death of a substantial proportion of the population, which it is suggested have a more rigid membrane (Section 5.2.5). This is supported by the observation that when cells are rehydrated at a reduced temperature (15°C) they exhibit a more rigid membrane than when cells are rehydrated at higher temperatures.

When rehydrated yeast was compared to control yeast the variations in membrane rigidity appeared to be strain specific. The lager yeast LAL1 exhibited a greater rigidity in rehydrated cells (at 30°C) compared to control yeast, whilst the ale yeast LAL2 exhibited a greater fluidity than its respective control yeast. For each of these strains two populations were immediately apparent with respect to fluidity potentially representing viable and non-viable cells. Interestingly LAL4 did not exhibit two populations and the reasons for this are not known.

### **5.3.2 The potential sources of the fluidity variations**

The fluidity of a membrane is influenced by many environmental and cellular factors. In the case of dried yeast the important environmental factors to consider are temperature variations (Simonin *et al.*, 2008) and the loss of water (Laroche *et al.*, 2001). Environmental factors may elicit adaptive cellular changes which alter the membrane fluidity (Arneborg *et al.*, 1995). Cellular factors which determine fluidity include trace elements, for example iron, copper or zinc (Garcia *et al.*, 2005), trehalose (Rudolph *et al.*, 1986), UFA (Rodriguez-Vargas *et al.*, 2007) and sterols (Abe and Hiraki, 2009).

Ergosterol, the predominant sterol in yeast, is found in high concentrations in the plasma membrane (Zinser *et al.*, 1993) and is thought to provide an important structural function. Work with model membranes demonstrated that ergosterol enhances rigidity in liquid membranes, but enhances fluidity in gel membranes (Arora *et al.*, 2004). The former observation appears to occur when the ergosterol biosynthetic pathway is disrupted (Section 5.2.3; Abe and Hiraki 2009). When relative concentrations of sterols were determined in control and dried yeast samples of LAL1, no significant difference in the levels of ergosterol could be determined, suggesting that this component was not responsible for the membrane fluidity differences observed with this strain. Differences in the concentrations of intermediate sterols in the ergosterol biosynthetic pathway were apparent, however these metabolites are normally located in other organelles (Zinser *et al.*, 1993) and it is therefore unlikely that they are responsible for the fluidity variation detected.

Sterols associate with other lipids to form structures termed lipid rafts (Bagnat *et al.*, 2000). These rafts have differing fluidity to other regions of the membrane and serve key functions, such as protein localisation (Bagnat *et al.*, 2000). The association of these rafts in dried yeast has not been investigated here, but could potentially play a role in the fluidity

variations observed. Furthermore the composition of non-sterol lipids associated with rafts was not determined and it is proposed that they could also influence membrane fluidity.

### 5.3.3 The impact of drying on the ability of yeast cells to resist stress

Whilst the cause of differences in fluidity are open to debate, a more immediate practical question is whether or not these differences have an impact on the function of the membrane and the cell. A key function of the plasma membrane is to act as a barrier to stress, therefore the ability of ADY to resist stresses targeted at the plasma membrane was assessed.

It was hypothesised that the fluidising effect of ethanol and SDS would have more of a detrimental effect on fluid membranes compared to membranes exhibiting more rigid properties. However, LAL1 dried yeast, which exhibited a more rigid membrane phenotype than the control yeast, appeared to be more sensitive to these fluidising stresses (Section 5.2.7). Ethanol stress causes fluidisation to the membrane, which on its own is not normally lethal, but leads to membrane disruption (Piper, 1995). This disruption manifests itself in cell volume decreases and roughening of the cell surface (Canetta *et al.*, 2006). Dried yeast is known to exhibit a rough, almost wrinkled appearance (Finn and Stewart, 2002). This suggests that the membrane, located directly inside the cell wall, may be perturbed prior to any additional stress. As might be expected, cell volume also decreases when a cell is exposed to dehydration stresses (Simonin *et al.*, 2007b). The combined effect of these stresses may have a synergistic effect on the cell's volume and structure which is detrimental to its fitness. Therefore, although the premise of this work was to target stresses to differences in fluidity, the fluidity of a structure may in fact be of secondary importance to resisting ethanol stress.

LAL2 and LAL4 did not demonstrate a reduction in redox potential to the extent of LAL1. This was not unexpected, as it has already been shown that LAL1, a lager yeast, suffers

greater losses in viability when dried suggesting a lower inherent stress resistance (Chapter 3).

### 5.3.4 The impact of drying on the proton efflux of the membrane bound H<sup>+</sup>-ATPase

The acidification power test is a measure of a yeast population's capacity to acidify its surrounding medium (Opekarova and Sigler, 1982; Kara *et al.*, 1988; Siddique and Smart, 2000). The acidification of the surrounding medium by yeast cells during the uptake of a substrate, such as glucose, is in part due to the action of the integral membrane enzyme H<sup>+</sup>-ATPase. The plasma membrane H<sup>+</sup>-ATPase found in *Saccharomyces cerevisiae* (Serrano, 1978) functions as a hydrogen ion pump (Serrano, 1984) which produces a proton gradient across the membrane responsible for the active transport of nutrients via H<sup>+</sup> symport (Serrano *et al.*, 1986). The addition of glucose causes the activation of H<sup>+</sup>-ATPase (Campetelli *et al.*, 2005) and proton efflux (Serrano, 1983). The resultant change in pH of the surrounding medium can be easily measured. The protocol used in this study followed the pH of a yeast suspension with the addition of water (WAP) and then the addition of glucose (GAP), the difference between these values producing the glucose induced proton efflux (GIPE). However, the proton efflux may be contributed to by cellular leakage through a permeable membrane and, in cases of severe membrane damage, leakage of organic acids. It is the WAP or spontaneous acidification power which has provided the greatest differences between dried and control cells.

The spontaneous proton efflux demonstrated by LAL4 when dried and rehydrated was greater than that demonstrated by the control yeast. This initial large decrease in pH without substrate addition has been noted previously (Sigler *et al.*, 2006), although no reason was suggested. Dried yeast is thought to contain high concentrations of trehalose (Powell and Fischborn, In press), with accumulation encouraged by mild heat stress (Attfield, 1987; Hottiger *et al.*, 1987) at the end of propagation immediately prior to the

drying process. It functions to protect the cell from some of the stresses associated with dehydration and rehydration (Gadd *et al.*, 1987; Sharma, 1997; Hounsa *et al.*, 1998; Cerrutti *et al.*, 2000). However, trehalose could be detrimental to cell upon resumption of normal metabolic activity (Sebollela *et al.*, 2004). As a consequence, after rehydration large amounts of trehalose are likely to be converted to glucose. It is proposed that high WAP values are indicative of trehalose catabolism. This hypothesis would suggest that high WAP values exhibited by LAL4 (dried sample) indirectly reflects trehalose catabolism. In contrast LAL2 which demonstrated comparable WAP profiles for dried and control yeast would exhibit negligible trehalose catabolism.

LAL1 however, showed a reduced WAP when the cells were dried and rehydrated compared to the control but this strain is also known to exhibit the lowest viability post rehydration. In this scenario, the reduced WAP may reflect a reduced activity of H<sup>+</sup>-ATPase enzyme, or perhaps an increase in ion leakage through the membrane. One potential cause of this could be heat shock protein 30 (Hsp30). Hsp30 is a stress induced inhibitor of H<sup>+</sup>-ATPase (Piper *et al.*, 1997). Glucose limitation, heat shock and severe osmostress can all induce Hsp30 (Piper *et al.*, 1997) and are all present in ADY production (Chapter 1). The inhibition of H<sup>+</sup>-ATPase activity is thought to act as an energy conservation mechanism (Piper *et al.*, 1997), as the action of the enzyme results in high ATP use and it is possible that it has been induced in LAL1.

#### **5.4 CONCLUSIONS**

Fluctuations in the fluidity of the plasma membrane have previously been highlighted as potential sources of cell death. However, little comment has been made regarding the fluidity of the membrane subsequent to rehydration and prior to utilisation in fermentation. In this chapter the fluidity of yeast after rehydration was compared to control yeast intended to represent propagated yeast. Whilst strain specific differences

have been highlighted, the cause of altered fluidity and, indeed, consequences remain unclear. Differences in the functionality of rehydrated yeast cells with regard to stress resistance are apparent, but these have not been linked to the property of membrane fluidity. There are also distinct differences between the strains with LAL1, the only lager strain investigated, appearing less fit-for-purpose than the two ale strains LAL2 and LAL4 following dehydration and rehydration. This suggests that the impact of dehydration and rehydration on membrane integrity may be strain dependent.

## CHAPTER 6: FERMENTATIONS USING DRIED YEAST

### 6.1 INTRODUCTION

Several studies have considered the suitability of replacing propagated yeast with dried yeast as the method of yeast supply for fermentation (Gosselin and Fels, 1998; Finn and Stewart, 2002; van den Berg and Van Landschoot, 2003; Cyr *et al.*, 2007; Powell and Fischborn, 2010). Gosselin and Fels (1998) conducted fermentations at 1 hl and 300 hl with a dried lager yeast. Comparisons with control yeast of the same strain (propagated or cropped) were made for attenuation rates, but no other fermentation properties were analyzed. Surprisingly the authors concluded that the beer produced was similar to that normally produced at the brewery, for which a different yeast strain was also utilised. Finn and Stewart (2002) conducted laboratory scale investigations in conical flasks (500 mL) and 1.5 l static fermentations using EBC tall tubes. These fermentations used two strains of lager yeast and two strains of ale yeast. Crucially they provided comparisons to propagated yeast of the same strain. Van den Berg and Van Landschoot (2003) compared the use of four dried ale yeasts in pilot plant brews varying between 0.6 and 1.2 hl to previous fermentations using these yeasts in a non-dry form. Cyr *et al* (2007) conducted 3 hl scale fermentations using two dried lager yeast and propagated samples of the same strain. Powell and Fischborn (2010) completed fermentations using dried and propagated lager yeast in 8.5 l squat shaped cylindroconical vessels.

The literature concerning the impact of dried yeast on fermentation performance is not comprehensive and indeed in some cases contradictory. For example, fermentation rate, which is a measure of how quickly a yeast population assimilates sugars present in wort has been examined. Van den Berg and Van Landschoot (2003) demonstrated that fermentation rates and uptake of sugars by dried yeast cultures were comparable to freshly propagated yeast, although they did encounter some hung fermentations in which the uptake of

maltose and maltotriose were impaired. Others have reported a greater lag in fermentation initiation exhibited by dried, in comparison to control, yeast (Powell and Fischborn, 2010), which may also result in a slower overall rate of fermentation and specific growth rate observed by others (Cyr *et al.*, 2007). A delay of approximately 24 hrs in the uptake of leucine, valine and isoleucine during dried yeast fermentations was also observed (Cyr *et al.*, 2007) indicating an extended lag phase. Furthermore, a higher concentration of diacetyl was observed during and at the end of dried yeast fermentation when compared to control fermentations. In contrast, 2,3-pentanedione was present at reduced levels during dried yeast fermentations (Cyr *et al.*, 2007) when compared to the control. High levels of diacetyl are a concern for the brewer since this component is considered an undesirable flavour in most beer styles, but especially lager, due to its more subtle flavour profile.

Concentrations of the flavour active compounds dimethyl sulfide (DMS) and higher alcohols have been shown to be comparable for propagated and dried yeast fermentations (Powell and Fischborn, 2010), but ester formation, most notably ethyl acetate and isoamyl acetate increased during dried yeast fermentations when compared to the control. Differences in the growth of the yeast have also been noted (Powell and Fischborn, 2010). Lager dried yeast has been associated with altered flocculation, increased beer haze and reduced foam (Finn and Stewart, 2002). In the latter study it was suggested that dead yeast in suspension contributed to the occurrence of haze and release of proteinase A which in turn adversely affected foam stability. Interestingly these modifications in fermentation performance appeared to occur for lager and not ale dried yeast fermentations, although this may have been a function of the impaired viability of the former when compared to the latter (Finn and Stewart, 2002). In a separate study no significant difference in the ability of dried and propagated yeast to form aggregates could be determined (Powell and Fischborn, 2010). Contradictions in the literature with regard to the impact of the use of dried yeast on fermentation performance necessitated a further study in this area. To address this, the

lager strain, LAL1, was assessed for fermentation performance attributes using small scale fermentations. Laboratory propagated yeast termed “control” and dried yeast were directly compared with respect to attenuation rate, yeast cell viability and cell growth. Throughout the fermentations the assimilation of amino acids and the uptake of fructose, sucrose, maltose, maltotriose and glucose were monitored. The production and subsequent uptake of the key flavour volatile diacetyl was also determined.

## 6.2 RESULTS

Fermentations were completed to compare the performance of YPD propagated yeast, fed-batch propagated yeast and dried yeast which were handled as described in the materials and methods (Section 1.8). YPD propagated yeast was used with the aim of modelling the yeast propagation which commonly occurs in breweries, whereby yeast is grown in increasing batch sizes. Although this would normally be completed in wort, YPD was used as it is suggested that growth on this medium yields a more reproducible yeast culture. Fermentations using this yeast were compared to fermentations using rehydrated dried yeast, handled as if it were being deployed in a brewery. Dried yeast and typical brewing yeast slurries are propagated very differently with the former derived from a combination of batch and fed batch propagation, while the latter is produced using a single batch propagation system. In addition to differences in propagation process, the media used to grow both types of yeast also differs. To eliminate pre-growth as a variable, an additional control, comprising fed batch yeast grown in a manner designed to mimic that used during dried yeast propagation, was also employed (Sections 2.8.1-2.8.3). The assignment of an entire fermentation vessel for each sample point (destructive sampling) enabled samples to be removed from an undisturbed system. Samples were removed at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hours for analysis. The population dynamics were followed using viable cell counts and budding indices (Section 6.2.1), whilst the metabolic activity of the yeast was followed using the change in specific gravity (Section 6.2.2), sugar utilisation (Section 6.2.3),

amino acid utilisation (Section 6.2.4), and diacetyl formation and assimilation (Section 6.2.6).

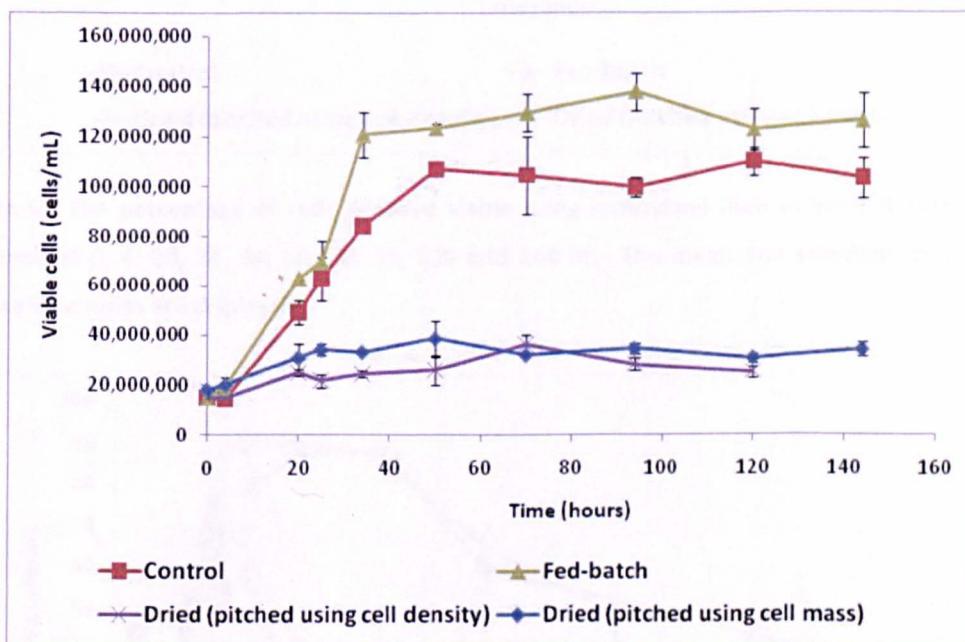
### **6.2.1 Variations in the size and viability of the yeast cell populations during fermentation**

Two techniques were used to follow yeast population dynamics during fermentation: viability (Section 2.4) and budding index (Section 2.4). These are markers of yeast physiological state which can be used when studying yeast during fermentations (Gibson *et al.*, 2010; Powell and Fischborn, 2010; Miller *et al.*, In Press). Initially fermentations were pitched at  $1.5 \times 10^7$  yeast cells/ml, with the exception of one of the dried yeast fermentations, for which a pitching regime based on the directions of the dried yeast manufacturer was employed, using a measure of cell mass, which resulted in a pitching rate of  $1.8 \times 10^7$  cells/ml.

Budding (Figure 6.3) was initiated by the first sampling point (4 hrs) in both the control and fed-batch yeast fermentations, but not in the two dried yeast fermentations. Budding in the dried yeast population was minimal at time zero and the first sampling point (4 hrs), before rapidly increasing to 88 % at 20 hrs. In contrast, control yeast exhibited a budding index of 30 % at pitching and this increased at 4 hrs reaching a peak value of 58 % by 25 hrs. After this, a steady decline to less than 10 % by 50 hrs was observed. By 20 hrs incubation all four populations of yeast exhibited maximal or near maximal budding indices.

Budding normally results in the increase of viable cells in the population. This was the case for the control and fed-batch yeast populations, but not the two dried yeast populations. This may be partially explained by the observation that many of the yeast cells which were budding, or the buds themselves, were stained blue by methylene blue indicating they were non-viable. Indeed the viability of the dried yeast populations remained between 50-

60 % throughout the fermentations (Figure 6.2). As a consequence a significantly lower cell density was observed in dried yeast fermentations (pitched using cell mass  $3.4 \times 10^7$  cells/ml) compared to that of control yeast ( $1.0 \times 10^8$  cells/ml), with fed-batch yeast significantly higher again ( $1.3 \times 10^8$  cells/ml) (significance determined using an analysis of variance,  $P \leq 0.05$  and Tukey-Kramer's pair wise comparisons) (Figure 6.1). Although the viable cell concentrations in the four fermentations demonstrated little or no increase after 50 hr, dried yeast fermentations continued to demonstrate a high budding index.



**Figure 6.1.** The mean number of viable cells present in fermentations at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr assessed using methylene blue staining. The standard deviation of triplicate samples is displayed.

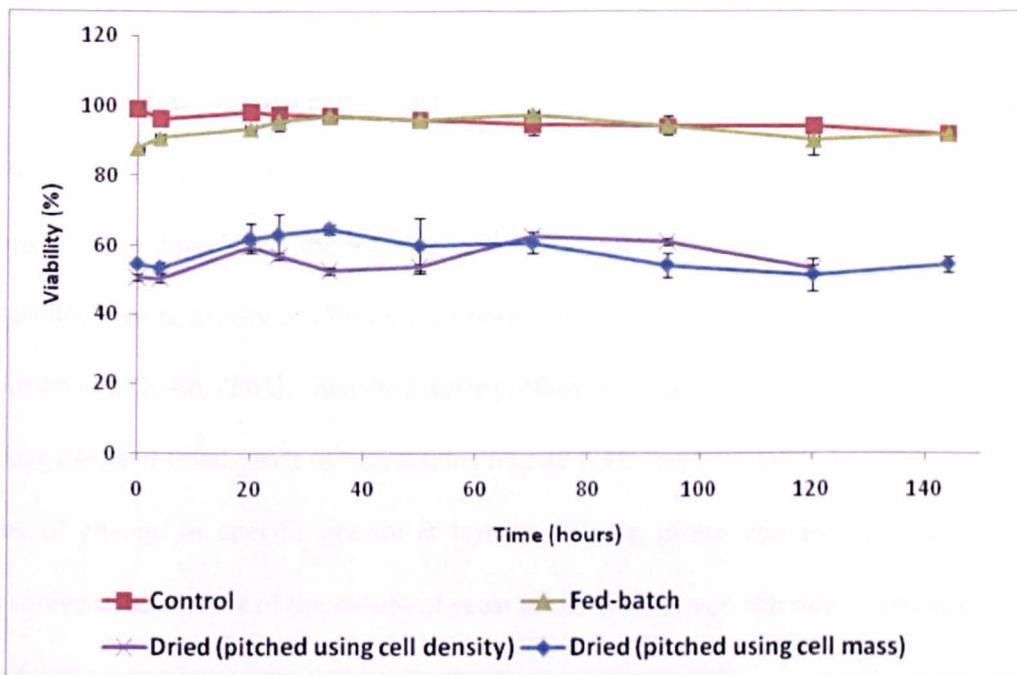


Figure 6.2. The percentage of cells deemed viable using methylene blue staining. Samples were removed at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr. The mean and standard deviation of triplicate samples are displayed.

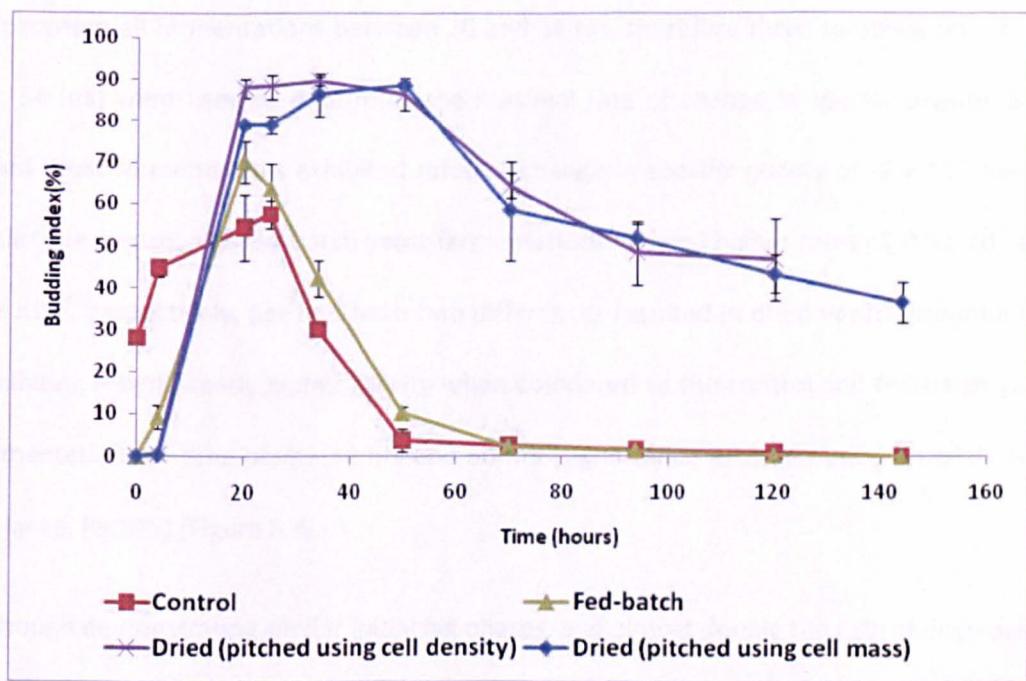


Figure 6.3. The percentage of cells exhibiting a bud, termed the budding index. Non-viable cells (identified using methylene blue staining) were excluded from the analysis. Samples were removed at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr. The mean and standard deviation of triplicate samples are displayed.

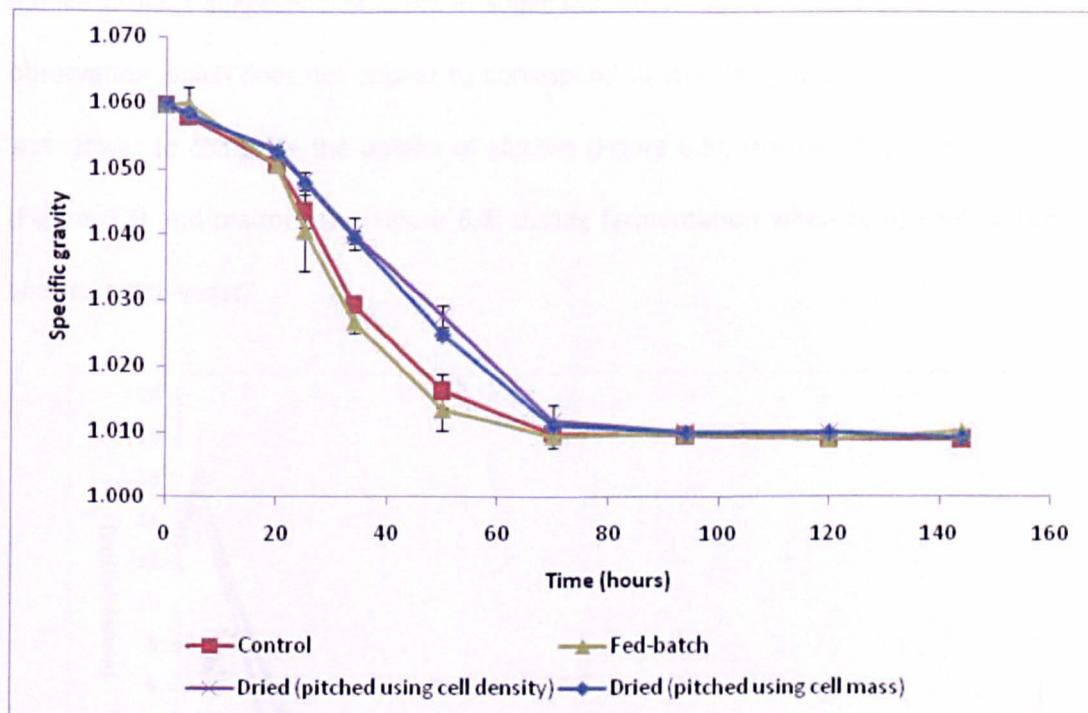
## 6.2.2 Time taken to reach final attenuation

Attenuation can be defined as the decrease in specific gravity of wort during fermentation (Boulton and Quain, 2001). The time taken to reach final attenuation is one of the key attributes that determines the length of fermentation. The rate of attenuation is typically determined using gravity profiles, which permit fermentation consistency to be assessed (Boulton and Quain, 2001). Specific gravity profiles were determined for control, fed batch propagated and dried yeast fermentations (Figure 6.4). The time taken to reach maximum rates of change in specific gravity is termed the lag phase and this appeared to be conserved independent of the nature of yeast applied. However, the rate of attenuation for dried yeast was slower than that of the control yeast (Figure 6.4).

The change in specific gravity would be expected to produce a sigmoidal curve when plotted against time exhibiting a central linear section. Linear attenuation profiles were apparent in all fermentations between 20 and 34 hrs, therefore three sampling points (20, 25, 34 hrs) were used to determine the maximal rate of change in specific gravity. Both dried yeast fermentations exhibited rates of change in specific gravity of  $-9 \times 10^{-4}$  per hr, whilst the control and fed-batch yeast fermentations showed higher rates of  $-15 \times 10^{-4}$  and  $-17 \times 10^{-4}$ , respectively, per hr. These rate differences resulted in dried yeast fermentations exhibiting a significantly higher gravity when compared to the control and fed-batch yeast fermentations at time points 34 hrs and 50 hrs (significance assessed using an analysis of variance,  $P \leq 0.05$ ) (Figure 6.4).

Although demonstrating similar initial lag phases, and almost double the rate of decrease in specific gravity in the linear phase of attenuation, the control yeast and fed-batch yeast reached attenuation at the same time point as the dried yeast. This was due to a shorter linear phase and a more gradual rate change towards the end of the sigmoid curve shown by the control and fed-batch yeast.

Importantly, there was no significant difference (significance assessed using an analysis of variance,  $P \leq 0.05$ ) between the final specific gravities. The final sample for dried (pitched using cell density) was taken at 120 hrs, as sample point 144 hrs was not included for this fermentation, but attenuation was deemed to have been reached prior to this.

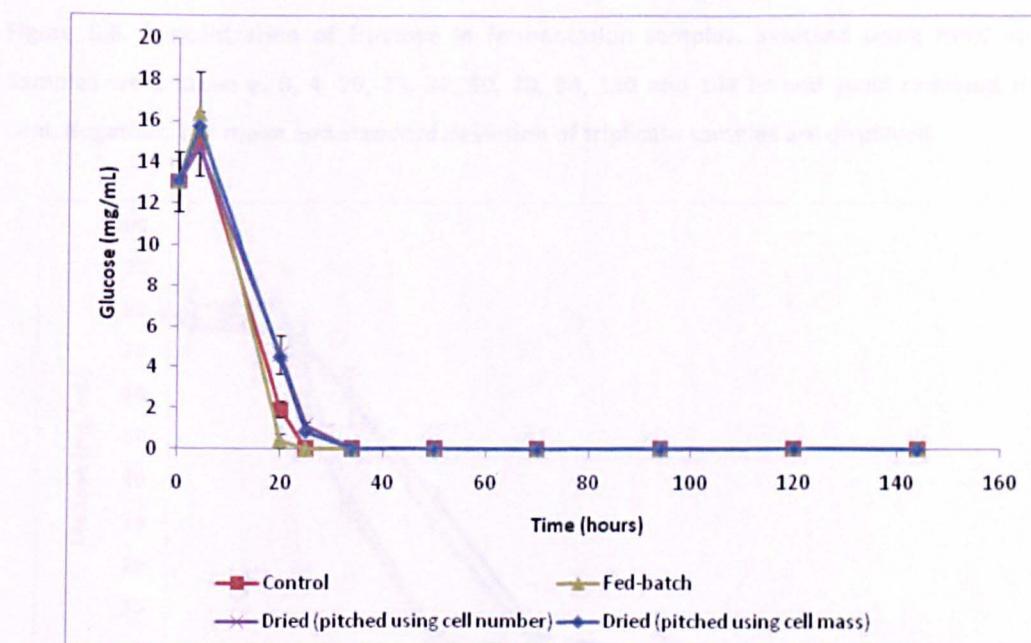


**Figure 6.4. The change in specific gravity exhibited by fermentations.** Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The standard deviation of triplicate samples is displayed.

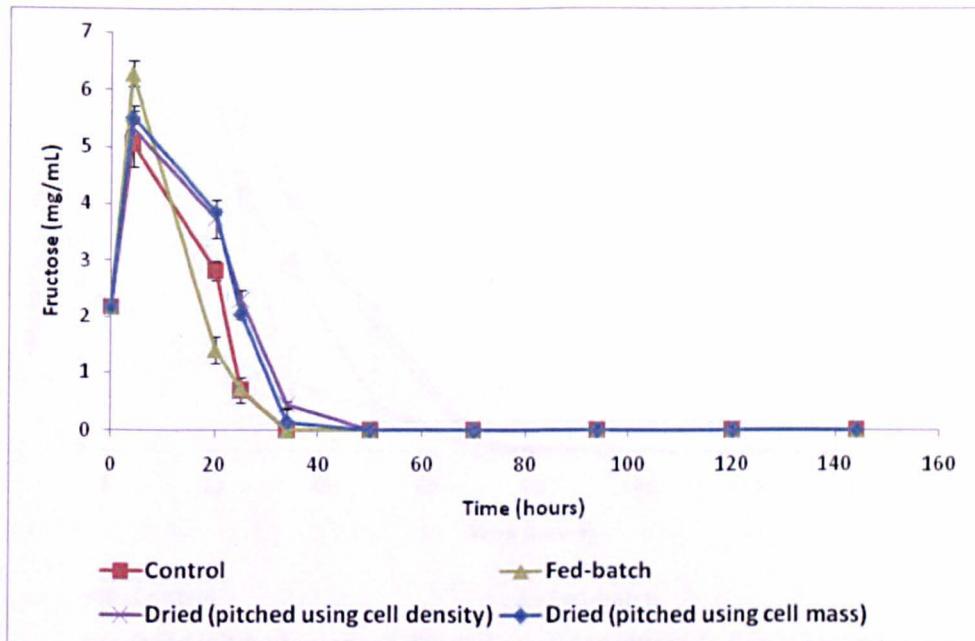
### 6.2.3 The assimilation of individual sugars during fermentation

Sugars are the most abundant dissolved matter in wort, and as such, play a significant determining role in specific gravity. Wort contains the fermentable sugars sucrose, fructose, glucose, maltose and maltotriose, as well as non-fermentable sugars such as maltotetraose and maltopentose (Ingledew, 1975). The concentration of individual sugars in samples taken during fermentations was determined, to indicate the rate at which they were being assimilated.

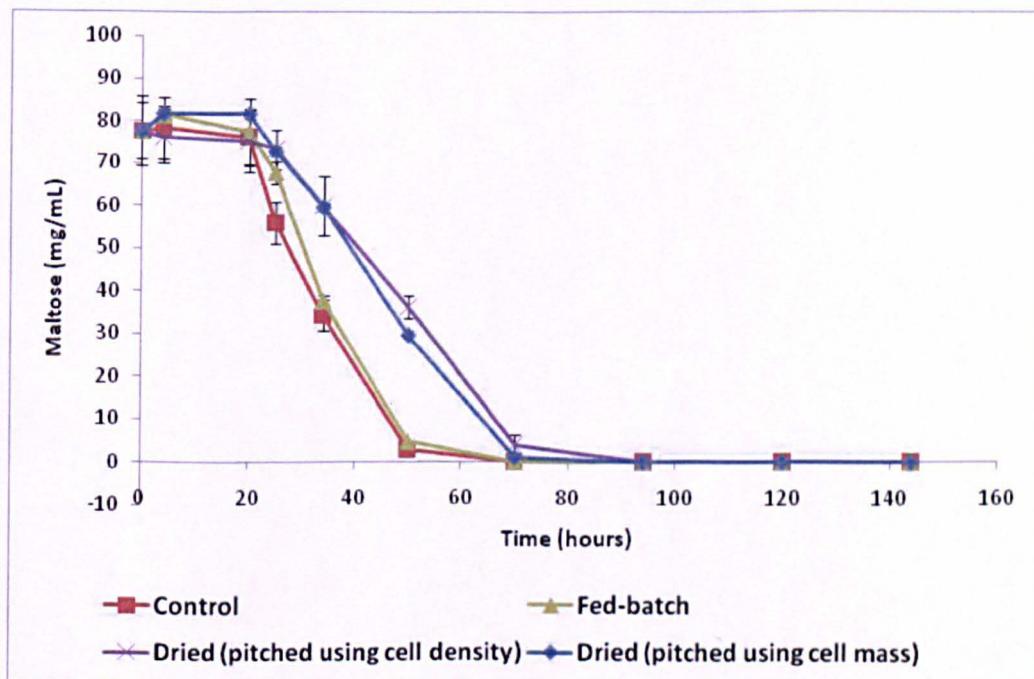
Sucrose was depleted from all fermentations by the first sample point (4 hr) (Data not shown). Control and fed-batch yeast fermentations showed similarities with respect to their sugar utilization profiles. The two dried yeast fermentations were slower to completely assimilate the sugars present in the medium. Comparison of individual sugar uptake profiles suggests that a lag in sugar utilisation was exhibited by dried yeast, an observation which does not appear to correspond to specific gravity decline. Dried yeast was slower to complete the uptake of glucose (Figure 6.5), fructose (Figure 6.6), maltose (Figure 6.7) and maltotriose (Figure 6.8) during fermentation when compared to control and fed batch yeast.



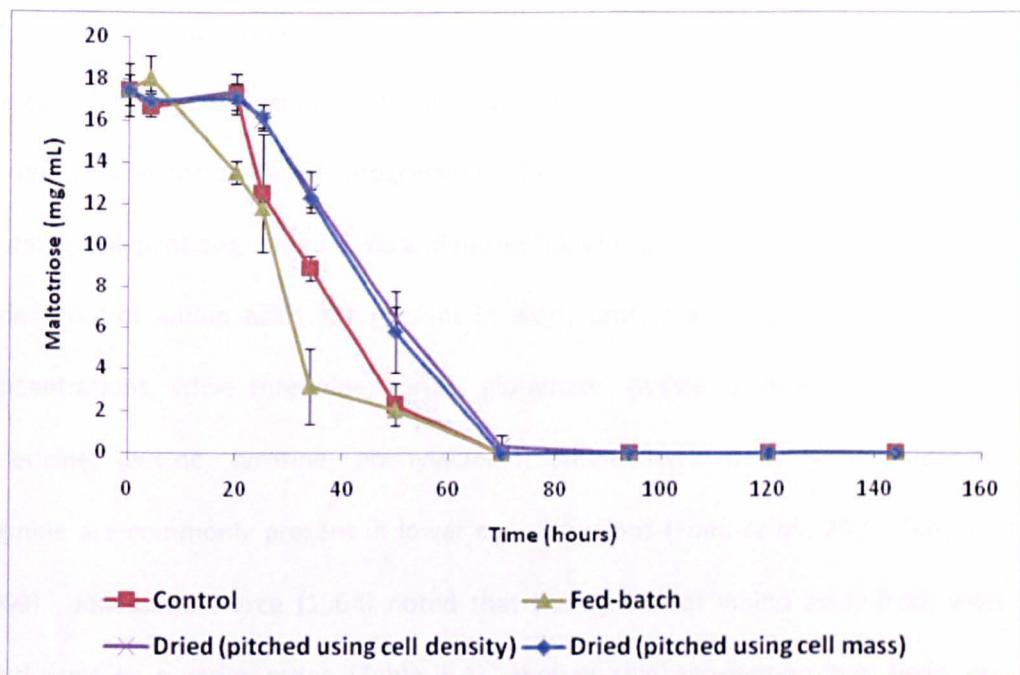
**Figure 6.5. Concentration of glucose in fermentation samples, assessed using HPLC analysis.** Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.



**Figure 6.6.** Concentration of fructose in fermentation samples, assessed using HPLC analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.



**Figure 6.7.** Concentration of maltose in fermentation samples, assessed using HPLC analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.



**Figure 6.8.** Concentration of maltotriose in fermentation samples, assessed using HPLC analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

considered to represent the maximum glucose uptake rate. The glucose uptake rate was determined using the concentration of the major or predominantly dominant substrate in each sample taken at various stages of fermentation growth. In the case of maltotriose, the glucose peak could not be detected using the analytical column.

Glucose uptake rates were mostly measured from the medium in the region following the initial lag phase up to the 34 hr sampling point (Figures 7.8-7.15), with several cases being measured up to 50 hr. The glucose peak (glucose and glucose-6-phosphate) was almost entirely removed by 25 hr. When compared to the fed-batch fermentation, the glucose uptake profile was similar to the control; the fed-batch yeast fermentations showed no significant difference in glucose uptake rates compared to the control. However, following the completion of uptake in excess of 24 hr, glucose disappearance in the fed-batch fermentations (those sterilized first) is clearly evident in the control and control-like (Figure 7.12) and fed-batch (Figure 7.11). The fed-batch yeast fermentations did not show an increase in the glucose disappearance starting with 24 hr, but 27 hr, and this

#### **6.2.4 Amino acid uptake**

The availability and utilisation of nitrogenous compounds (Casey *et al.*, 1984) is a key factor in determining fermentation progression. The nitrogenous content of wort comprises proteins, polypeptides, amino acids and nucleotides in varying amounts (Ingledew, 1975). A wide array of amino acids are present in wort, proline and asparagine in the highest concentrations, while threonine, serine, glutamate, glycine, alanine, valine methionine, isoleucine, leucine, tyrosine, phenylalanine, aminobutyric acid, lysine, histidine and arginine are commonly present in lower concentrations (Fumi *et al.*, 2009; Gibson *et al.*, 2009). Jones and Pierce (1964) noted that the uptake of amino acids from wort was conducted in a strict order (Table 6.1), though this assumption has been recently challenged (Gibson *et al.*, 2009). According to Jones and Pierce (1964) group A amino acids are assimilated immediately, group B more slowly, whilst group C are only assimilated once groups A and B are depleted. Group D, consisting only of the imino acid proline, is normally considered to remain un-utilised.

In this study the concentrations of the majority of physiologically important amino acids were determined in various samples of fermentation broth, with the caveat that arginine could not be detected using the protocol adopted.

Class A amino acids were mostly removed from the medium in the control fermentation prior to the 34 hr sampling point (Figures 7.9-7.15), with several amino acids (threonine, serine, lysine and asparagine) removed by 25 hr. Whilst the fed-batch yeast fermentation demonstrated a similar profile to the control, the two dried yeast fermentations showed a lag in the completion of uptake in excess of 24 hr. Glutamine, a member of the class A amino acids (those assimilated first) is poorly assimilated in the control yeast fermentation (Figure 6.12), with 92 % remaining (Table 6.1). The two dried yeast fermentations in fact show an increase in the amount of glutamine, finishing with 178 % and 211 % of the

original wort content. These high percentages are partly explained by the relatively low amounts initially present in the wort.

Class B amino acids (Figures 7.16-7.20), thought to be assimilated more slowly than Class A amino acids, were depleted from the medium during control fermentations by 34 hr. One class B amino acid, methionine, was assimilated by 25 hr, a profile more akin to class A amino acids. Again, fed-batch profiles were comparable to the control fermentations, whereas dried yeast demonstrated lags of around 36 hr.

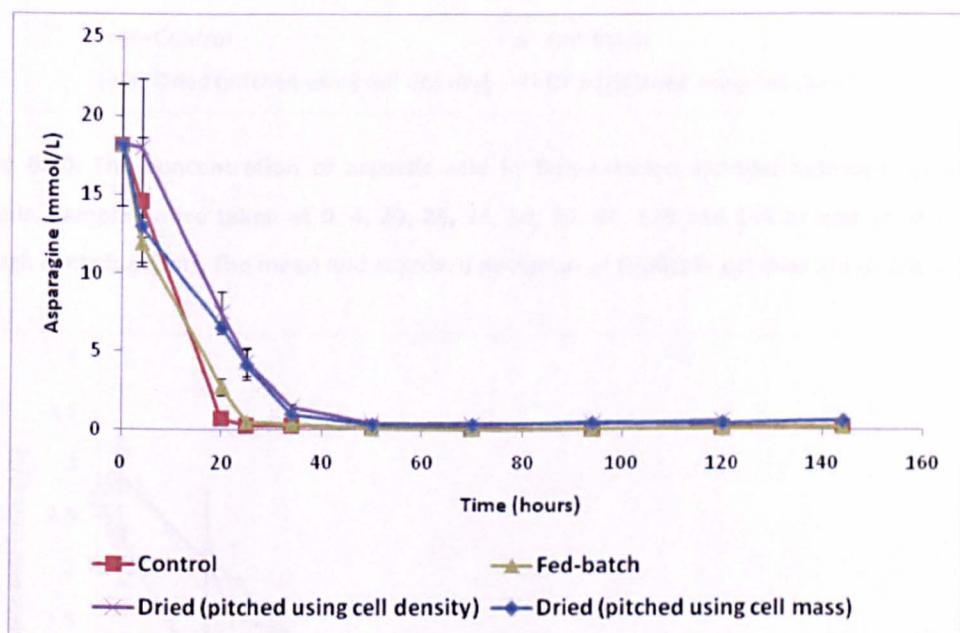
Class C amino acids (Figures 7.21-7.25), thought to be assimilated after class A and B amino acids are nearing depletion, were assimilated during the control fermentation by 34 hr. Surprisingly, the onset of assimilation was evident at 20 hr, when class A and B amino acids were still present. Similar lags, of approximately 34 hr, were again apparent in dried yeast fermentations, however the pattern of utilisation was similar to that observed for control yeast. Glycine (Figure 6.22), like glutamine, is also initially present in wort in relatively low concentrations, and remains at a relatively high concentration during control and dried yeast fermentations. In contrast, this was not observed during fed-batch propagated yeast fermentations.

Proline, the only member of group D, is present in the highest concentration in wort, although its assimilation is usually limited or absent in fermentation. However, in the fed-batch and control fermentations described here it appears there was some limited assimilation of the amino acid (Figure 6.26).

In addition to the lag demonstrated in the assimilation of amino acids, there was also a high percentage of several amino acids (valine, alanine, phenylalanine, tyrosine and tryptophan) which remained in the two fermentations completed using dried yeast. This was in contrast to the fermentations performed using control and fed-batch yeast in which they were

almost completely assimilated. This more complete assimilation supports the observations of Gibson *et al.* (2009). A previous study (Cyr *et al.*, 2007) investigating the use of dried yeast identified a similar lag in the uptake of the amino acids valine and isoleucine as those described in the current study. However, the high residual levels of some amino acids have not previously been reported and may be of concern to brewers.

### **Class A**



**Figure 6.9.** The concentration of asparagine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

Figure 6.10 shows the concentration of valine and isoleucine in samples taken during the growth of yeast. Valine and isoleucine concentrations increased rapidly until approximately 20 hours, after which they decreased. The concentration of valine was higher than that of isoleucine throughout most of the growth phase, except for the final 24 hours, when the two amino acids were present in approximately equal amounts.

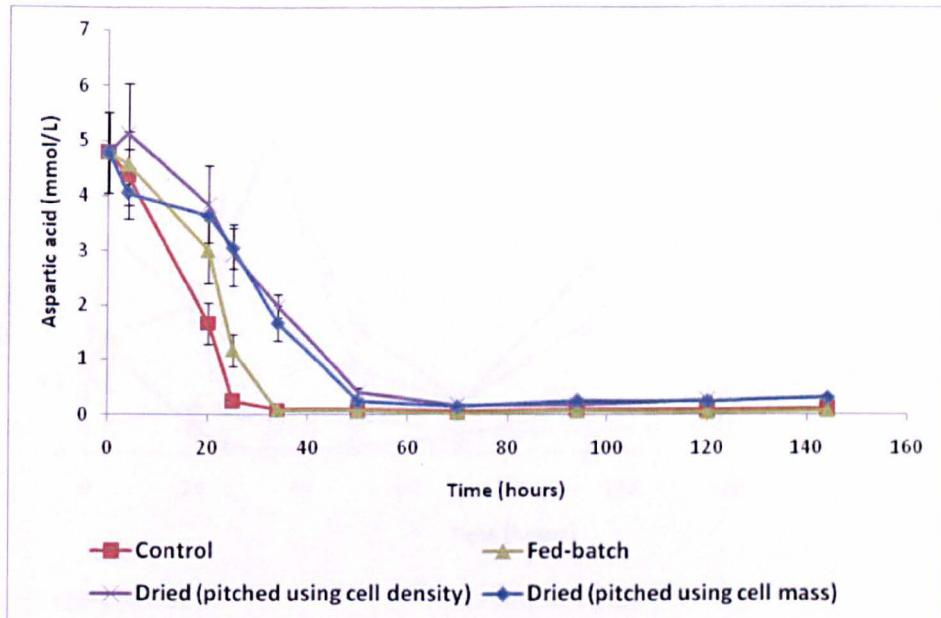


Figure 6.10. The concentration of aspartic acid in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

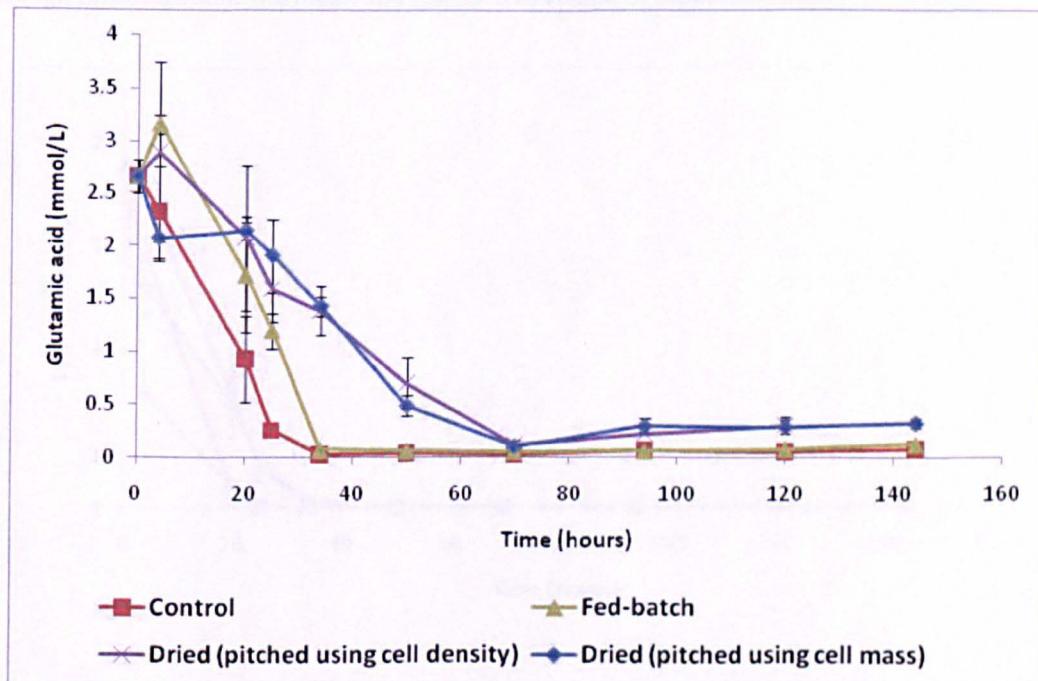


Figure 6.11. The concentration of glutamic acid in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

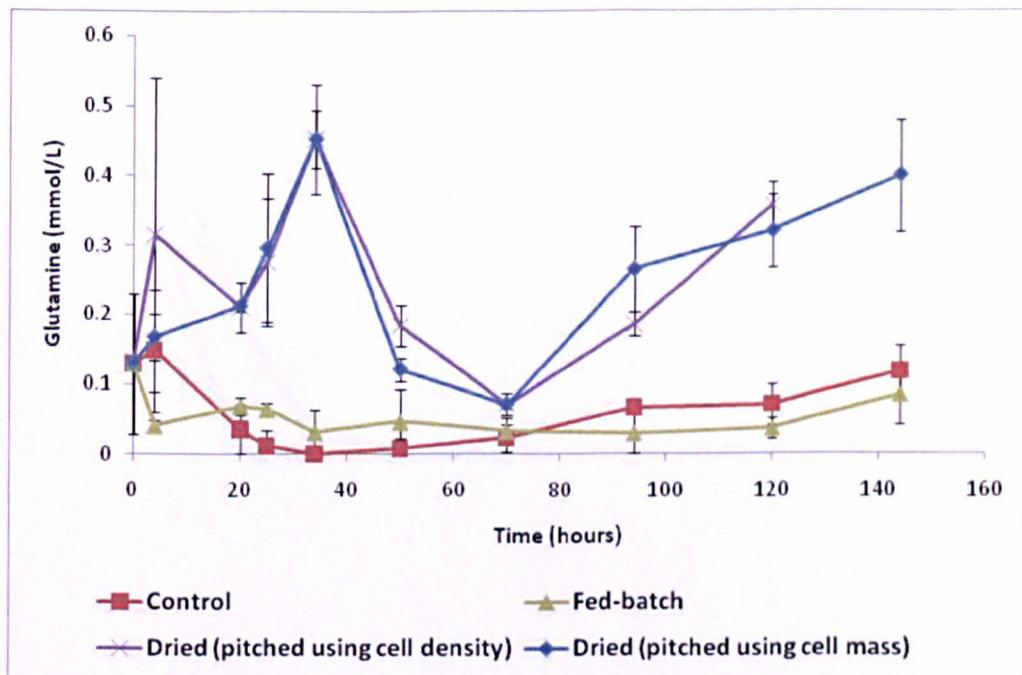


Figure 6.12. The concentration of glutamine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

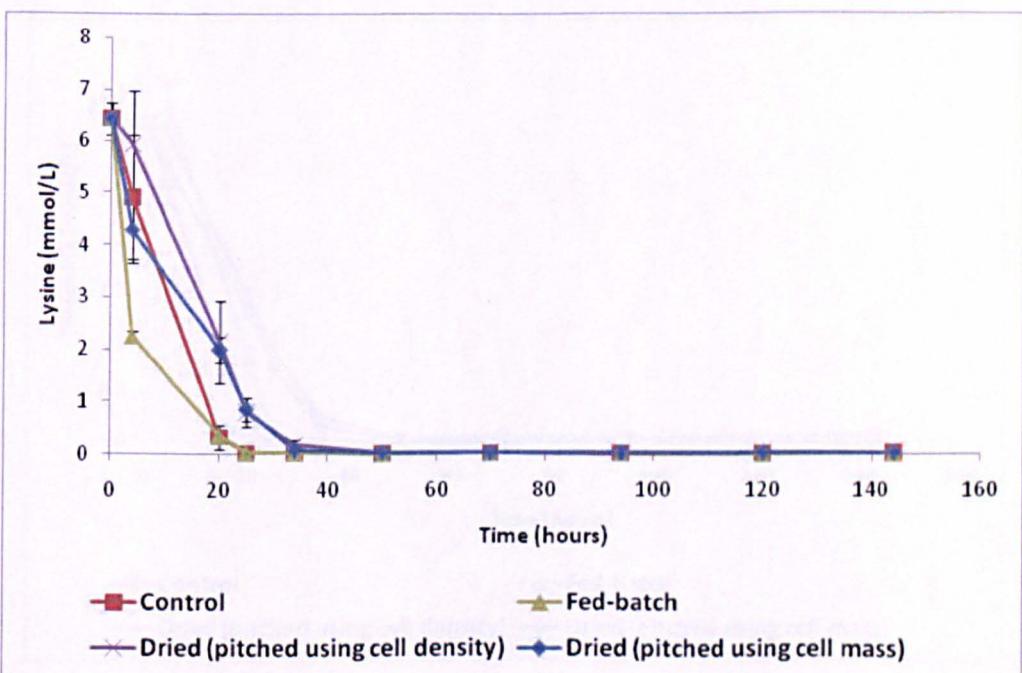


Figure 6.13. The concentration of lysine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

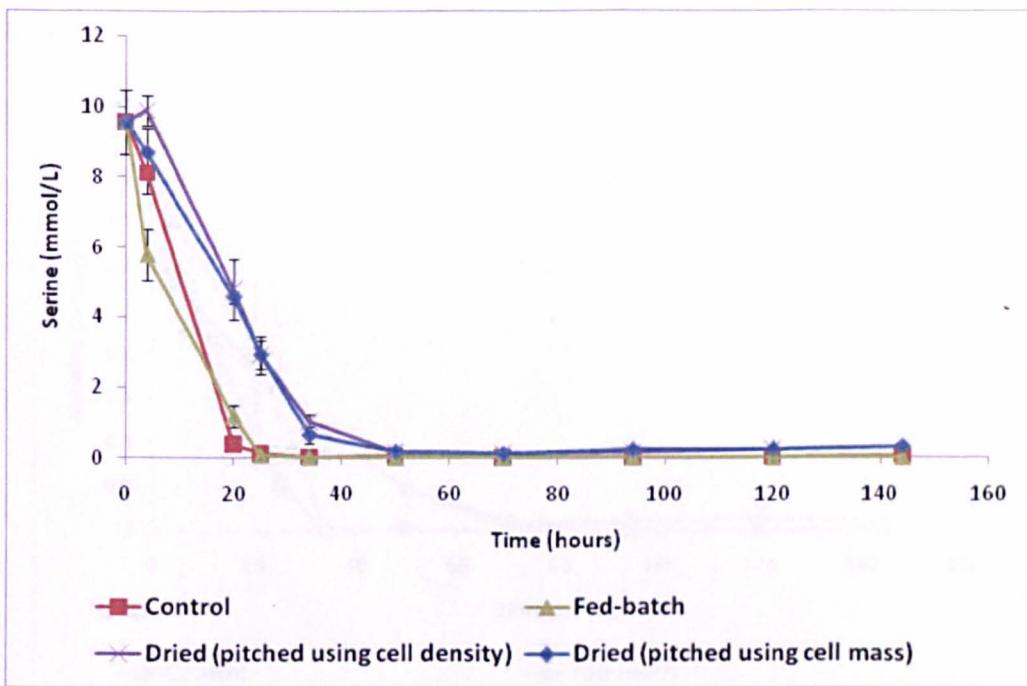


Figure 6.14. The concentration of serine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

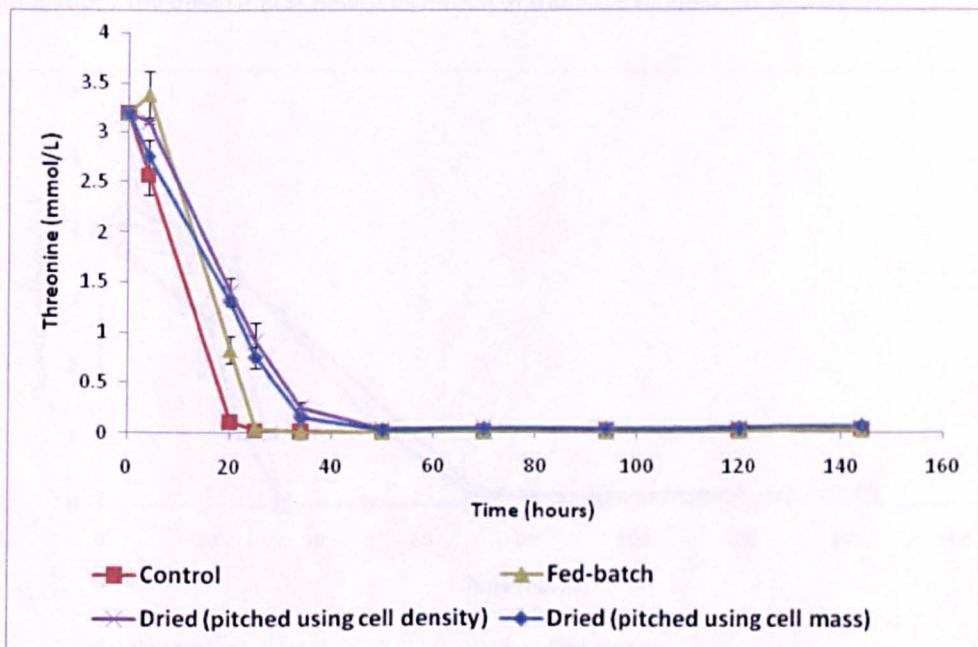
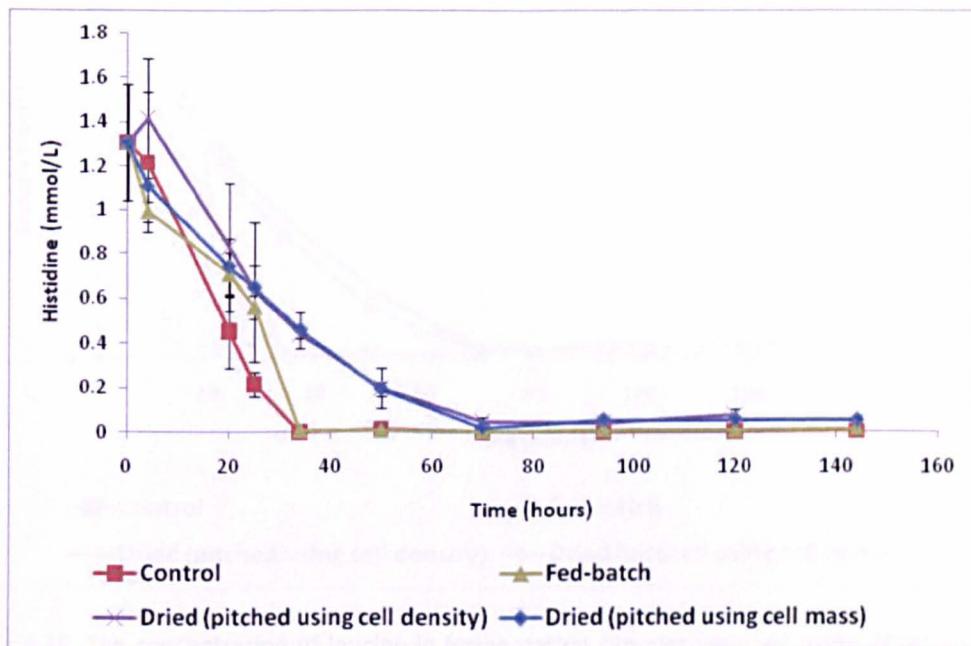
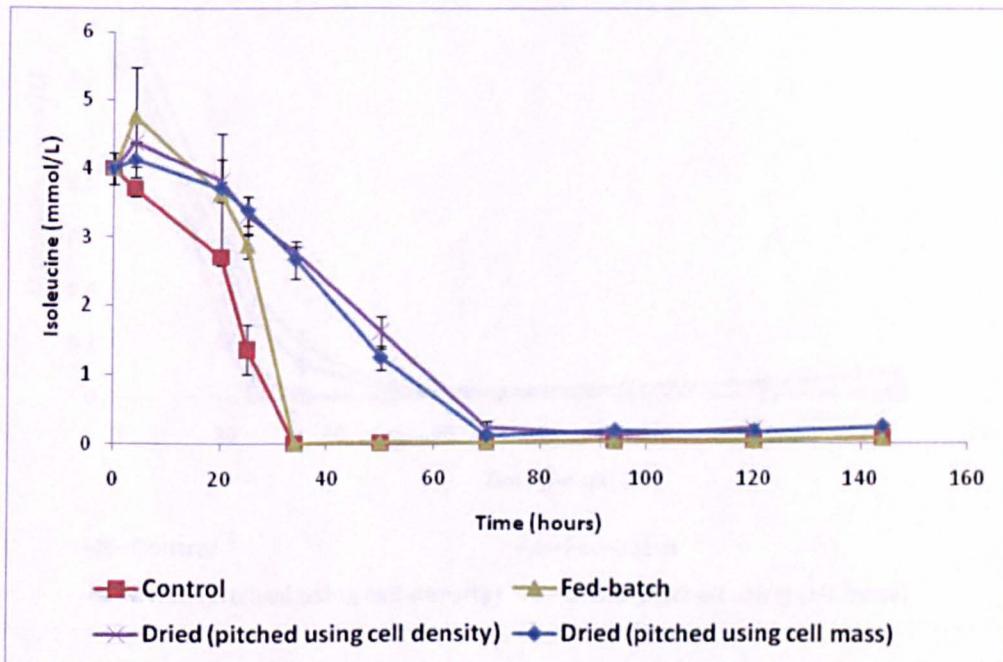


Figure 6.15. The concentration of threonine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

**Class B**



**Figure 6.16.** The concentration of histidine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.



**Figure 6.17.** The concentration of isoleucine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

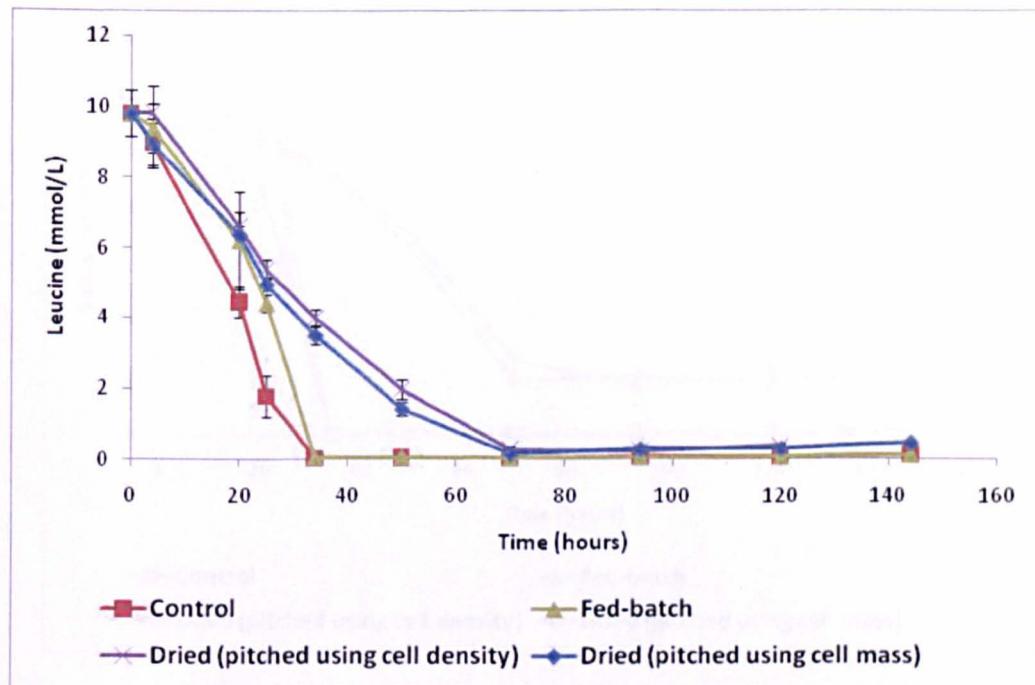


Figure 6.18. The concentration of leucine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

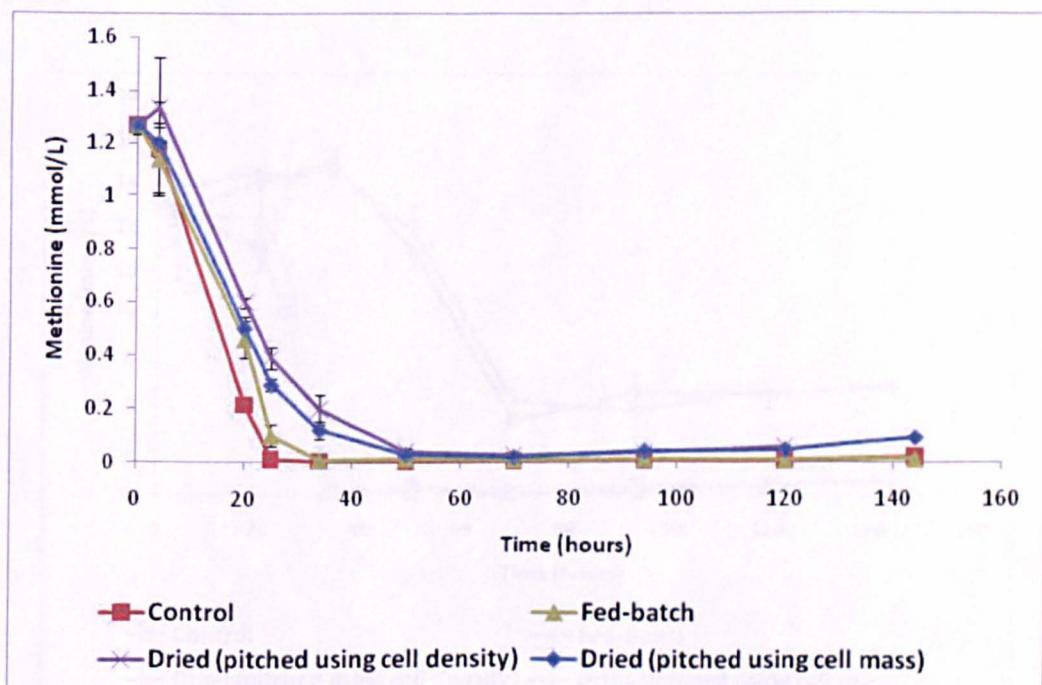


Figure 6.19. The concentration of methionine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

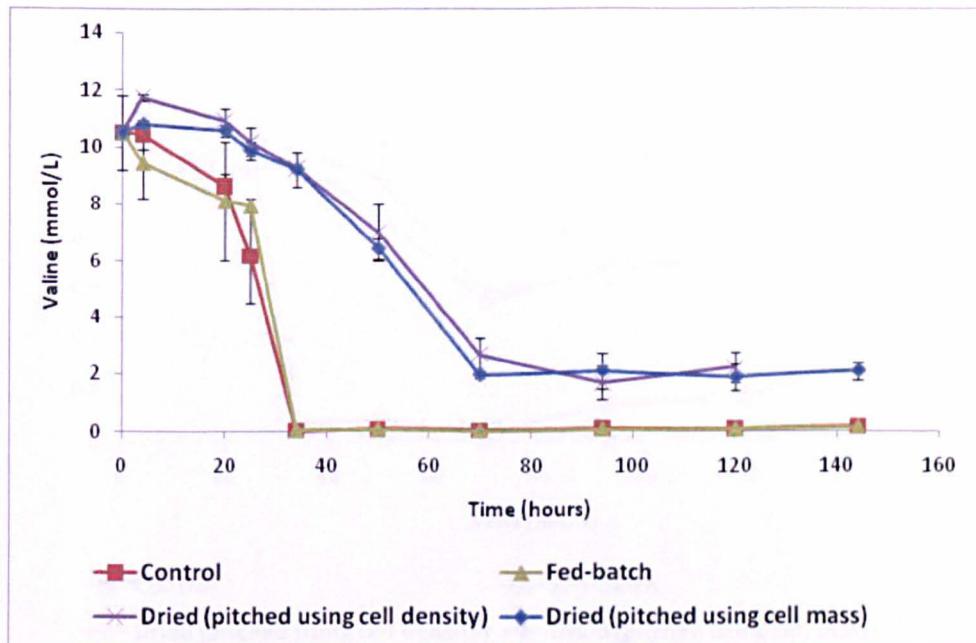


Figure 6.20. The concentration of valine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

### Class C

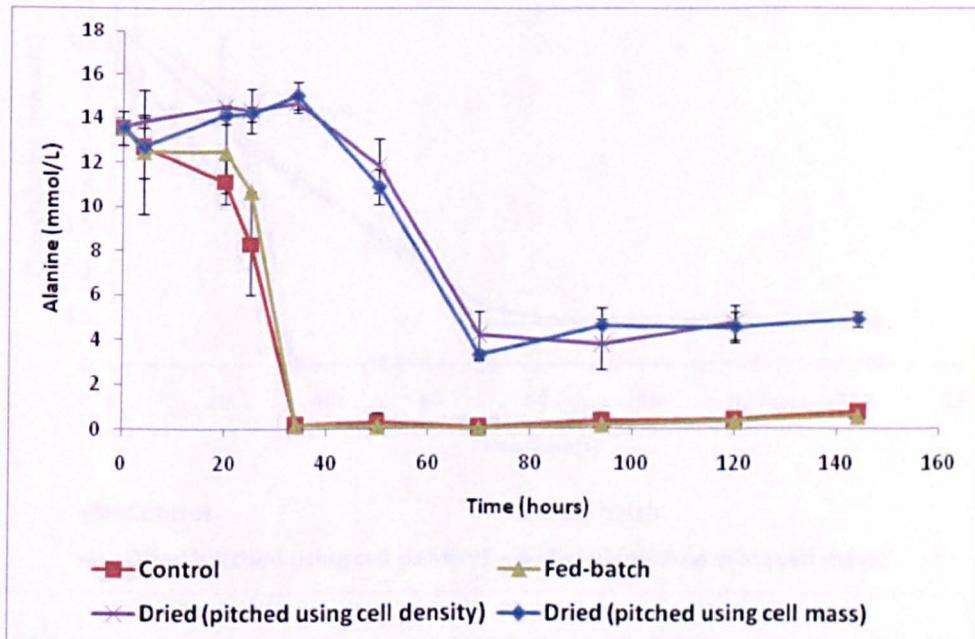


Figure 6.21. The concentration of alanine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

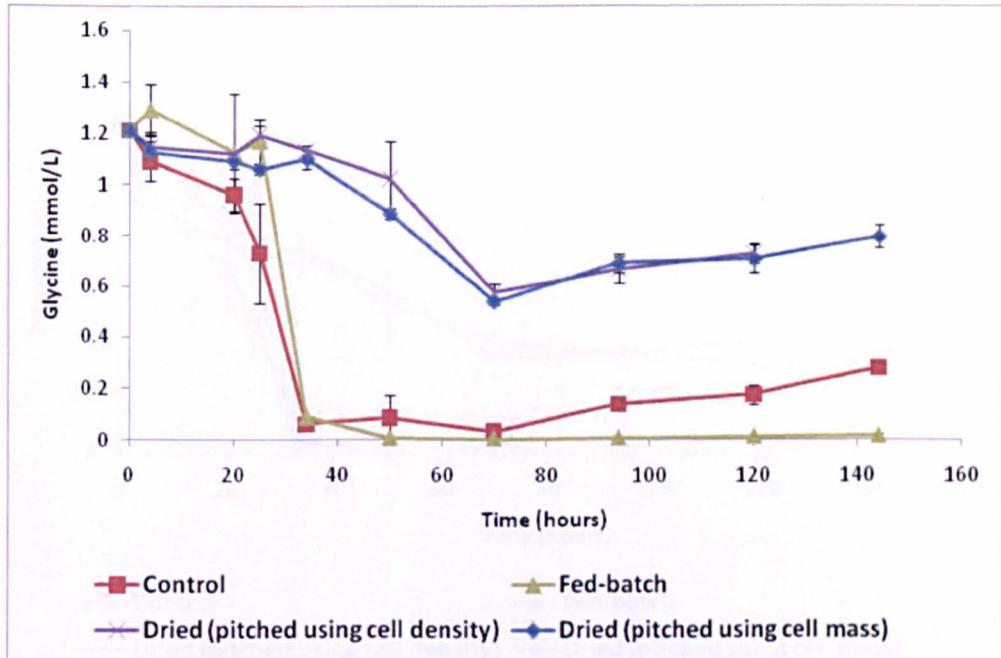


Figure 6.22. The concentration of glycine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

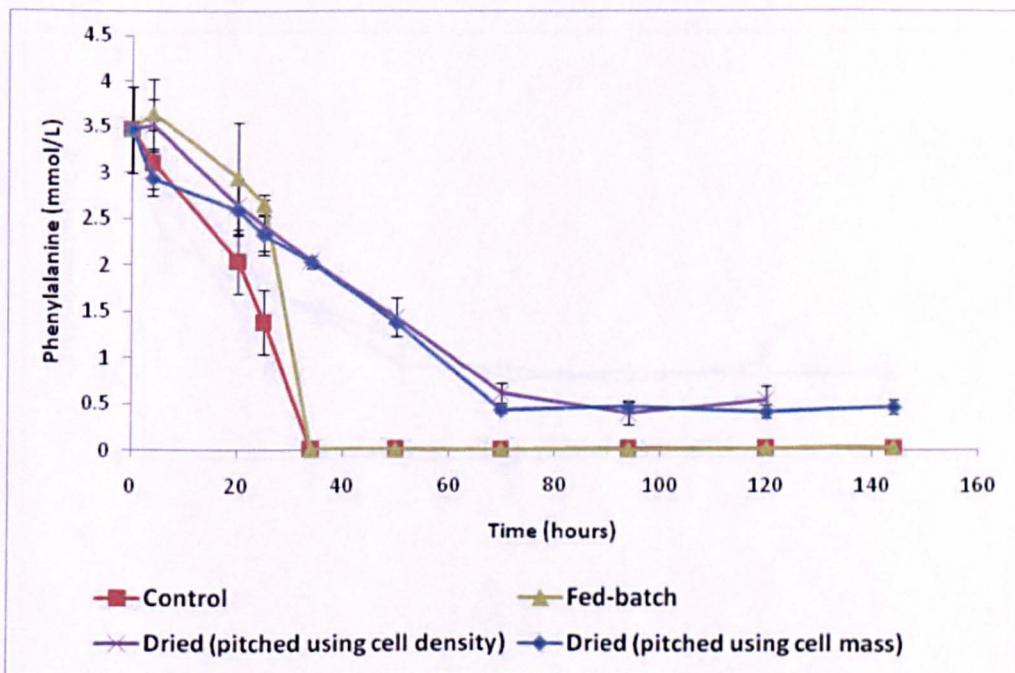
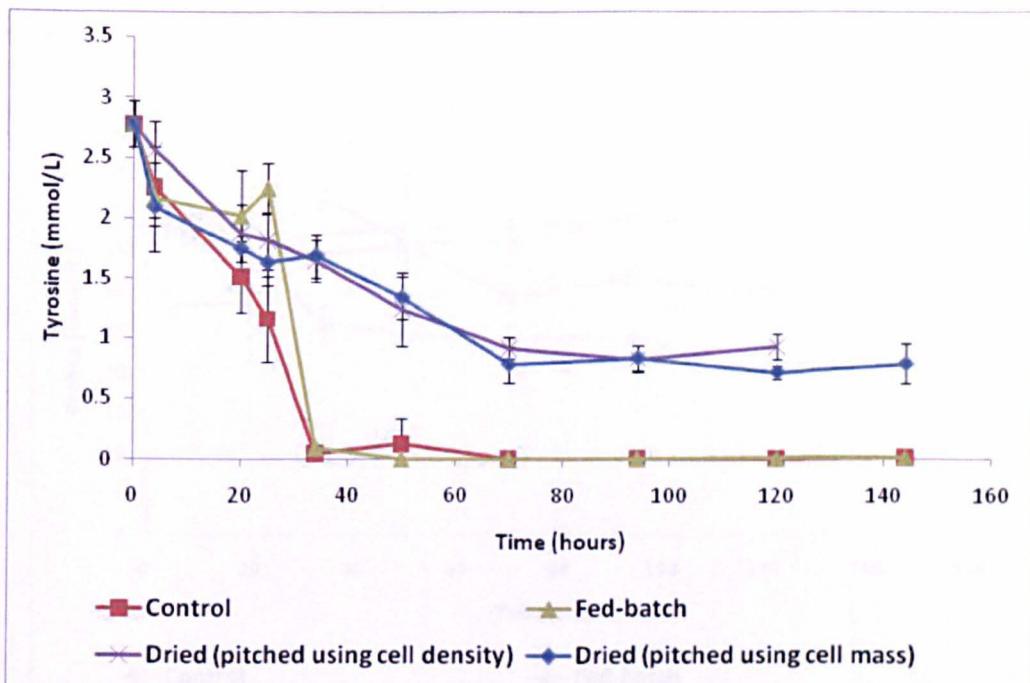
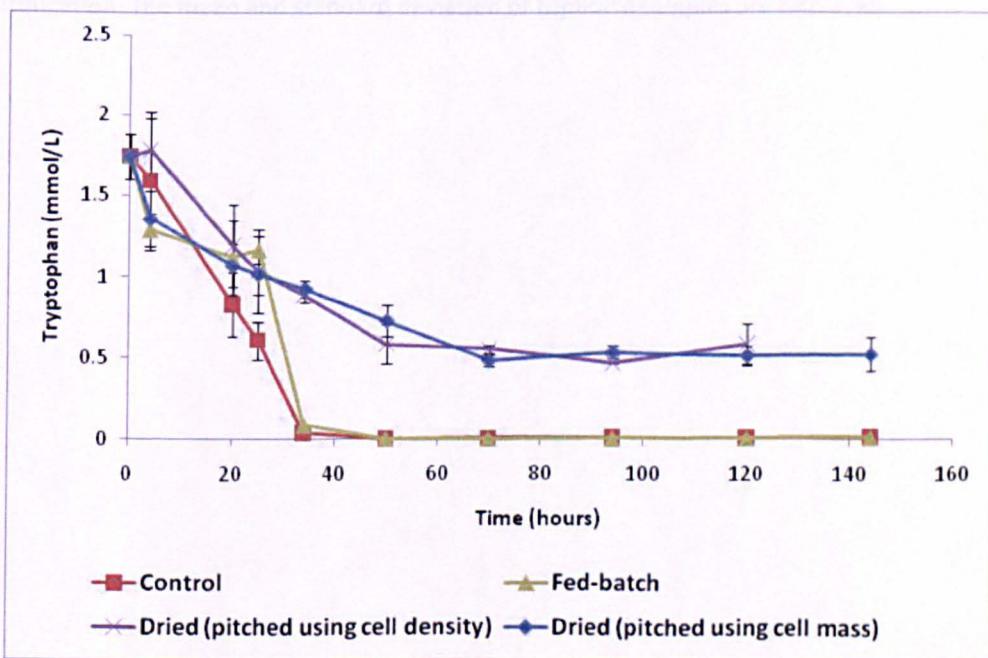


Figure 6.23. The concentration of phenylalanine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

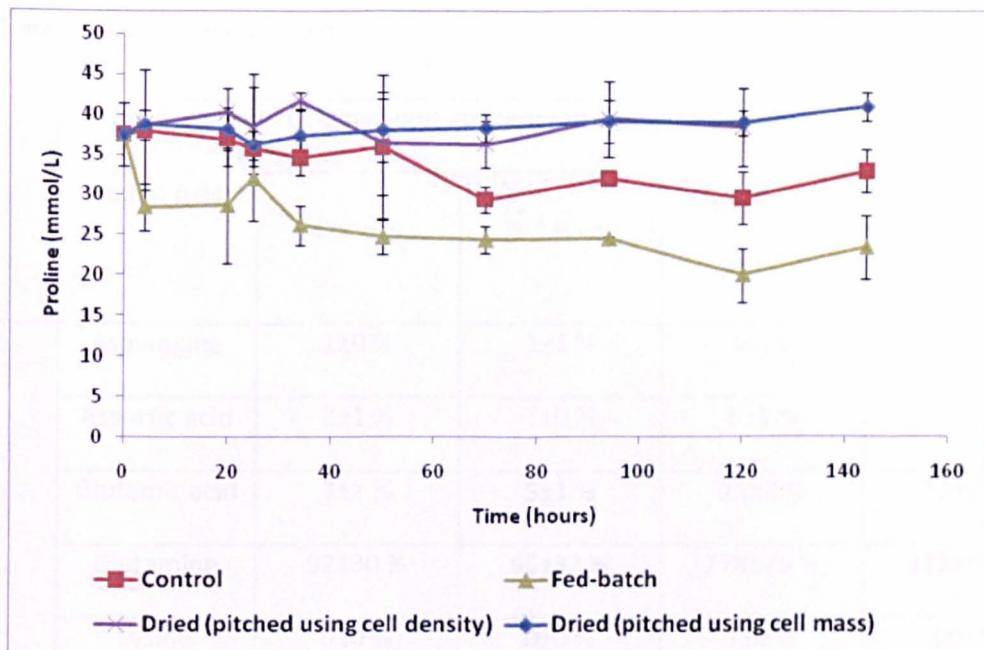


**Figure 6.24.** The concentration of tyrosine in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.



**Figure 6.25.** The concentration of tryptophan in fermentation samples assessed using GCMS analysis. Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

**Class D**



**Figure 6.26. The concentration of proline in fermentation samples assessed using GCMS analysis.**  
Samples were taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr and yeast removed through centrifugation. The mean and standard deviation of triplicate samples are displayed.

**Table 6.1. The percentage of the original concentration of amino acids remaining at the end of fermentation, assessed using GCMS analysis. The mean percentage and standard deviation of triplicate fermentations are shown.**

Amino Acid	Original wort content remaining at the end of fermentation (%)				
	Control	Fed-batch	Dried (cell density)	Dried (cell mass)	
Class A	Asparagine	1±0 %	1±1 %	3±1 %	3±0 %
	Aspartic acid	3±1 %	1±0 %	6±1 %	7±0 %
	Glutamic acid	3±1 %	5±1 %	11±3 %	12±2 %
	Glutamine	92±30 %	65±32 %	278±25 %	311±62 %
	Lysine	0±0 %	0±0 %	1±0 %	1±0 %
	Serine	1±1 %	0±0 %	2±1 %	3±1 %
	Threonine	1±0 %	1±0 %	1±0 %	2±0 %
Class B	Histidine	0±1 %	0±0 %	6±2 %	4±1 %
	Isoleucine	2±0 %	2±0 %	5±1 %	6±0 %
	Leucine	1±0 %	1±0 %	4±1 %	5±0 %
	Methionine	2±1 %	1±1 %	5±1 %	7±1 %
	Valine	2±0 %	2±1 %	21±5 %	20±3 %
Class C	Alanine	6±1 %	4±1 %	35±3 %	37±3 %
	Glycine	23±2 %	1±1 %	61±3 %	67±4 %
	Phenylalanine	1±0 %	1±0 %	16±4 %	13±2 %
	Tyrosine	0±0 %	1±0 %	34±4 %	29±6 %
	Tryptophan	1±0 %	1±0 %	37±7 %	30±6 %
Class D	Proline	88±7 %	63±11 %	102±13 %	109±5 %

### **6.2.5 Intracellular amino acid content**

The initial assimilation of amino acids is a selective process, which it is likely is influenced by the existing concentrations of amino acids within the yeast cell. These will vary depending on the previous conditions of the yeast (Martinez-Force and Benitez, 1995). Potential differences were probed by assessing the relative proportions of amino acids detected in whole cell extractions, from YPD, fed-batch and dried yeast cells. The data from these analyses are shown in Figure 6.27. Laboratory (YPD media) and fed-batch (molasses based media) grown yeast were compared to investigate the affect of growth medium and regime on the amino acid cell content. Glutamic acid is present in the highest concentration in laboratory (YPD) grown cells, accounting for almost half (47 %) of all amino acids, compared to 29 % in fed-batch (molasses based media). In contrast the next most abundant amino acid, alanine, is present at only a third of the amount found in fed-batch grown cells. The affect of drying was assessed by comparisons between fed-batch grown cells and rehydrated dried yeast cells. Significant differences (significance assessed using an analysis of variance  $P \leq 0.05$ ) were found between the concentrations of glycine, lysine, valine (proportions lower in dried cells), aspartic acid and methionine in control and dried yeast fermentations with the latter exhibiting higher levels. There were no significant differences in the other amino acid concentrations between control and dried yeast fermentations.

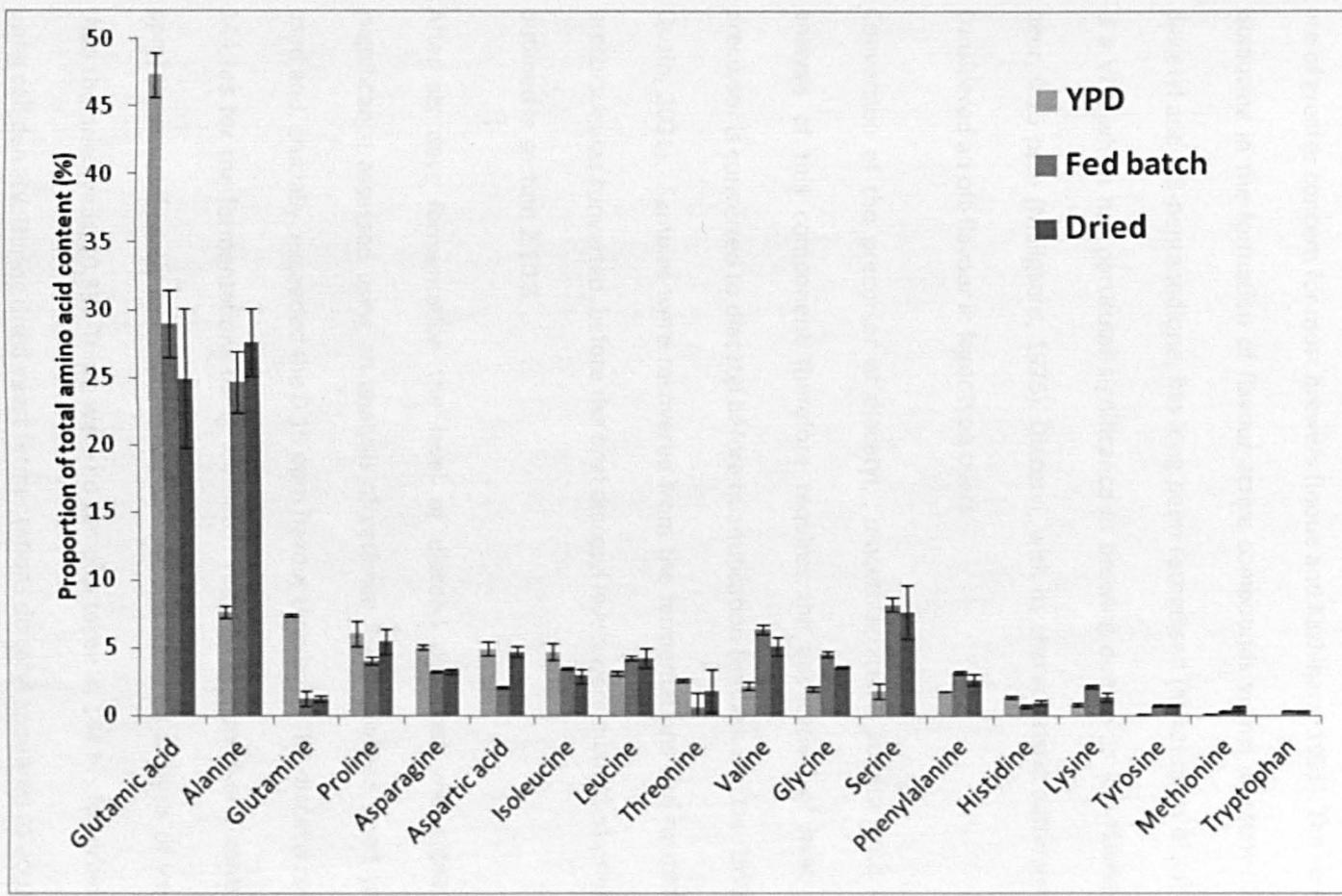


Figure 6.27. The proportion of individual amino acids in the total amino acid content extracted from whole cell populations. Cells were boiled to extract amino acids, which were subsequently detected and identified using GCMS analysis. Displayed, left to right, based on the relative abundance within YPD grown cells. The standard deviation of triplicate samples is displayed.

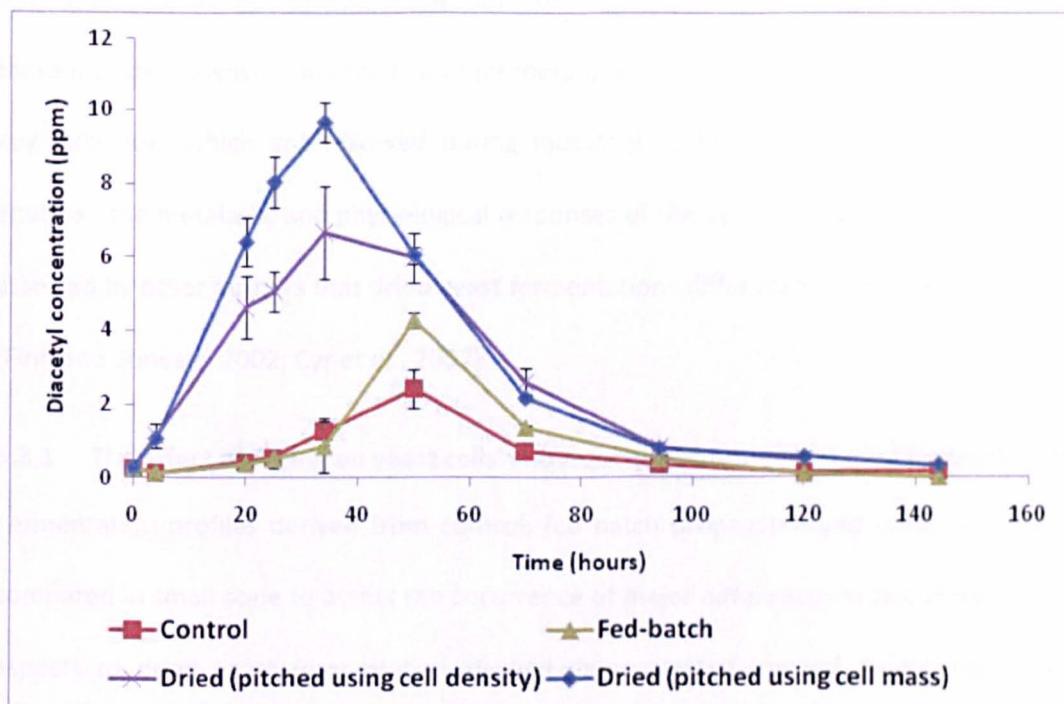
## **6.2.6 The potential concentration of the flavour active compound diacetyl during fermentation**

Whilst nitrogen is integral for yeast growth (Casey *et al.*, 1984; Manginot *et al.*, 1998), the effects of its metabolism by yeast are key to the final beer's organoleptic properties and are of greater concern for most brewers (Inoue and Kashihara, 1995). The role of valine and isoleucine in the formation of flavour active compounds vicinal diketones (VDK), such as diacetyl and 2,3-pentanedione, has long been recognised (Nakatani *et al.*, 1984a). Diacetyl is a VDK which has particular significance in brewing due to its low flavour threshold in beer, 0.15 ppm (Meilgaard, 1975). Diacetyl, with its characteristic butterscotch flavour, is considered an off-flavour in lager type beers.

Conversion of the precursor of diacetyl,  $\alpha$ -acetolactate, is spontaneous and adequate analysis of this component therefore requires the application of heat to ensure all precursor is converted to diacetyl before quantification (Haukeli and Lie, 1978; Boulton and Quain, 2001). Samples were recovered from the fermentations and heated to ensure all precursor was converted, before the final diacetyl levels were quantified using the methods outlined in section 2.10.8.

After six days fermentation the level of diacetyl observed was significantly higher (significance assessed using an analysis of variance  $P \leq 0.05$ ) where dried yeast had been used and, crucially, exceeded the 0.15 ppm flavour threshold. The diacetyl concentration at 144 hrs for the fermentations using fed-batch yeast (0.129 ppm) and control yeast (0.17 ppm) were significantly lower (significance assessed using an analysis of variance  $P \leq 0.05$ ) than the dried yeast (0.33). There were no samples taken at 144 hrs for dried yeast pitched using cell density. During dried yeast fermentations diacetyl appeared to accumulate within a few hours of pitching, rapidly increasing until 34 hrs at which point the concentrations peaked. In the control fermentation the onset of diacetyl formation was later than that observed for dried yeast (Figure 6.28). The diacetyl peaks for dried yeast fermentations

were higher (9.66 ppm pitched using cell mass and 6.67 ppm pitched using cell density) when compared to fermentations completed using fed-batch produced yeast (4.27 ppm) and control yeast (2.40ppm) (Figure 6.28).



**Figure 6.28.** The presence of diacetyl in fermentation samples taken at 0, 4, 20, 25, 34, 50, 70, 94, 120 and 144 hr. Volatiles were isolated using headspace sampling and subsequently separated and identified using GCMS. The mean and standard deviation of triplicate samples are displayed.

## **6.3 DISCUSSION**

Brewers aspire to consistent fermentation profiles even though batch to batch variation in wort composition and differences in pitching yeast physiological state can occur. Typically only a limited number of parameters are routinely monitored during fermentation and these include: downshift in gravity and temperature. In this study we have recorded some key indicators which are followed during industrial scale fermentation, but have also analysed the metabolic and physiological responses of the yeast to address the hypothesis asserted by other authors that dried yeast fermentations differ from control fermentations (Finn and Stewart, 2002; Cyr *et al.*, 2007).

### **6.3.1 The affect of drying on yeast cells' subsequent performance during fermentation**

Fermentation profiles derived from control, fed batch propagated and dried yeast were compared in small scale to assess the occurrence of major differences in performance. The aspects of dried yeast fermentation studied demonstrated marked differences to the control fermentation.

Dried yeast exhibited an impaired growth profile when compared to that observed for control and fed batch yeast. Furthermore dried yeast exhibited reduced viability during fermentation. An increase in pitching rate resulted in a small increase in the final crop yield, but even with this adjustment final cell numbers were lower than those observed at the end of control fermentations. It should be noted that although a reduced cell number would suggest reduced cell growth, cell growth can still occur without cell division. Reduced cell growth may create problems should the cropped yeast be required for serial repitching, a subject which will be discussed in more detail in Chapter 7. The reasons for these differences in cell viability and yield are unknown, but it seems likely that they are related to other differences noted during dried yeast fermentations.

Dried yeast cells exhibited a delay in sugar and amino acid uptake either causing, or occurring as a consequence of, poor growth. A lag in nutrient uptake can delay the onset of replication by effectively stalling cell division (Casey *et al.*, 1984; Manginot *et al.*, 1998), however dried yeast exhibiting a reduced viability would also be expected to exhibit a reduced sugar uptake rate until cell divisions had occurred and biomass levels were restored. The observed delay may also reflect a requirement for dried yeast to rehydrate and possibly repair damage caused by the dehydration / rehydration process before initiating efficient fermentation.

In addition to the lag in the uptake of amino acids, it was noted that some amino acids were not fully assimilated. One possible reason for this may lie in the cellular reserves of amino acids, which can affect the ability of cells to assimilate amino acids (Woodward and Cirillo, 1977). Differences in cell growth and division profiles have been demonstrated to lead to altered intracellular amino acid profiles (Martinez-Force and Benitez, 1995). Interestingly the control and fed batch yeast utilised in this study were pre-grown using differing methods but failed to exhibit differences in amino acid uptake. This suggests that in this instance pre-growth conditions do not affect amino acid uptake.

The majority of the amino acids that were not fully utilised by the dried yeast are classified as class C amino acids and, as such, would not be expected to be utilised until later in the fermentation (Jones and Pierce, 1964). Indeed it has been suggested by these authors that Class C amino acid uptake is repressed by Class A amino acids (although the data shown in Section 6.2.4 may challenge this assumption) and a delay in the utilisation of Class A amino acids could delay Class C amino acid uptake. The relatively low viability exhibited by the dried yeast population during fermentation could also be a cause of the apparent stall in the uptake of these amino acids.

A difference noted which may potentially impact the final product is the apparent early onset of diacetyl formation and high peak and residual levels of this flavour active compound during dried yeast fermentation. Levels of the flavour active compound were above the flavour threshold (0.15 ppm) after six days of fermentation. When the number of dried yeast cells was increased, a corresponding increase in the maximum concentration of diacetyl was also observed. This is an important observation, since it suggests that compensation of low viability by over pitching dried yeast may not benefit the brewer. The addition of a maturation period, perhaps at an elevated temperature, may result in the reduction of diacetyl to acceptable levels. Indeed, it is not uncommon when using propagated yeast for fermentation times to be extended because of such issues. However, for brewers, this is undesirable as it would result in lengthier fermentation times and higher costs, mitigating some of the potential benefits proposed for ADY.

Diacetyl is formed during fermentation as a consequence of normal yeast metabolism and is a by-product of the valine anabolic pathway (Figure 6.29). Pyruvate, originating from glycolysis, is converted to  $\alpha$ -acetolactate, then two further intermediates before finally producing valine. However, if  $\alpha$ -acetolactate is leaked from the cell it undergoes an oxidative decarboxylation forming diacetyl. The majority of diacetyl does not persist in beer, instead it is taken up by yeast and converted to acetoin, and subsequently 2,3-butanediol. This vicinal diketone does persist in beer, but has a much higher taste threshold and therefore is unlikely to impact on the final flavour. Whilst most steps are under enzymatic control, the decarboxylation of  $\alpha$ -acetolactate is not, and is thought to be the rate limiting step in most fermentations (Boulton and Quain, 2001). The action of this pathway during fermentation typically results in a peak of diacetyl formation, which is then reduced to levels below the flavour threshold towards the end of fermentation.

One rationale to explain the higher levels of diacetyl in beer produced using dried yeast is that ADY populations may exhibit elevated production of  $\alpha$ -acetolactate, which subsequently forms diacetyl. The enzyme responsible for the conversion of pyruvate to  $\alpha$ -acetolactate is acetohydroxy acid synthase. As this is an anabolic pathway for valine, acetohydroxy synthase is inhibited by the presence of this amino acid, and is also inhibited by the presence of alanine, threonine and glutamate (Barton and Slaughter, 1992). In the current study the same batch of wort was used for all fermentations thereby eliminating wort amino acid composition as a cause. When yeast is pitched into wort it contains an intracellular pool of amino acids. To assess whether this differed between the pitching yeast investigated in this study, the relative abundance of the internal amino acids was determined. Although differences were apparent between dried and control yeast, possibly reflecting their differing growth parameters, fed batch and dried yeast were similar. Despite this, fed batch yeast demonstrated a diacetyl profile similar to control yeast; it may therefore be asserted that the intracellular pool of amino acids is not the cause of the differences in diacetyl formation observed.

Early production of diacetyl during fermentations using dried yeast has been noted previously (Cyr *et al.*, 2007). It was suggested that this was due to an impaired ability to assimilate amino acids, leading to synthesis of valine and therefore diacetyl (Cyr *et al.*, 2007). Delayed uptake of amino acids was noted in the fermentations described in the current study, including valine. Diacetyl production began almost immediately peaking at approximately 40 hrs, closely matching the time at which valine assimilation began. With the assimilation of valine causing the suppression of diacetyl formation (Nakatani *et al.*, 1984b), this may explain the profiles obtained.

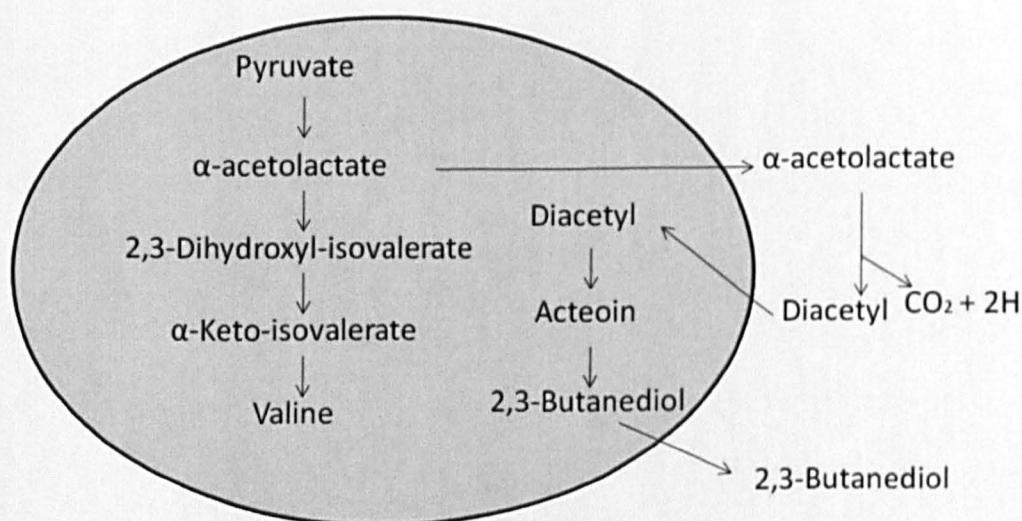
Yeast growth and VDK production have been strongly linked (Nakatani *et al.*, 1984a) primarily because both are strongly influenced by amino acid uptake and synthesis. It has

been suggested that increased yeast growth may lead to production of more precursors for diacetyl formation, due to increased amino acid synthesis (Lekkas *et al.*, 2007). There was, however, less yeast growth in dried yeast fermentations (Section 6.2.1), which produced higher levels of diacetyl in the medium. This suggests that whilst amino acid synthesis may be involved in the abnormal diacetyl profile, it is by no means the only factor to consider.

Another potential cause of the aberrant diacetyl profile observed is that dried yeast cells have an increased leakage of  $\alpha$ -acetolactate or a reduced rate of assimilation for diacetyl. This would result in the accumulation of diacetyl in the fermentation medium, giving the appearance of more diacetyl in total because of the requirement to accelerate diacetyl formation during the assay. Whilst the non-enzymatic decarboxylation is often considered the rate limiting step in vicinal diketone pathways (Boulton and Quain, 2001), it is clear that the fermentations using dried yeast described here are not what could be considered normal. Boulton *et al.* (2001) have described an assay for yeast vitality based on the assimilation of diacetyl, which they suggest is reliant on a competent membrane. As discussed in Chapter 5, the competence of the dried yeast membrane is questionable. Leakage of cellular components has also been noted in dried yeast (Rapoport *et al.*, 1997; Attfield *et al.*, 2000). Indeed, during the dried yeast fermentations high residual concentrations of amino acids were demonstrated to be present. These higher concentrations are suggested to be due to cellular leakage from the high percentage of non-viable cells present in dried yeast cultures. In addition, viable cells generating  $\alpha$ -acetolactate, but exhibiting a leaky membrane may also increase the extracellular pool of the compound.

Given that a perception exists of dried yeast being prone to brewery contaminants, it would be remiss not to mention their possible contribution to diacetyl. The metabolism of contaminate lactic acid bacteria, particularly *Pediococcus* species can produce diacetyl

during fermentation (McCaig and Weaver, 1983). *Pediococcus* contamination can lead to elevated levels of diacetyl at the end of fermentation, due to production by the bacteria and also the reduced uptake by yeast associated with the contamination (McCaig and Weaver, 1983). Often, in cases of contamination, fermentations do not fully attenuate (McCaig and Weaver, 1983). As this was not the case and there was no other evidence for contaminant microorganisms, it is suggest that the elevated levels of diacetyl were not due to *Pediococcus* contamination.



**Figure 6.29.** Valine anabolic pathway, which results in the production of diacetyl during fermentation. Adapted from Boulton and Quain (2001) and Barton and Slaughter (1992). The contents of the grey oval represent reactions occurring within yeast cells.

#### 6.4 CONCLUSIONS

In this chapter significant differences between dried and control yeast fermentations have been demonstrated. Fermentations pitched using dried yeast take longer to reach final attenuation and may require a longer diacetyl stand than conventional propagated yeast. These problems may be associated with the yeast population dynamics which do appear to be different. The fact that these differences are not present in fed batch yeast would indicate that the differences are due to the effects of dehydration and rehydration, rather than variations in growth parameters.

No sensory analysis was completed on the products of these fermentations as the beer produced was not further processed, however, it is unlikely that a beer with an altered amino acid content and diacetyl levels would be accepted as comparable to regular product. The reduced growth and low viability is problematic when considering the use of dried yeast for the repitching of a subsequent fermentation. The question of whether these issues persist in subsequent fermentations is a pertinent one and will be addressed in Chapter 7.

## CHAPTER 7: THE FERMENTATION CAPACITY OF DRIED YEAST WHEN IT IS SERIALLY REPITCHED

### 7.1 INTRODUCTION

Towards the end of fermentation yeast cells aggregate in a process termed flocculation. Once flocs (cell aggregates) are formed they separate from the beer, ale yeast rising to the surface and lager yeast sinking to the bottom providing the vessel type permits. This separation is important to remove the yeast from the final beer, but it also allows the yeast to be collected. This collected, or cropped, yeast can then be used to pitch further fermentations in a practice termed serial repitching. Freshly propagated yeast utilised for fermentation performs differently to yeast that has been used for a previous fermentation (Miller, 2010). In some instances yeast only begins to perform towards its optimal level after a few serial repitchings have been completed. However, in many breweries repitching does not occur indefinitely and the yeast is periodically replaced with freshly propagated yeast. Although historically continuous repitching was problematic primarily due to contamination, it can also impact on the fermentation performance of yeast. It is accepted that there is often a general decline in yeast quality as the number of fermentations (termed generation number) increases, particularly when lager strains are used. This decline may begin to manifest itself in a loss in viability (Smart and Whisker, 1996; Jenkins *et al.*, 2003), altered flocculation (Smart and Whisker, 1996; Sato *et al.*, 2001), genetic damage (Sato *et al.*, 2001) and membrane damage (Jenkins *et al.*, 2003). This damage can take many generations to appear and repitched yeast is expected to perform adequately for several fermentations, although the precise number is often strain specific.

As discussed in Section 6.1.1 there have been several reports on the direct use of dried yeast in fermentation (Gosselin and Fels, 1998; Fels, 1999; Finn and Stewart, 2002; Cyr *et al.*, 2007), but there are only a limited number of reports concerning the suitability of dried

yeast for repitching into multiple fermentations (Reckelbus *et al.*, 2000; Powell and Fischborn, 2010). Powell and Fischborn (2010) compared dried yeast fermentations to freshly propagated yeast over five successive fermentations. When first utilised, dried yeast was found to exhibit a greater lag and altered rate of attenuation when compared to control yeast. The final beer from these fermentations showed similar alcohol contents, specific gravities and pH. Despite the observation that dried yeast exhibited lower viability when compared to that of the control yeast at pitching (64 % and 98 %, respectively), by the end of fermentation the viability of the two yeast batches were comparable (97 % and 96 %). After repitching, both fermentations demonstrated a shorter lag phase than observed in the corresponding initial fermentations and although dried yeast may have demonstrated a slightly longer lag phase than control yeast of the same generation number, after several fermentations the two populations demonstrated similar profiles. Powell and Fischborn (2010) also examined the concentration of several flavour compounds in the final beer produced from the first and fifth generation control and dried yeast fermentations. Taste tests suggested no impact on final product flavour. Furthermore serially repitching dried yeast did not lead to the accumulation of genetic variants. Analysis of the flocculation potential of the yeast, a commonly altered phenotype during serial repitching (Sato *et al.*, 2001), demonstrated that in this instance dried yeast exhibited comparable flocculation to that observed for the control yeast throughout the fermentations.

Reckelbus *et al.* (2000) described serial repitching trials of dried yeast completed at two breweries operating with 60 hL and 400 hL capacities. Differences were noted in the change in specific gravity over time; dried yeast demonstrating a lag with regard to this parameter. It was suggested that this may be due to pitching rate differences, as the dried product was pitched according to its weight and not the number of cells. This difference was considered to be diminished when yeast was repitched. Beers produced using dried

yeast in the first instance and after repitching were considered to be acceptable with regard to their final taste profiles as assessed by tasters, although specific flavour compound concentrations were not assessed (Reckelbus *et al.*, 2000).

Whilst these studies (Reckelbus *et al.*, 2000; Powell and Fischborn, 2010) suggest that dried yeast is suitable for serial repitching, the initial fermentations did not display (or were not assessed for) some of the aberrant characteristics observed in other studies, including the potential to flocculate, haze formation (Finn and Stewart, 2002), lags in amino acid uptake and altered vicinal diketone formation (Cyr *et al.*, 2007). In Chapter 6 of the current study, fermentations using LAL1 dried yeast were shown to display lags in attenuation, sugar and amino acid assimilation as well as altered yeast growth. In this Chapter, the question of whether these particular traits continue when the yeast is utilised for a subsequent fermentation is addressed.

Fermentations were conducted in bioreactors (5 L capacity) to assess similarities and differences between laboratory propagated yeast termed “control” and dried yeast. Yeast from this first fermentation was then collected and stored at 4°C before being repitched into a second fermentation. Fermentations were compared with respect to time to attenuation, yeast cell viability and budding indices. At time points throughout the fermentations the assimilation of amino acids, and the uptake of fructose, sucrose, maltose, maltotriose and glucose were monitored. The production and subsequent uptake of the key flavour volatile diacetyl was also followed.

## 7.2 RESULTS

Fermentations were conducted in mechanically agitated bioreactors (5 L). The primary reason for this increase in scale from the miniature fermentations (100 ml wort volume) described in Chapter 6 was to ensure a sufficient crop yield and recovery for subsequent repitching. Control yeast was propagated using YPD medium in an aerated batch system

(Section 2.9.1), whilst dried yeast was rehydrated using tap water (Section 2.9.2) (Figure 7.1). After the completion of one fermentation yeast was recovered and stored under beer at 4°C for two days prior to repitching (Figure 7.2). All fermentations were pitched at 1.5 x 10<sup>7</sup> viable cells per ml.

The use of the bioreactors permitted greater control of oxygenation but the exact oxygen content of the wort at pitching was not measured. The bioreactors enabled pure oxygen to be passed through the wort to achieve a dissolved oxygen content of approximately 18 ppm.

Figure 7.1. Schematic of the yeast preparation prior to pitching into fermentations. Propagated yeast was grown aerobically for a total of 7 days. Dried yeast was rehydrated for 60 min immediately prior to pitching.

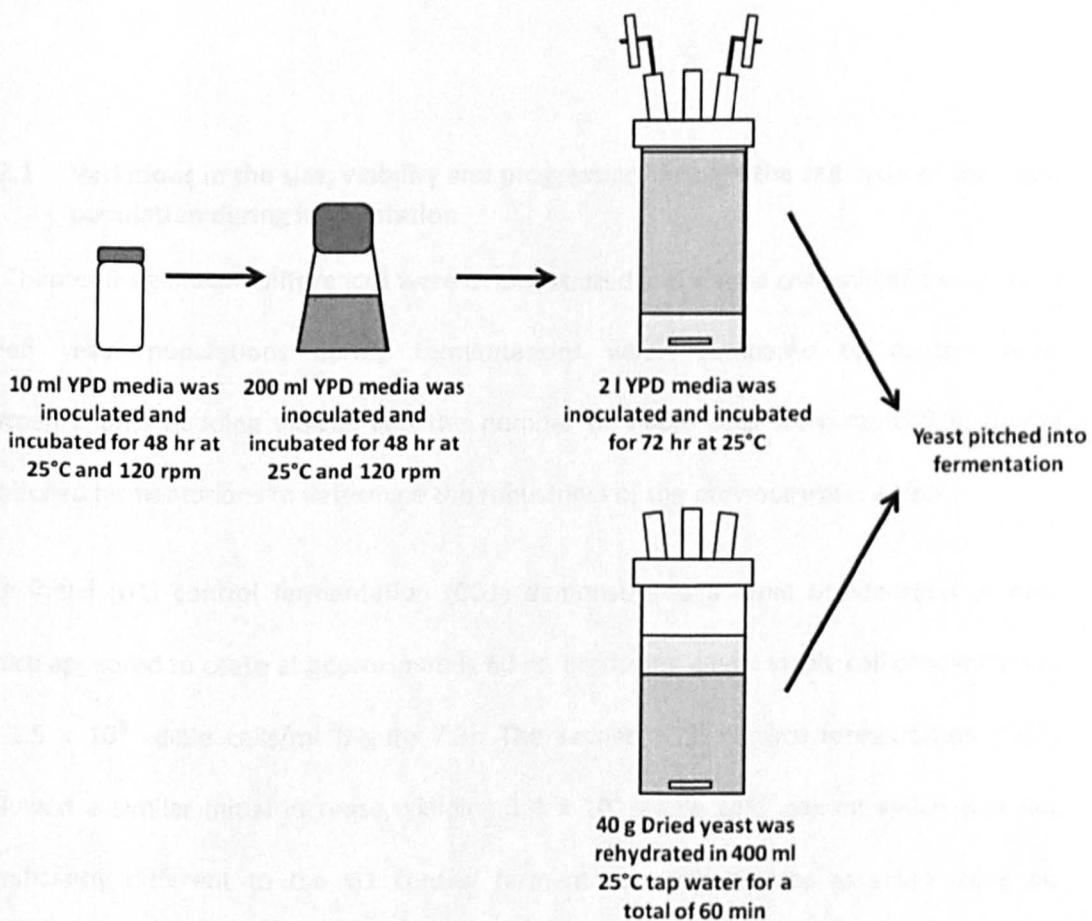
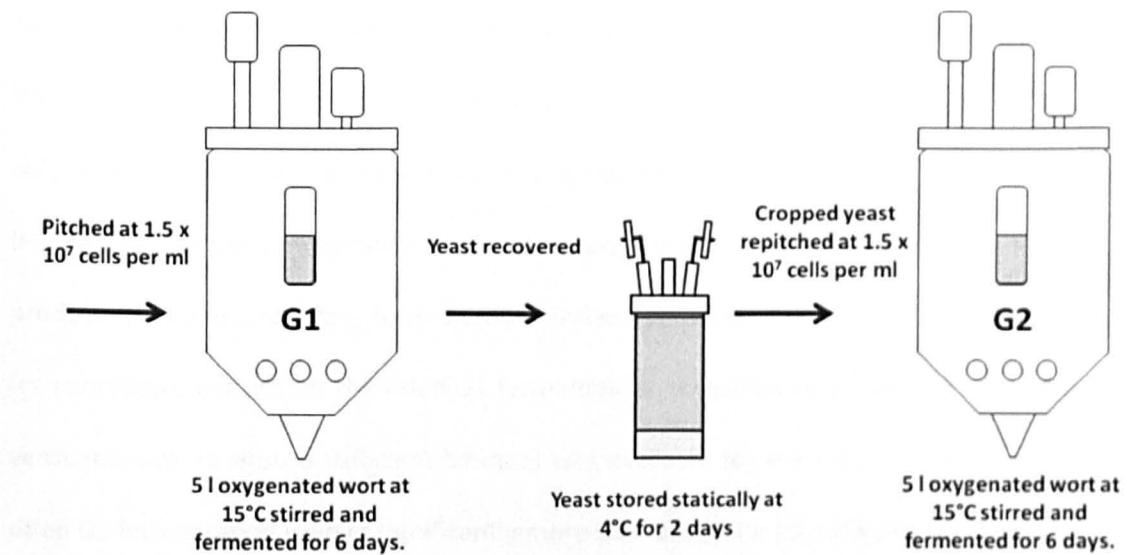


Figure 7.1. Schematic of the yeast preparation prior to pitching into fermentations. Propagated yeast was grown aerobically for a total of 7 days. Dried yeast was rehydrated for 60 min immediately prior to pitching.



**Figure 7.2. Schematic of the fermentation cycle employed.** 5 l oxygenated wort were pitched at  $1.5 \times 10^7$  cells per ml and fermented for 6 days. Yeast were recovered and stored for two days prior to repitching.

### 7.2.1 Variations in the size, viability and progression through the cell cycle of the yeast population during fermentation

In Chapter 6 significant differences were demonstrated in the crop cell yield and viability of dried yeast populations during fermentations when compared to control yeast fermentations. Budding indices and the number of viable cells were monitored during repitched fermentations to determine the robustness of the previous observation.

The initial (G1) control fermentation (CG1) demonstrated a rapid proliferation of cells which appeared to cease at approximately 60 hr, producing a final viable cell concentration of  $1.5 \times 10^8$  viable cells/ml (Figure 7.3). The second (G2) control fermentation (CG2) followed a similar initial increase, yielding  $1.4 \times 10^8$  viable cells per ml which was not significantly different to the G1 control fermentation (significance assessed using an analysis of variance  $P \leq 0.05$ ). These profiles are in stark contrast to those observed during fermentations completed with dried yeast. Initial dried yeast fermentation (Dried G1) demonstrated impaired growth, yielding a viable cell concentration of  $3.3 \times 10^7$  cells/ml.

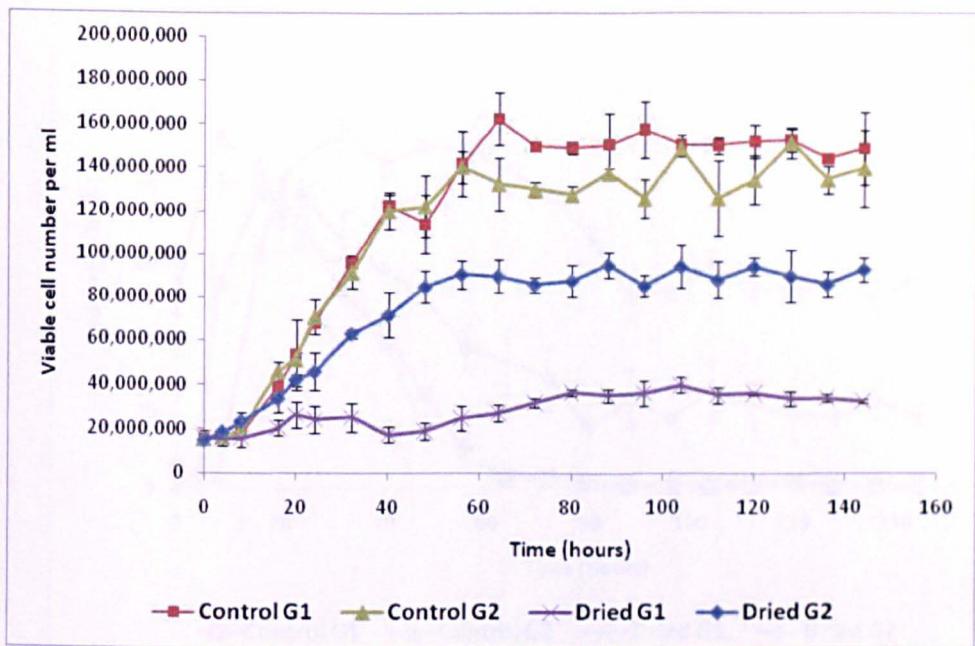
The growth was slower but also persisted for longer during fermentation ceasing at 80 hr (Figure 7.3). There was a reduction in the number of viable cells (40 hr), which appeared to be caused by cell death with the percent viability decreasing rapidly from 70 % to 39 % (Figure 7.4). When fermentations are repitched there is normally an excess of yeast product from the preceding fermentation. Indeed, this was the case for control yeast fermentations, but not for the dried G1 fermentation necessitating a high rate of cropped yeast recovery to ensure sufficient biomass was available for the next fermentation. The dried G2 fermentation yielded significantly more biomass ( $9.4 \times 10^7$  cells per ml) than its G1 counterpart (significance assessed using an analysis of variance  $P \leq 0.05$ ) indicating that phenotype recovery occurred following serial repitching. However, crop yield was still lower than that achieved during control fermentations.

The high residual budding index observed at the end of dried G1 fermentation (Figure 7.5) may indicate that cell growth persisted throughout fermentation. However, the lack of increase in cell number would suggest this is not the case leading to the hypothesis that cytokinesis was impaired for dried yeast populations. To investigate this hypothesis, DNA content of cell populations during fermentation was assessed (Section 2.10.1) and compared to the budding index of the cell populations. The synthesis of DNA is commonly used as an indicator to the stage of the cell cycle (Burke *et al.*, 2000; Raithatha and Stuart, 2009), and has been used to assess propagation of brewing yeast (Novak *et al.*, 2007) as well as fermentation (Müller *et al.*, 1997; Miller *et al.*, In Press). Actively dividing cells exhibit a doubling in their DNA content prior to cell budding.

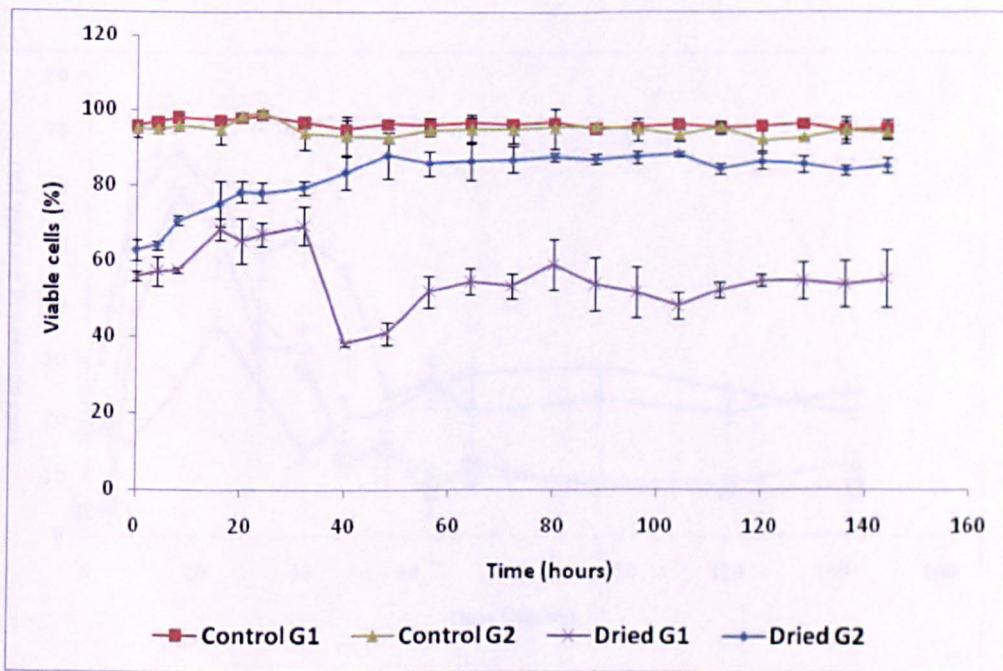
Histograms were generated based on the intensity of fluorescence from each cell when stained with the DNA binding compound propidium iodide (Table 7.1). Initially only one peak was evident (Representing 1 x DNA or G1), as cells progressed through the fermentations a second peak emerged (representing 2 x DNA or G2). Using the peak reflect

method (Givan, 1992) the number of cells containing twice the normal compliment of DNA were estimated and this value was plotted against time (Figure 7.6). This provided an indication of the proportion of cells in G2 and thus the progression of mitosis. The individual histogram plots (Table 7.1) indicate that the initial Control fermentation (CG1) displayed a significant number of cells which had duplicated their DNA by 4 hr. The peak in DNA synthesis mirrored the peak in budding, indicating that cell division had ceased by approximately 60 hr. When dried yeast was analysed the histograms were heavily influenced by non-viable yeast which could not be identified. The peak of the percentage of cells replicating was always less than 40 % (Figure 7.6) and, as budding indices peaked around 90 % (Figure 7.5), this would suggest that many of the non-viable cells contained the normal compliment of DNA. There was also an indication that there were two major periods of DNA synthesis, at 24 and 80 hr, which was not apparent from the budding index data alone.

In all fermentations a portion of the population of cells appeared not to complete cytokinesis exhibiting twice the normal compliment of DNA. However, Dried G1 fermentations exhibited a greater proportion of cells with this phenotype than control G1 fermentations, 20 % and 8 % respectively.



**Figure 7.3.** The number of viable cells present in fermentations, assessed using methylene blue staining. The standard deviation of triplicate samples is displayed. Samples were taken every 8 hr (every four hr at the beginning of fermentation).



**Figure 7.4.** The percentage of viable cells present in fermentations, assessed using methylene blue staining. The standard deviation of triplicate samples is displayed. Samples were taken every 8 hr (every four hr at the beginning of fermentation).

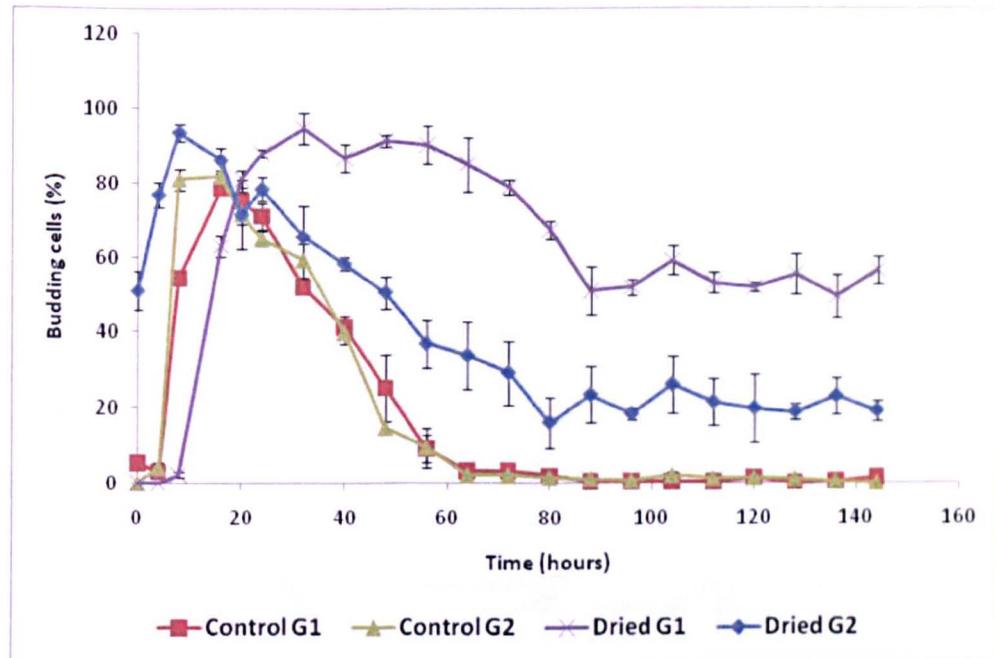


Figure 7.5. Percentage of cells exhibiting a bud present in fermentations. The standard deviation of triplicate samples is displayed. Samples were taken every 8 hr (every four at the beginning of fermentation). Cells were deemed to be budding if there was evidence of a bud or cell still attached to the mother cell.

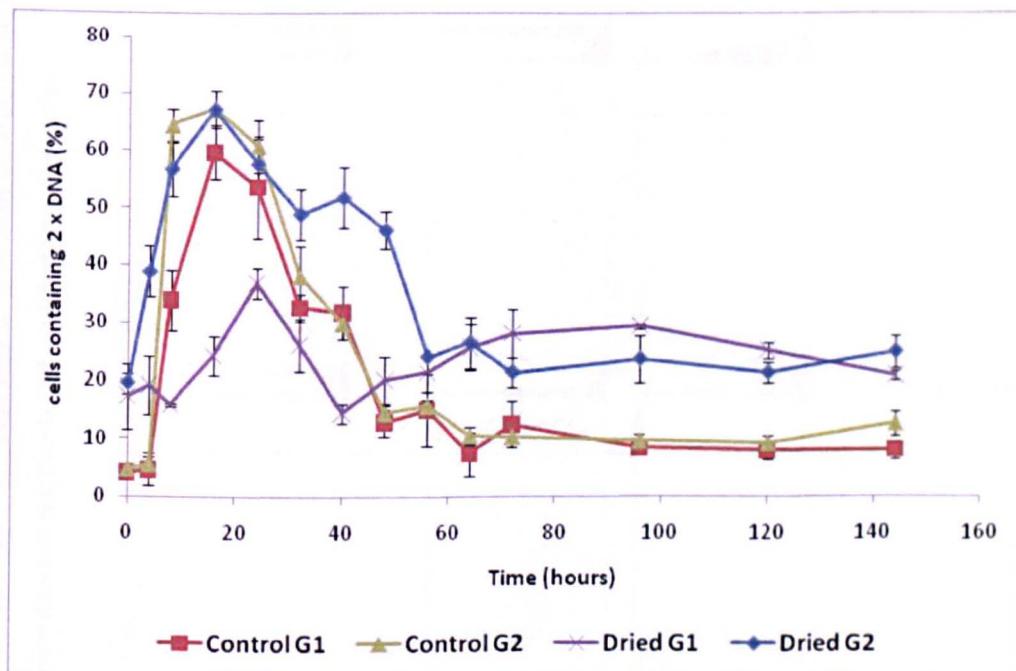
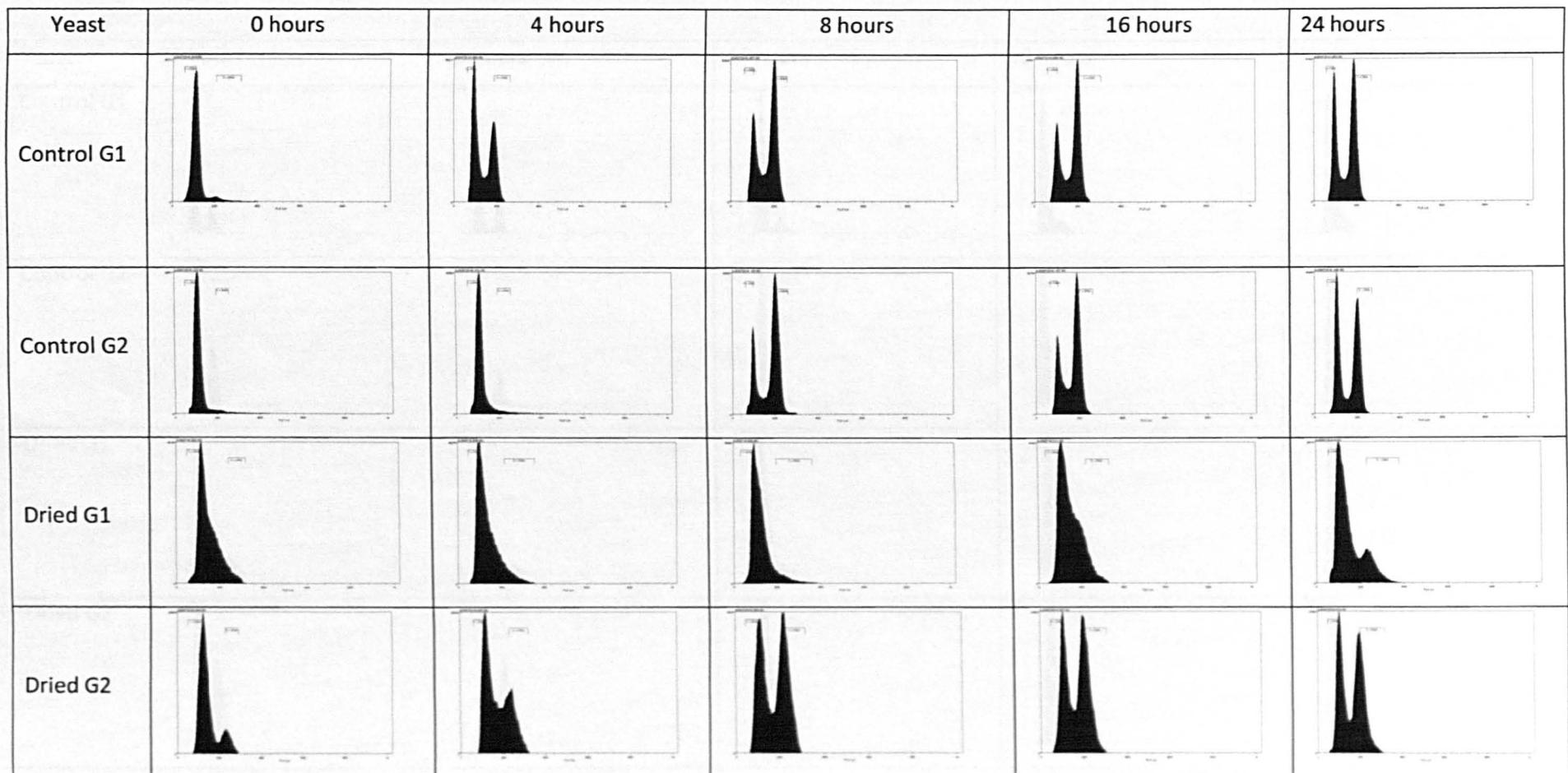
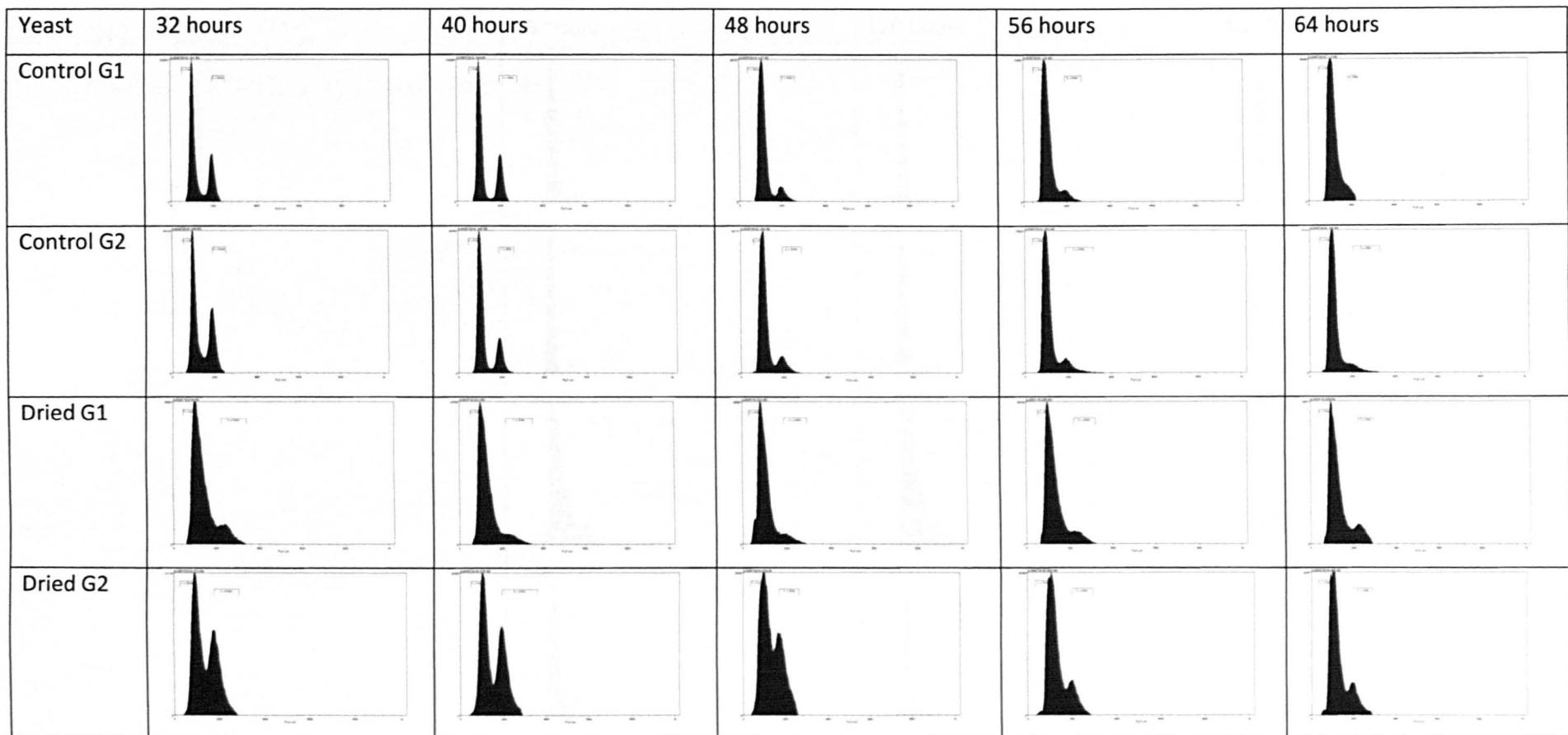


Figure 7.6 The percentage of the cell population deemed to contain two copies of DNA. The standard deviation of triplicate samples is displayed. Samples were taken at 0, 4, 8, 16, 24, 32, 40, 48, 56, 64, 72, 96, 120 and 144 hr. DNA content of each cell was estimated using propidium iodide staining and flow cytometry analysis. The percentage of cells with two times the amount of DNA was estimated using the peak reflect method.

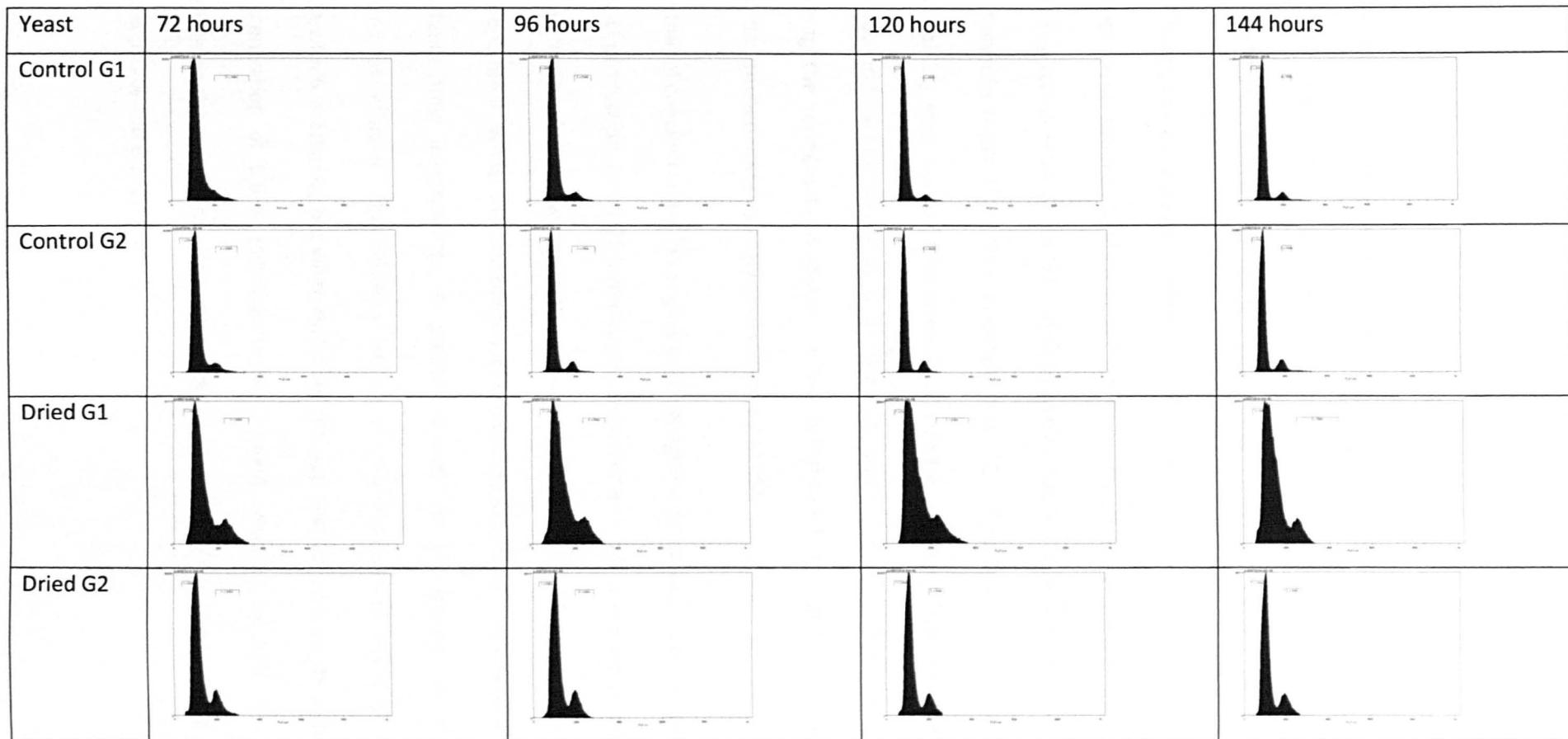
**Table 7.1.** Example of histograms (one included from a triplicate of samples) showing the light (675/30 nm) intensity when fixed yeast cells were stained with propidium iodide and illuminated with light (488 nm) generated by an argon laser. Single events, representing one yeast cell, were recorded using a flow cytometer. Events with low scatter were deemed cell debris and excluded from the analysis.



**Table 7.1 (continued) Example of histograms (one included from a triplicate of samples) showing the light (675/30 nm) intensity when fixed yeast cells were stained with propidium iodide and illuminated with light (488 nm) generated by an argon laser. Single events, representing one yeast cell, were recorded using a flow cytometer. Events with low scatter were deemed cell debris and excluded from the analysis.**



**Table 7.1 (continued) Example of histograms (one included from a triplicate of samples) showing the light (675/30 nm) intensity when fixed yeast cells were stained with propidium iodide and illuminated with light (488 nm) generated by an argon laser. Single events, representing one yeast cell, were recorded using a flow cytometer. Events with low scatter were deemed cell debris and excluded from the analysis.**

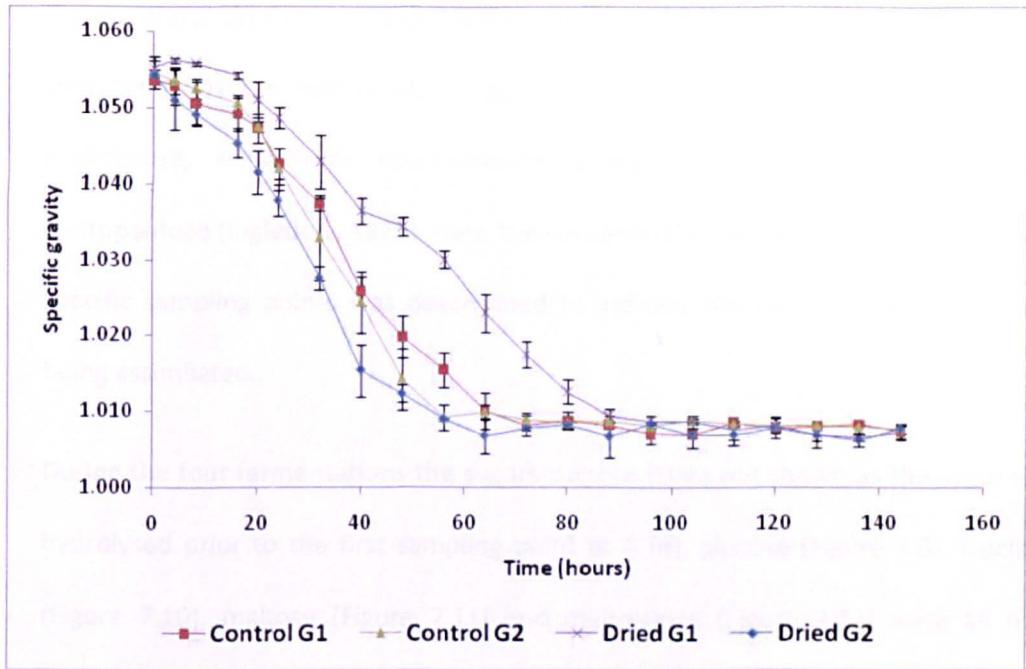


### **7.2.2 Time taken to reach final attenuation and the production of ethanol**

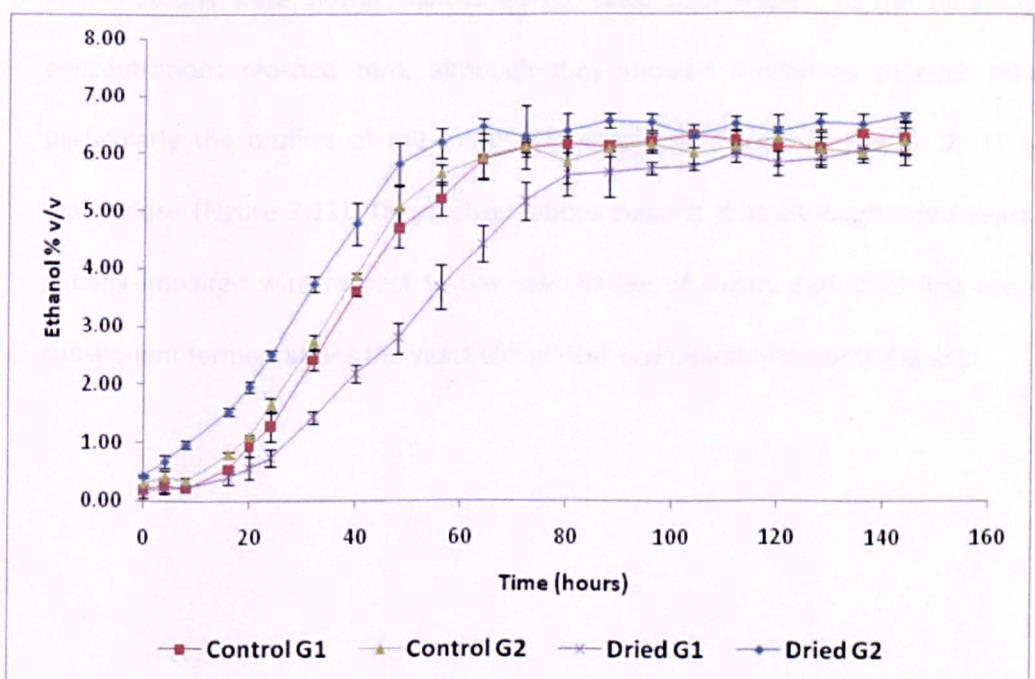
Attenuation can be defined as the decrease in specific gravity of wort during fermentation (Boulton and Quain, 2001). It is intimately related to the concentration of sugars and the production of ethanol. The time taken to reach the final attenuation and ethanol production are two important determining factors that contribute to the length of fermentation and as such are commonly monitored by brewers.

As early as 8 hours, the specific gravity of the dried (G1) fermentation was significantly higher than the other fermentations (significance determined using an analysis of variance  $P \leq 0.05$ ) (Figure 7.7). Repitched yeast (Dried G2 and Control G2) reached final attenuation by 56 hr and were therefore faster than their corresponding G1 fermentations. The propagated control (Control G1) yeast reached final attenuation by 64 hr and the rehydrated dried yeast (Dried G1) reached final attenuation by 88 hr. The final specific gravity (approximately 1.008) reached during the fermentations (taken at 144 hr) showed no significant differences (significance determined using an analysis of variance  $P \leq 0.05$ ).

Analysis of ethanol concentrations in samples also highlighted differences with respect to the rate of ethanol production. Dried G1 fermentation exhibited a slower rate when compared to that observed for Dried G2. However, with control yeast ethanol production rates were not generation dependent. Whilst the rate of ethanol production may be of concern with respect to the residence time in fermenter, of greater concern for the brewer are the final concentrations of ethanol. The initial dried yeast fermentation (Dried G1) finished fermentation with 6 % ethanol, but when repitched the yeast produced a significantly higher ethanol concentration of 6.6 % (significance determined using an analysis of variance  $P \leq 0.05$ ). Neither of these concentrations were significantly different to either of the control fermentations (6.3 % and 6.2 %).



**Figure 7.7.** Change in specific gravity during fermentation. Standard deviation of triplicate samples shown. Samples were taken every 8 hr (every four at the beginning of fermentation) and yeast removed through centrifugation.



**Figure 7.8.** Change in ethanol concentration during fermentation. Standard deviation of triplicate samples shown. Samples were taken every 8 hr (every four at the beginning of fermentation) and yeast removed through centrifugation.

### **7.2.3 The assimilation of individual sugars during fermentation**

Wort contains the fermentable sugars sucrose, fructose, glucose, maltose and maltotriose, as well as non-fermentable sugars such as maltotetraose and maltopentose (Ingledew, 1975). Here the concentration of the fermentable sugars at specific sampling points was determined to indicate the rate at which they were being assimilated.

During the four fermentations the sugars sucrose (Data not shown as the sugar was hydrolysed prior to the first sampling point at 4 hr), glucose (Figure 7.9), fructose (Figure 7.10), maltose (Figure 7.11) and maltotriose (Figure 7.12) were all fully assimilated, although the sampling point at which the residual concentration of each sugar in wort was reduced to zero varied. Dried G2 yeast was the most rapid at fully utilising all sugars, and was markedly faster than dried G1 yeast. The control yeast fermentations were slower than dried G2 yeast with respect to the time sugar concentrations reached zero, although they showed similarities to each other, particularly the profiles of the more abundant sugars maltose (Figure 7.11) and maltotriose (Figure 7.12). These observations suggest that although dried yeast is initially impaired with respect to the assimilation of sugars during its first use, in subsequent fermentations the yeast will perform comparably to control yeast.

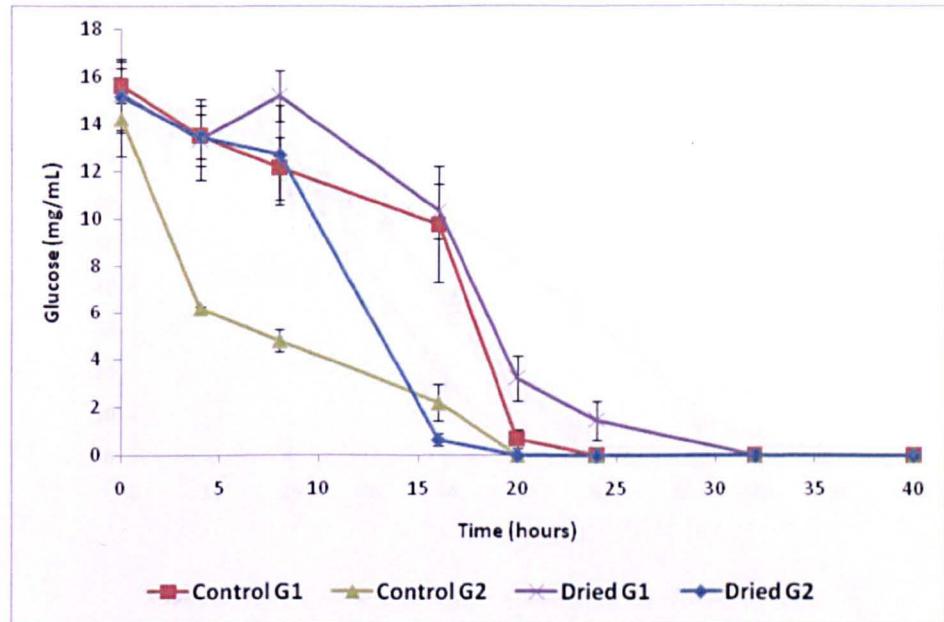


Figure 7.9. The presence of glucose in fermentation samples (assessed using HPLC analysis). Standard error of triplicate samples shown. Samples were taken every 8 hr (every four at the beginning of fermentation) and yeast removed through centrifugation. As glucose was absent in all fermentations after 40 hr, only early data points are shown.

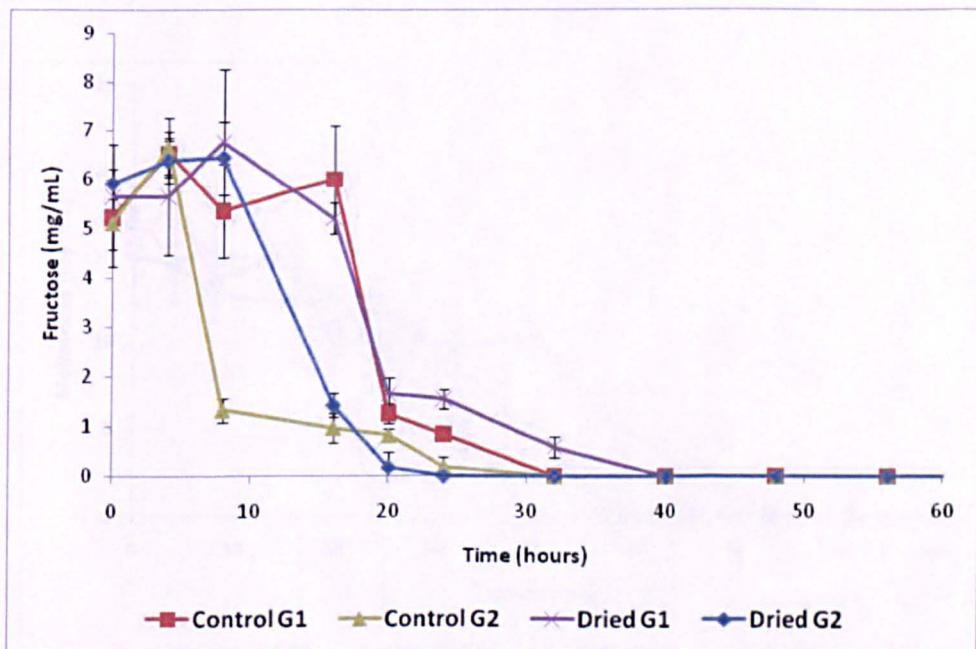


Figure 7.10. The presence of fructose in fermentation samples (assessed using HPLC analysis). Standard error of triplicate samples shown. Samples were taken every 8 hr (every four at the beginning of fermentation) and yeast removed through centrifugation. As fructose was absent in all fermentations after 40 hr, only early data points are shown.

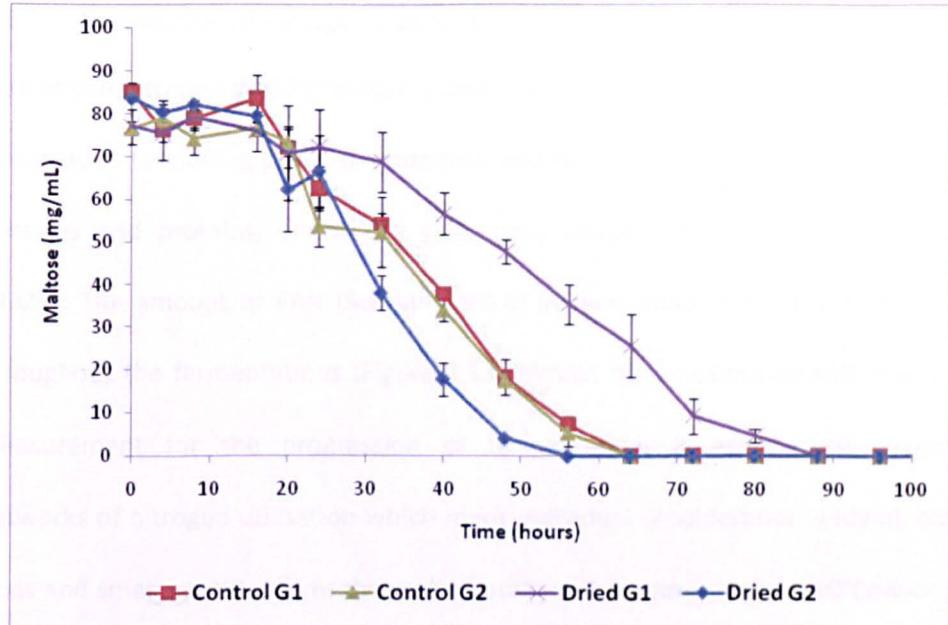


Figure 7.11. The presence of maltose in fermentation samples (assessed using HPLC analysis). Standard error of triplicate samples shown. Samples were taken every 8 hr (every four at the beginning of fermentation) and yeast removed through centrifugation. As maltose was absent in all fermentations after 96 hr, only data points up-to this time are shown.

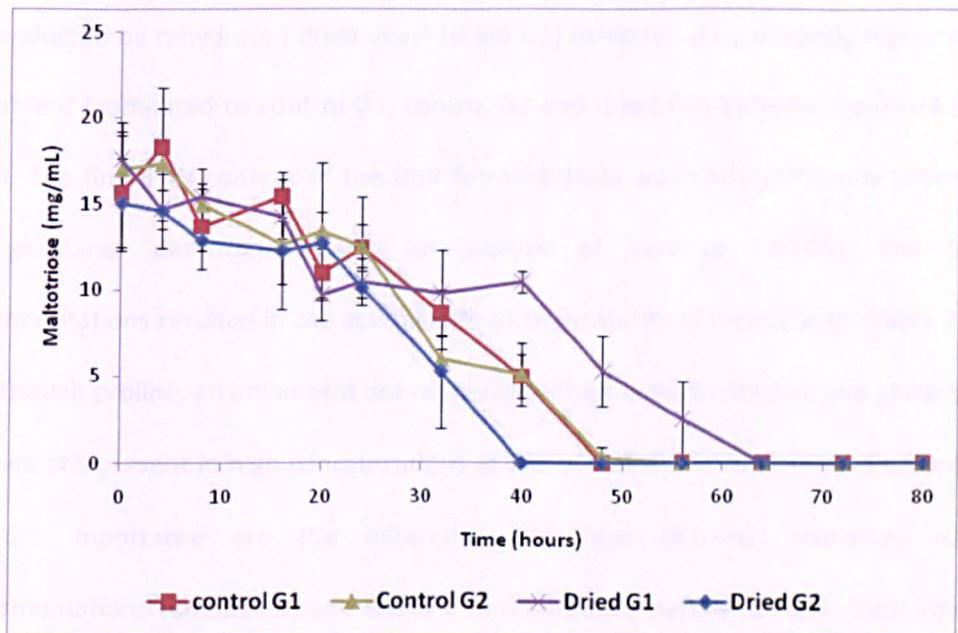


Figure 7.12. The presence of maltotriose in fermentation samples (assessed using HPLC analysis). Standard error of triplicate samples shown. Samples were taken every 8 hr (every four at the beginning of fermentation) and yeast removed through centrifugation. As maltotriose was absent in all fermentations after 80 hr, only data points up-to this time are shown.

#### **7.2.4 Assimilation of nitrogen during fermentation**

Free amino nitrogen (FAN) provides a convenient measure of the available nitrogen, amino acids (excluding proline), ammonia, and to some extent,  $\alpha$ -amino nitrogen in peptides and proteins, which the yeast may utilise during fermentation (ASBC, 1992b). The amount of FAN (Section 2.10.4) in fermentation broth was measured throughout the fermentations (Figure 7.13). Whilst the presence of FAN is a useful measurement for the progression of fermentation, it encompasses complex networks of nitrogen utilisation which merit individual consideration. Indeed, amino acids and small peptides form the bulk of usable nitrogenous sources (O'Connor-Cox 1989). In this study the concentrations of the majority of physiologically important amino acids (arginine could not be detected using the protocol chosen) were determined.

Comparisons of the FAN assimilation (Figure 7.13) show that the fermentation conducted by rehydrated dried yeast (dried G1) exhibited a significantly higher FAN content (compared to control G1, control G2 and dried G2) between hours 24 and 64. The final FAN content of the four fermentations was not significantly different (significance determined using an analysis of variance  $P \leq 0.05$ ). The four fermentations resulted in the assimilation of the majority of amino acids (Table 7.2), although proline, an imino acid not normally utilised in fermentation, and glutamine were still present in high concentrations at the end of the fermentation. Perhaps of more importance are the differences observed between miniature scale fermentations (Chapter 6) and the 5 L fermentations described here. Final amino acid concentrations in miniature scale dried yeast fermentations were considerably higher for valine (21 %), alanine (35 %), phenylalanine (16 %), tyrosine (34 %) and tryptophan (37 %) compared to the final levels in 5 L fermentations using the same dried yeast (<8 %). The uptake kinetics of each amino acid demonstrate that the

initial pitching of dried yeast (Dried G1) was consistently slower than for the other three fermentations (Dried G2, Control G1 and Control G2) which show similar profiles to each other.

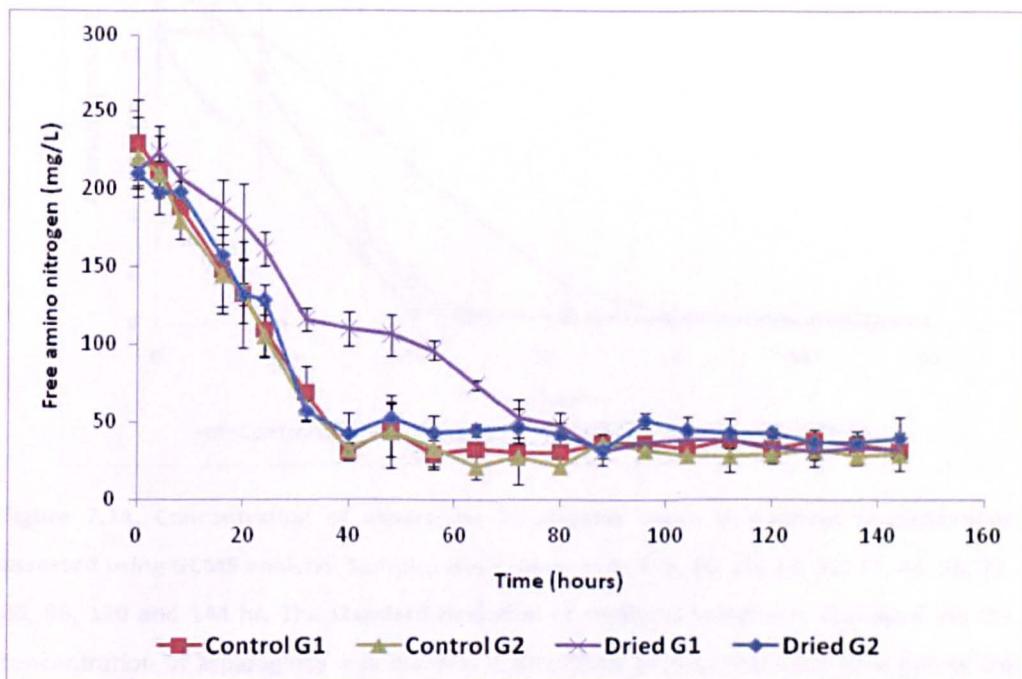


Figure 7.13. Free amino nitrogen assessed using the ninhydrin method. Samples were taken every 8 hr (every four at the beginning of fermentation) and yeast removed through centrifugation. The standard deviations of triplicate samples are shown.

## Class A

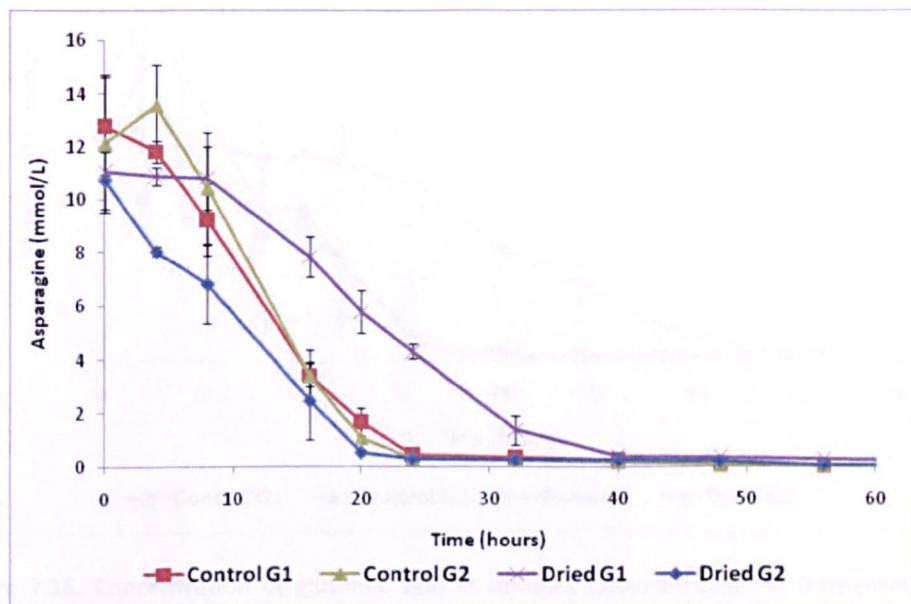


Figure 7.14. Concentration of asparagine in samples taken throughout fermentations assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of asparagines was minimal in later time points, only early time points are shown.

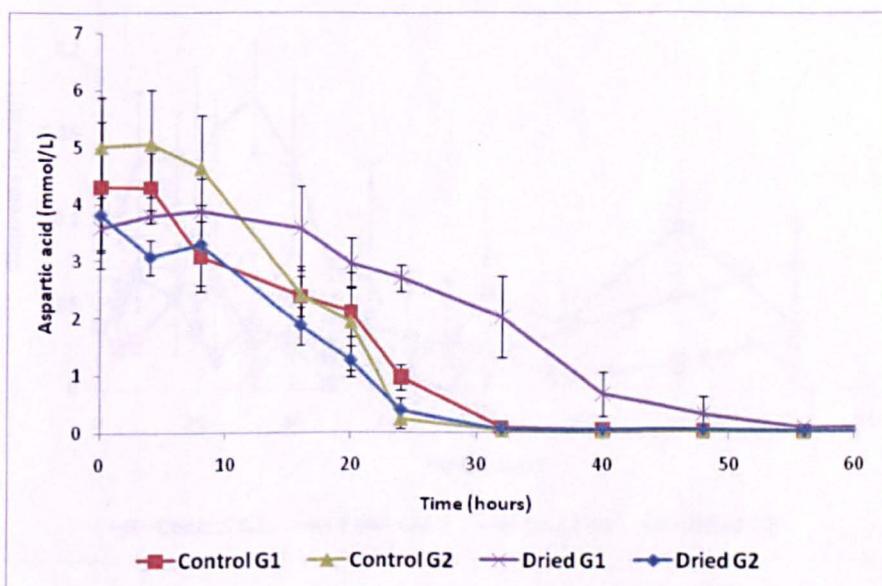


Figure 7.15. Concentration of aspartic acid in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of aspartic acid was minimal in later time points, only early time points are shown.

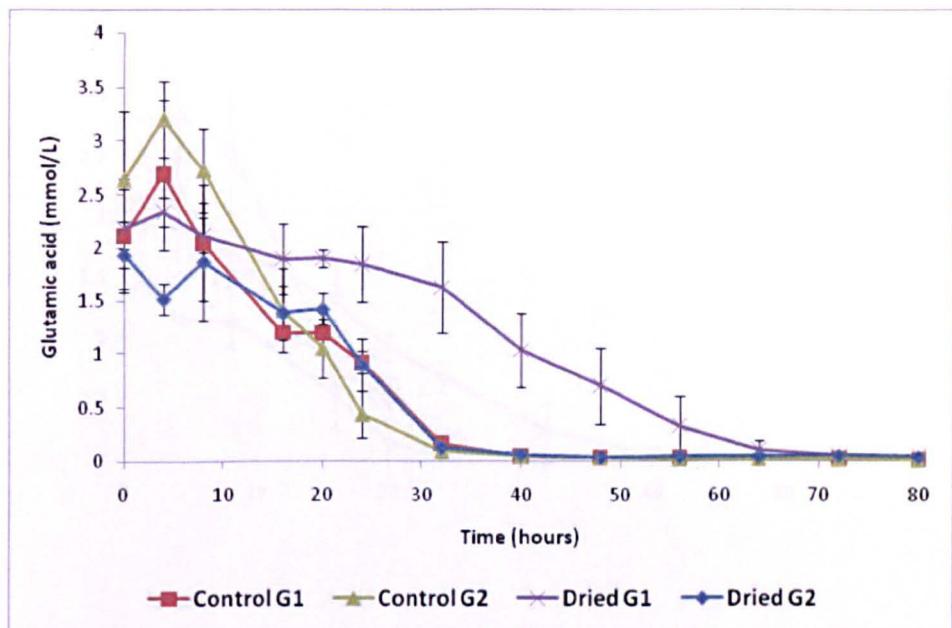


Figure 7.16. Concentration of glutamic acid in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of glutamic acid was minimal in later time points, only early time points are shown.

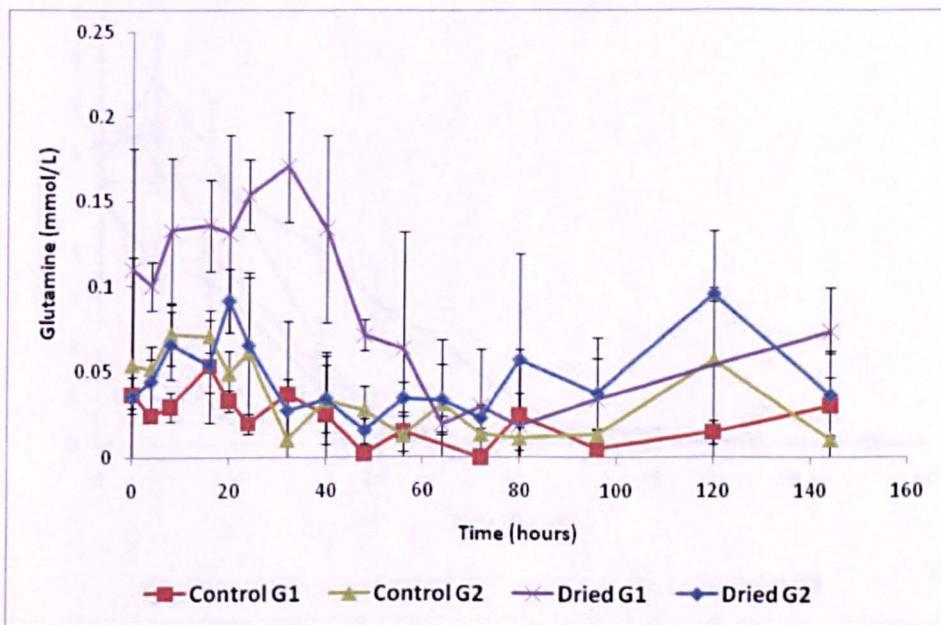


Figure 7.17. Concentration of glutamine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed.

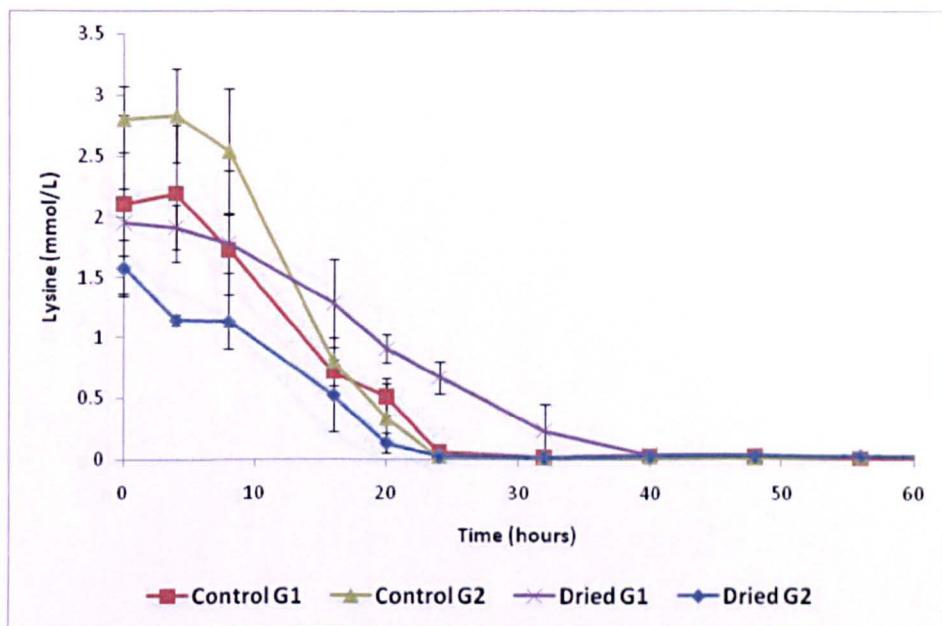


Figure 7.18. Concentration of lysine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of lysine was minimal in later time points, only early time points are shown.

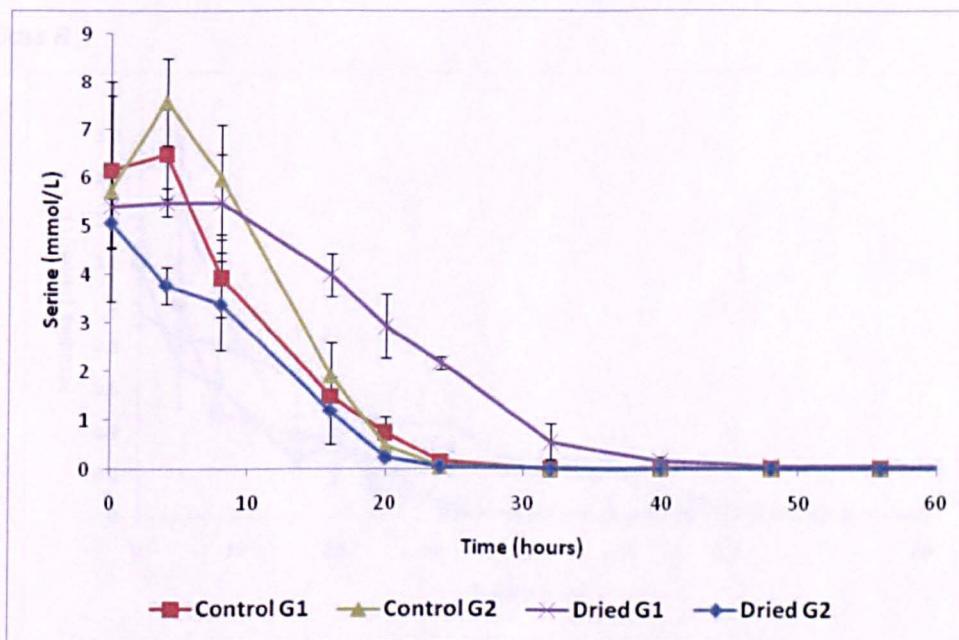


Figure 7.19. Concentration of serine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of serine was minimal in later time points, only early time points are shown.

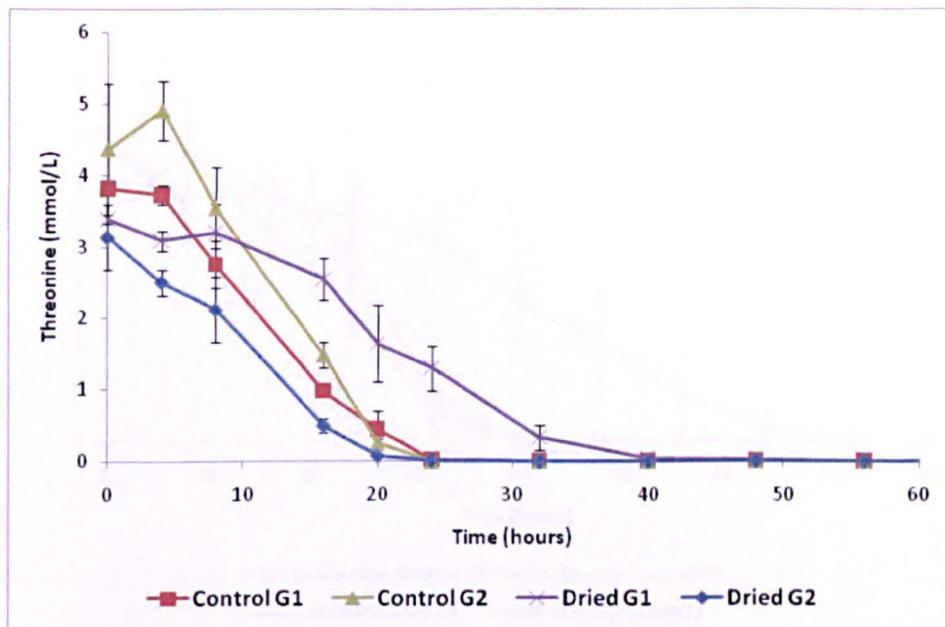


Figure 7.20. Concentration of threonine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of threonine was minimal in later time points, only early time points are shown.

#### Class B

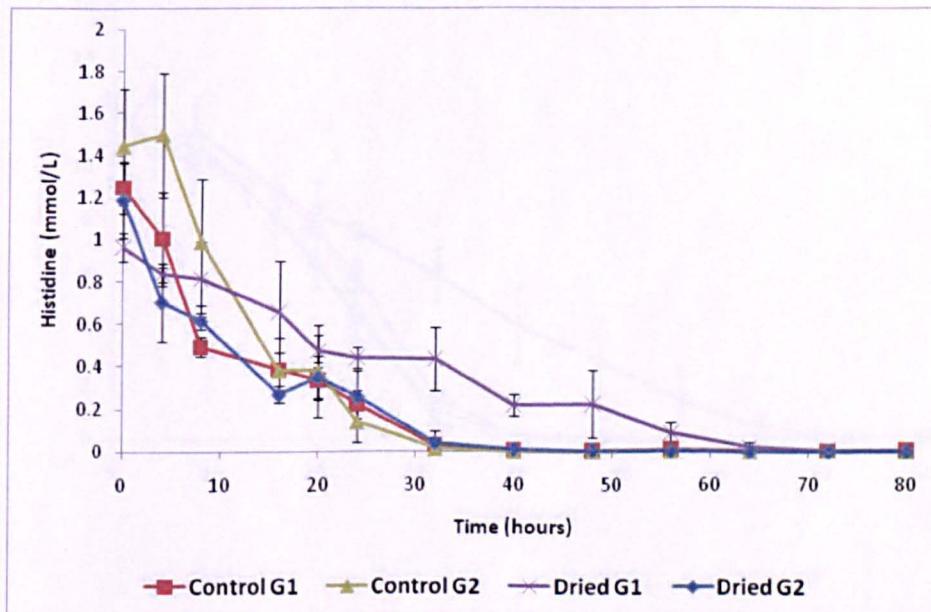


Figure 7.21. Concentration of histidine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of histidine was minimal in later time points, only early time points are shown.

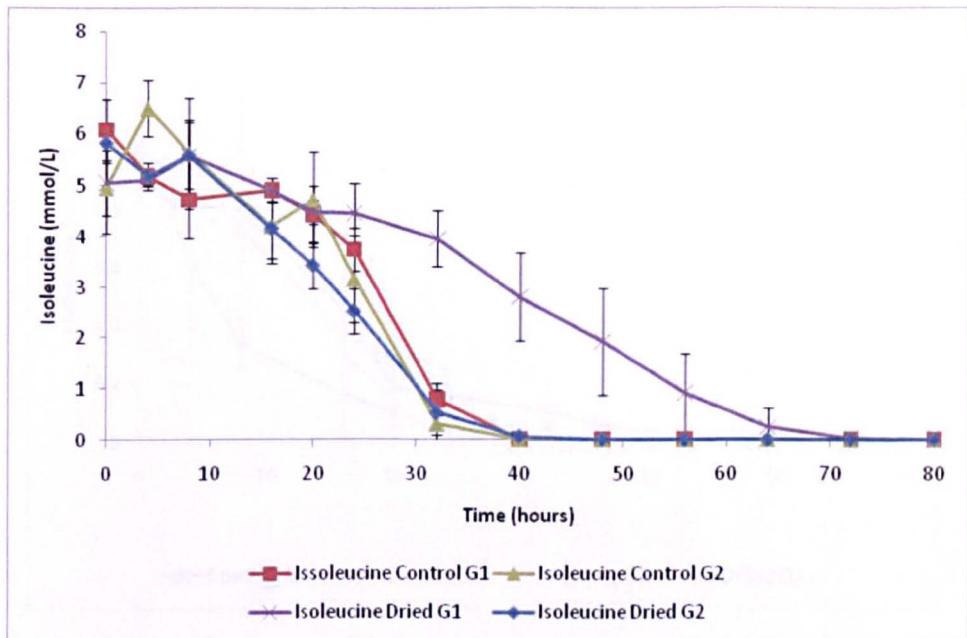


Figure 7.22. Concentration of isoleucine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of isoleucine was minimal in later time points, only early time points are shown.

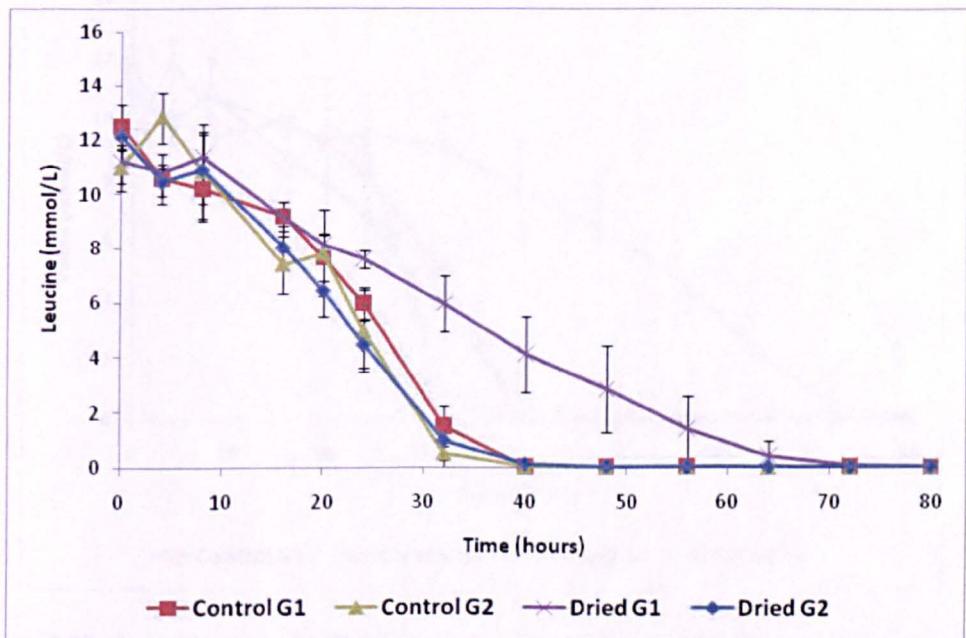
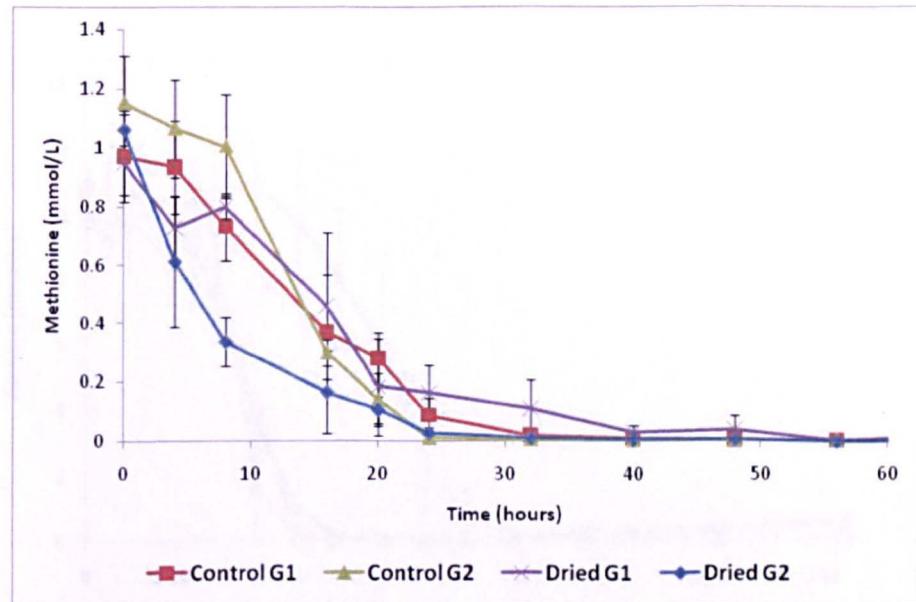
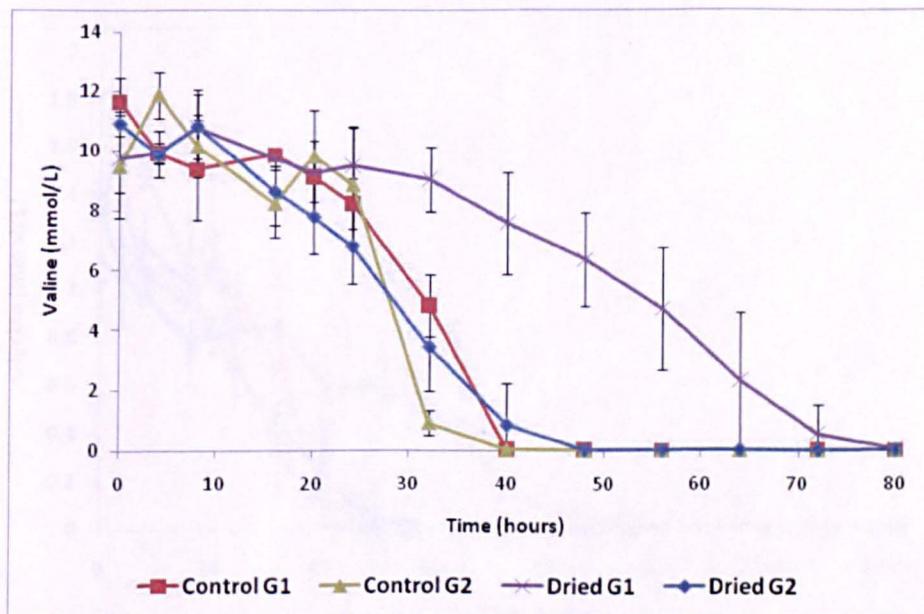


Figure 7.23. Concentration of leucine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of leucine was minimal in later time points, only early time points are shown.



**Figure 7.24.** Concentration of methionine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of methionine was minimal in later time points, only early time points are shown.



**Figure 7.25.** Concentration of valine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed. As the concentration of methionine was minimal in later time points, only early time points are shown.

### Class C

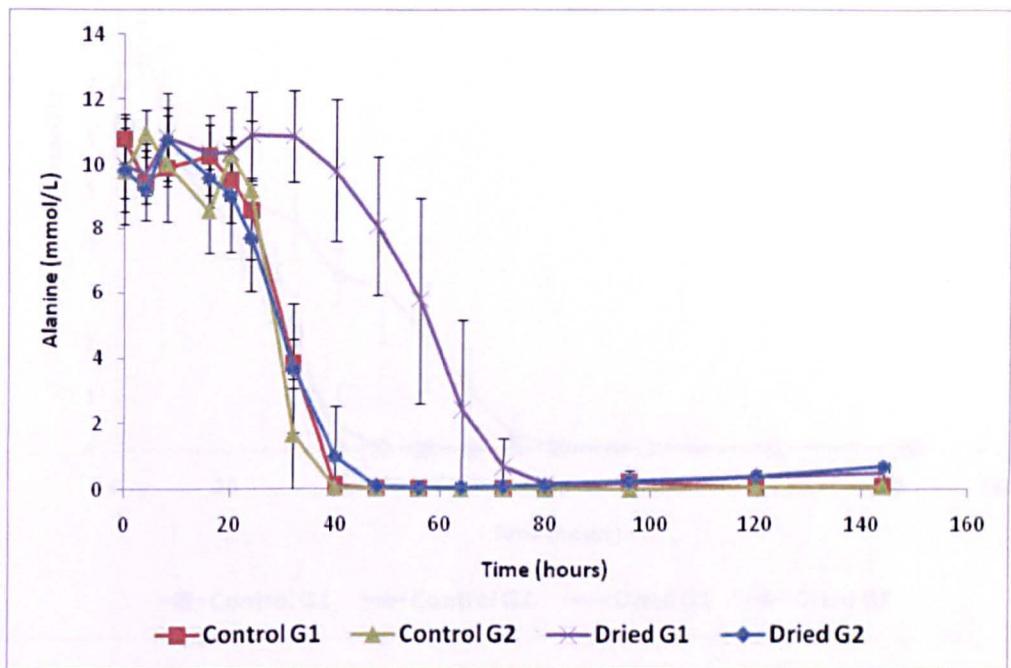


Figure 7.26. Concentration of alanine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed.

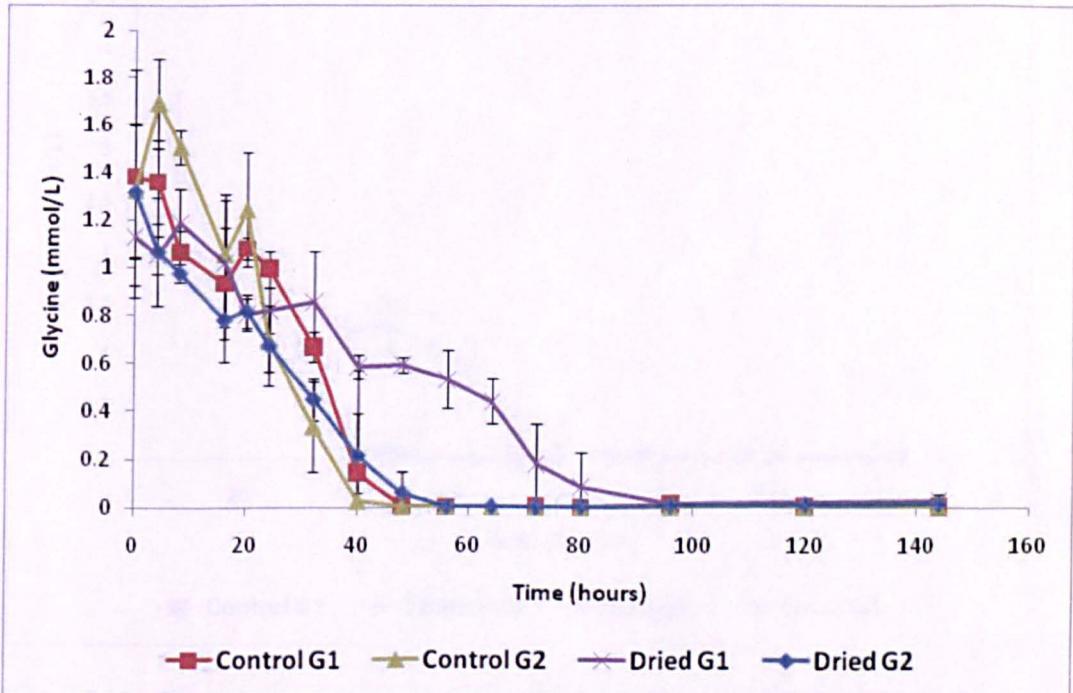


Figure 7.27. Concentration of glycine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed.

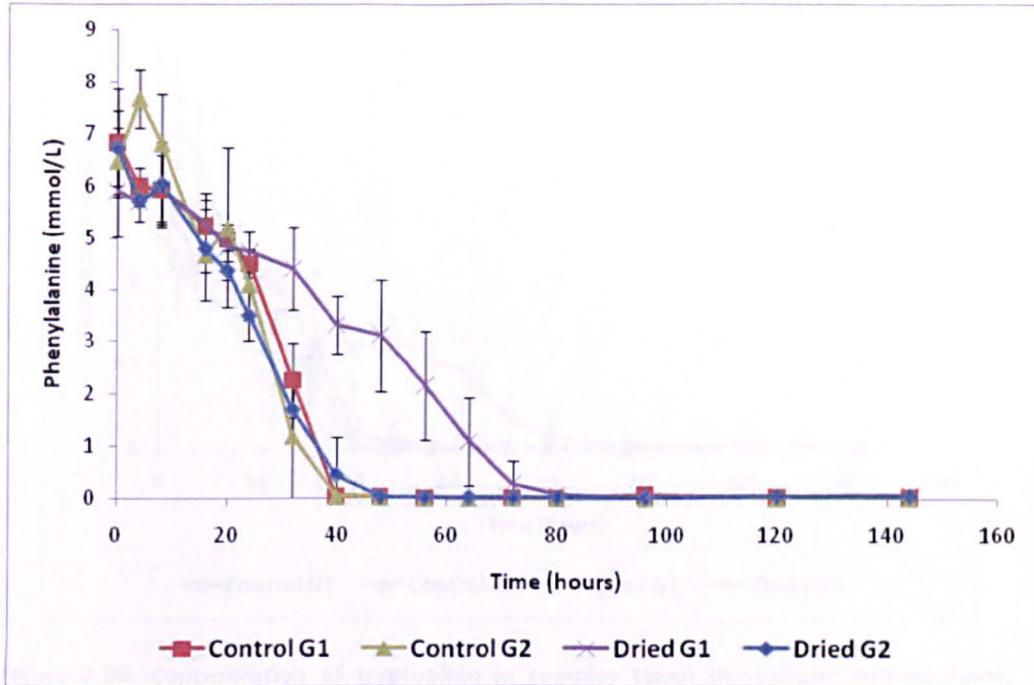


Figure 7.28. Concentration of phenylalanine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed.

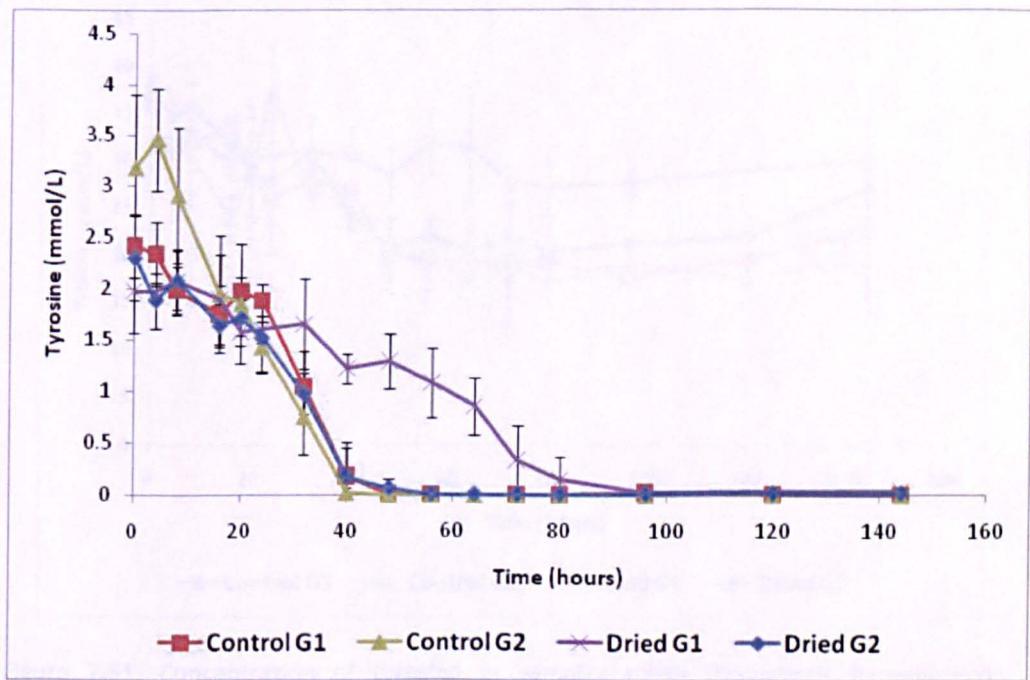


Figure 7.29. Concentration of tyrosine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed.

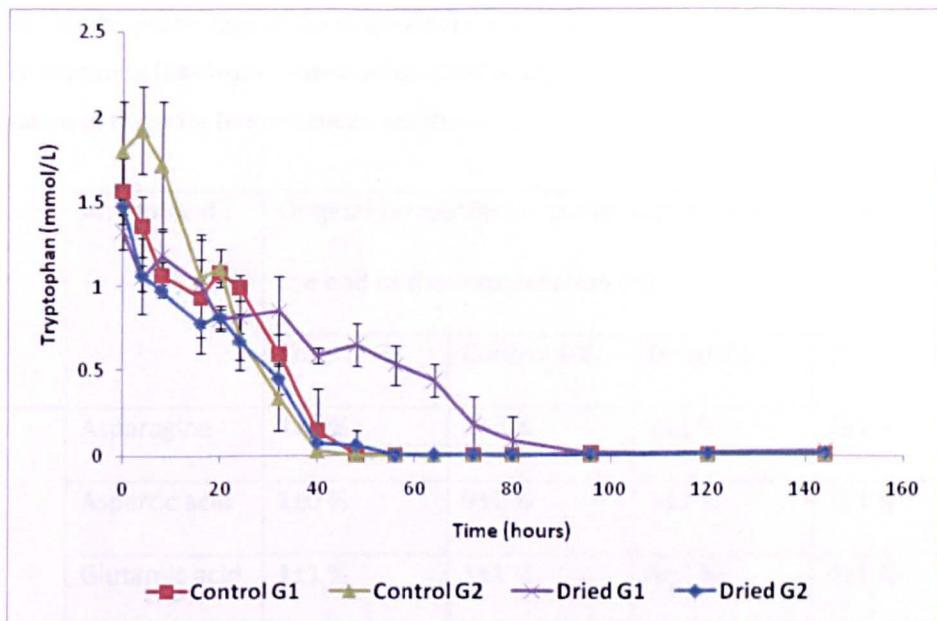


Figure 7.30. Concentration of tryptophan in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed.

#### Class D

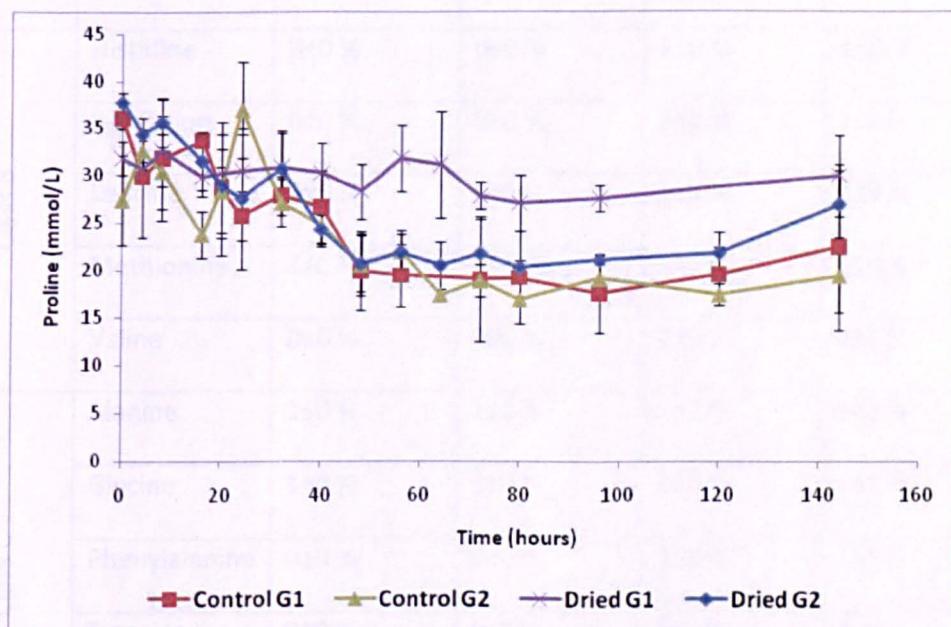


Figure 7.31. Concentration of tyrosine in samples taken throughout fermentations, assessed using GCMS analysis. Samples were taken at 0, 4, 8, 16, 20, 24, 32, 40, 48, 56, 72, 80, 96, 120 and 144 hr. The standard deviation of triplicate samples is displayed.

**Table 7.2. The percentage of the original concentration of amino acids remaining at the end of fermentation (144 hr), assessed using GCMS analysis. The mean percentage and standard deviation of triplicate fermentations are shown.**

	Amino Acid	Original fermentation content of amino acid remaining at the end of the fermentation (%)			
		Control G1	Control G2	Dried G1	Dried G2
Class A	Asparagine	1±0 %	1±0 %	2±1 %	2±1 %
	Aspartic acid	1±0 %	0±0 %	3±1 %	2±1 %
	Glutamic acid	1±1 %	1±1 %	5±2 %	4±1 %
	Glutamine	82±5 %	19±5 %	66±23 %	81±70 %
	Lysine	1±1 %	1±0 %	1±0 %	1±0 %
	Serine	0±0 %	0±0 %	0±0 %	0±0 %
	Threonine	0±0 %	0±0 %	0±0 %	0±0 %
Class B	Histidine	0±0 %	0±0 %	1±0 %	1±0 %
	Isoleucine	0±0 %	0±0 %	2±0 %	2±0 %
	Leucine	0±0 %	0±0 %	2±0 %	1±0 %
	Methionine	1±0 %	0±0 %	3±1 %	2±0 %
	Valine	0±0 %	0±0 %	2±1 %	2±1 %
Class C	Alanine	1±0 %	1±1 %	5±2 %	8±3 %
	Glycine	1±0 %	1±0 %	3±2 %	1±1 %
	Phenylalanine	0±0 %	0±0 %	1±1 %	1±1 %
	Tyrosine	0±0 %	0±0 %	2±2 %	1±1 %
	Tryptophan	0±0 %	0±0 %	2±2 %	1±1 %
Class D	Proline	62±24 %	60±14 %	94±14 %	79±9 %

### **7.2.5 The “potential” concentration of the flavour active compounds diacetyl and 2,3 pentanedione during fermentation**

The differences in nitrogen metabolism exhibited by dried yeast in this study (Chapter 6), and others (Cyr *et al.*, 2007) have been associated with abnormal profiles of flavour active compounds, such as vicinal diketones (VDKs). In addition to the determination of diacetyl concentration (Figure 7.32), the presence of 2,3-pentanedione was also monitored (Figure 7.33). These vicinal diketones exhibit low flavour thresholds in beer, 0.15 ppm and 0.9 ppm respectively (Meilgaard, 1975).

Dried G1 yeast displayed differing profiles of both diacetyl and 2,3-pentanedione compared to fermentations conducted either with control or repitched control or dried yeast. The fermentation profile obtained when using dried yeast (Dried G1) showed consistently high levels of diacetyl (Figure 7.32). It demonstrated a peak that was more than four times that of the control fermentation and, crucially, finished on the 6th day of fermentation with a diacetyl value of 0.49 ppm, which is greater than the compound’s flavour threshold (0.15 ppm). This observation supports those presented in Chapter 6 and in a previous study conducted by other researchers (Cyr *et al.*, 2007). When repitched, dried yeast displayed a comparable diacetyl profile to that of control fermentations, indicating that poor performance was not maintained.

Unlike diacetyl, levels of 2,3-pentanedione at the end of the 6 days of fermentation were below the flavour threshold in all dried and control fermentations (Table 7.3), with dried yeast exhibiting a significantly lower level than that obtained during fermentations with control yeast (significance assessed using an analysis of variance  $P \leq 0.05$ ). Interestingly the peak of this flavour compound occurred later in dried yeast G1 fermentation, an observation not previously reported and potentially linked to delayed completion of amino acid assimilation.

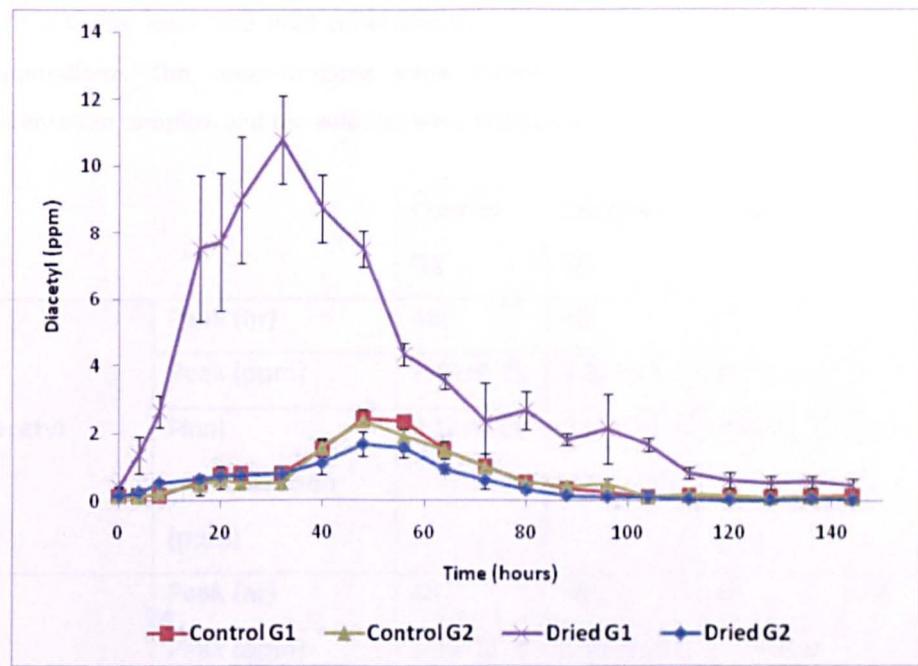


Figure 7.32. The presence of diacetyl in fermentation samples taken every 8 hr (every four at the beginning of fermentation). Volatiles were isolated using headspace sampling and subsequently separated and identified using GCMS. Standard deviation of triplicate samples shown.

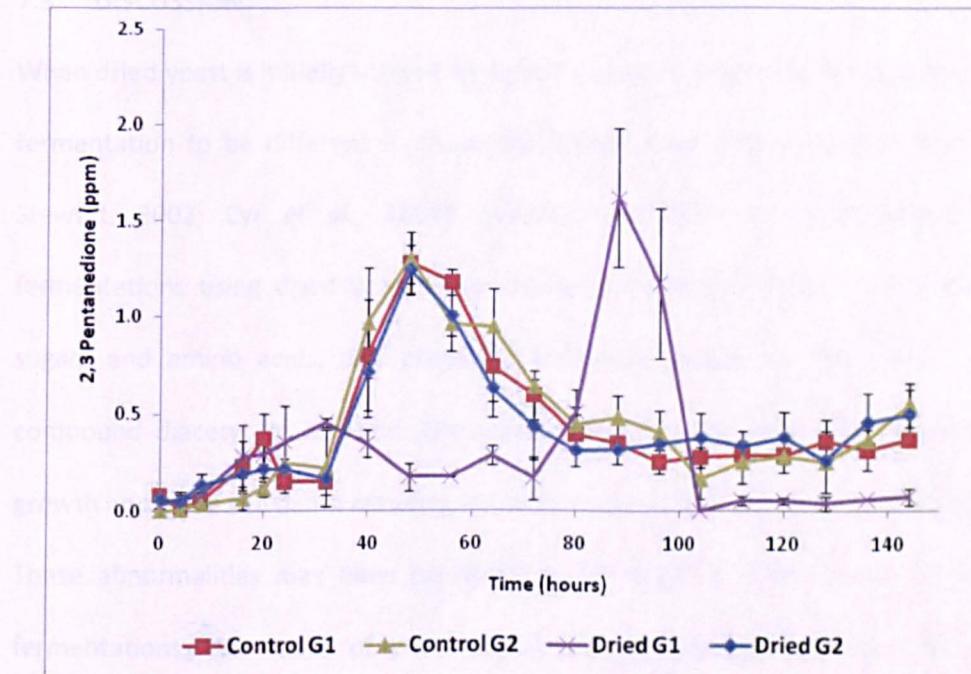


Figure 7.33. The presence of 2,3 Pentanedione in fermentation samples taken every 8 hr (every four at the beginning of fermentation). Volatiles were isolated using headspace sampling and subsequently separated and identified using GCMS. Standard deviation of triplicate samples shown.

**Table 7.3. The peak and final concentrations of the vicinal diketones diacetyl and 2,3 pentanedione. The concentrations were measured by sampling the headspace of fermentation samples, and the volatiles were isolated and identified using GCMS analysis.**

		Control G1	Control G2	Dried G1	Dried G2
Diacetyl	<b>Peak (hr)</b>	48	48	32	48
	<b>Peak (ppm)</b>	2.43±0.25	2.36±0.13	10.8±1.32	1.68±0.36
	<b>Final concentration (ppm)</b>	0.14±0.08	0.12±0.12	0.49±0.18	0.14±0.03
2,3 Pentanedione	<b>Peak (hr)</b>	48	48	88	48
	<b>Peak (ppm)</b>	1.35±0.16	1.35±0.13	1.62±0.36	1.25±0.12
	<b>Final concentration (ppm)</b>	0.36±0.02	0.54±0.11	0.07±0.04	0.49±0.13

### 7.3 DISCUSSION

When dried yeast is initially utilised for fermentation, it is common for aspects of the fermentation to be different to those the brewer may normally expect (Finn and Stewart, 2002; Cyr *et al.*, 2007). Indeed, in Chapter 6 it was shown that fermentations using dried yeast were slower with respect to the assimilation of sugars and amino acids, and produced abnormal profiles of the flavour active compound diacetyl. In addition, the population dynamics were altered, with less growth and more cell death resulting in fewer viable cells at the end of fermentation. These abnormalities may have ramifications for the use of this yeast in further fermentations. The reuse of yeast populations in subsequent fermentations, a practice termed repitching, is common in breweries and therefore assessment of performance of dried yeast during serial repitching is important to the brewer.

### **7.3.1 Changes in cell yield and viability when dried yeast is repitched**

When fermentations are repitched there is normally an excess of yeast product from the preceding fermentation. Indeed this was the case for control yeast which provided a final concentration of  $1.5 \times 10^8$  viable cells/ml, whereas dried yeast produced only  $3.3 \times 10^7$  cells/ml. This meant that a high rate of recovery of yeast cells was required to ensure sufficient yeast for the next fermentation.

During industrial fermentations the majority of lager yeast would be expected to form aggregates which then accumulate in the bottom of the fermentation vessel, these are not all routinely recovered. The concentration of trub is often greatest at the bottom, thus this section is often avoided when selecting yeast for repitching (Boulton and Quain, 2001). Warm cropping may also be applied, in which case only a portion of yeast for repitching is recovered prior to the diacetyl stand which requires some yeast to remain in the fermenter (Powell *et al.*, 2004). With the limited cell growth demonstrated by dried yeast during its first use, and depending on the choice of cropping regime, sufficient recovery of yeast may not be possible in industrial brewery conditions potentially making dried yeast unsuitable for repitching.

It is important to note that these results may not be typical for dried yeast. In a separate study, by Powell and Fischborn (2010), it was suggested that although dried yeast may initially begin with a low percentage viability, this can be recovered by the end of the first fermentation and remain high (>90 %) in subsequent fermentations, providing sufficient yeast for repitching (Powell and Fischborn, 2010). Whilst Finn and Stewart (2002) demonstrated similar profiles between dried and control yeast regarding the number of cells in suspension, indicating comparable growth. The difference in population dynamics demonstrated in this study, compared to previous ones, may be an indication that the yeast was damaged, possibly due to inadequate

storage conditions or oxygen ingress. Differences may also reflect variations in strain, batch or fermentation conditions. Indeed, the change in fermentation parameters from the miniature scale fermentation system (Chapter 6) to 5 L fermentations in bioreactors described here resulted in an increase in cell growth from  $2.6 \times 10^7$  cells/ml (miniature scale) to  $3.3 \times 10^7$  cells/ml (5 L) despite the same batch of dried yeast being utilised (significance determined using an analysis of variance). This increased growth may result in the complete utilisation of amino acids seen in the first use of dried yeast in 5 L fermentations (Section 7.2.4), compared to the high residual concentrations observed in miniature scale fermentations (Chapter 6).

The main purpose of using 5 L bioreactors was to enable collection of yeast after fermentation was complete to allow subsequent repitching. Scaling up the fermentations from 100 ml volume to 5 L resulted in the adjustment of several other parameters, perhaps most relevant to these current findings was oxygenation of the wort. Miniature scale fermentations were agitated in the presence of air for 24 hr, although the exact level of oxygenation was not measured. The bioreactors were oxygenated to approximately 18 ppm, to replicate typical brewery regimes. This potential disparity in oxygen levels in the two scales of fermentations deployed within the thesis may explain the differences in cell cycle and nitrogen utilisation observed.

Oxygen is required for the synthesis of sterols and other unsaturated fatty acids (David and Kirsop, 1973), which are integral for membrane composition (discussed in chapter 5). After this synthesis early in fermentation, there is no new addition of sterols to the yeast population. When budding occurs, sterols are split between the new cells reducing the sterol concentration each time. As sterol concentration becomes limited, cell growth is reduced or stalled (Straver *et al.*, 1993). Differences in

sterol content of yeast prior to pitching have been discussed earlier, but differences in the preliminary stages of fermentation were not probed. The higher concentration of oxygen in the 5 L fermentations may have resulted in more sterol synthesis in the yeast and, consequently, higher cell growth which was seen in both control and dried yeast compared to miniature scale fermentations (Sections 6.2.1 and 7.2.1). Despite increased oxygen alleviating some of the growth issues observed with dried yeast, the issue of poorer growth persisted and the fundamental cause is unknown.

As well as reduced growth, there was also a high level of cell death in the dried yeast fermentations. This cell death may be related to the increased residual budding index, potentially caused by the lack of cell separation during mitosis. Although the cause of this is unclear, some cell death was observed in budding cells. The cell cycle, the term given to the series of budding events of *S. cerevisiae*, is highly regulated with the expression of approximately 800 genes varied during its progression (Spellman *et al.*, 1998). START (Hartwell *et al.*, 1970; Hartwell, 1974) is the point during the cell cycle at which the yeast cell is committed to completing mitosis (cell division). START is only initiated once a cell has completed its previous cell-cycle (Hartwell, 1974), attained a certain size (Hartwell and Unger, 1977; Johnston *et al.*, 1977) and if there are sufficient nutrients available (Hartwell, 1974). Once START has been initiated, division must be completed regardless of any change in environmental conditions which may occur (Wheales, 1987). If the conditions required for START are not met, then no division occurs; instead the cells enter a stationary phase, referred to as G0 (Werner-Washburne *et al.*, 1996). Given the multiple checkpoints or safeguards which are in place within the cell cycle it would seem that the death caused is necrotic and therefore could be attributed to substantial and rapid cell damage. Although it is not clear what causes this cell

damage, it is clear that the dried yeast, or at least a proportion of the population, was more susceptible to fermentation stress than the control yeast.

### 7.3.2 Persistence of undesired fermentation traits when dried yeast is reused

Fermentation performance is a function of generation number, although the extent of the impact is strain dependent (Jenkins *et al.*, 2003; Powell and Diacetis, 2007).

The repitching of dried yeast which has already performed poorly in its first fermentation raises the question of whether the yeast has irreparable damage or could recover a more typical fermentation performance.

Not all the traits which were exhibited during the initial use of dried yeast previously (Chapter 6) were repeated in the initial dried yeast fermentation described here. The incomplete uptake of some amino acids, which was observed during mini-fermentations (Section 6.2.4), was not observed in larger scale fermentations. It is likely that this is due to the increased growth discussed in Section (Section 7.3.1). Despite this observation, G1 dried yeast fermentations did exhibit a lag in attenuation, assimilation of FAN, amino acids, sugars and the production of ethanol. Furthermore, G1 dried yeast fermentations exhibited higher diacetyl levels, and an altered profile of 2,3-pentanedione formation. The reasons for these observations are unclear (discussed in Chapter 6), although serial repitching minimises these phenotypes with G2 dried yeast fermentations exhibited profiles which were comparable with control yeast G2 fermentations. This is despite the lower number of viable cells found at the end of the G2 dried yeast fermentation.

Whilst brewing yeast is often referred to as if it is a homogenous cell population, it is in fact distinctly heterogeneous (Porro *et al.*, 1997; Minois *et al.*, 2009). These varying phenotypes within the population can determine the extent of a cell's resistance to certain stresses (Summer *et al.*, 2003). It is possible that the stresses

involved in drying, rehydration and fermentation acted as a selective force (substantial cell death was recorded). The subsequent yeast population may be better equipped to cope with fermentation.

#### 7.4 CONCLUSIONS

It is suggested that, despite potentially problematic initial G1 fermentations, dried yeast recovers to yield comparable G2 fermentations. The key issue for brewers then will be that G1 fermentations may be impaired and crop yields from G1 may be insufficient for serial repitching. There are obvious benefits to the use of dried yeast and as the associated problems appear to be transient, and limited to G1 fermentations, the potential of ADY is clear. One possible route to its deployment would be to use dried yeast as an inoculum to pitch propagation vessels in the brewery. Employing dried yeast as an inoculating culture for propagation vessels rather than for direct pitching has been investigated previously (Reckelbus *et al.*, 2000) and would still provide a substantial saving in time over propagation. Alternatively, the beer produced during the first use of dried yeast could be blended with other batches to generate a more acceptable product, a common practice in some breweries. It is also important to note that this study has investigated a single batch of dried lager yeast and for the fermentation analyses only one strain has been assessed. The differences in the literature regarding dried yeast utilisation for fermentation may represent differences in protocol of rehydration and fermentation or indeed differences relating to strain.

## CHAPTER 8: CONCLUSION AND FUTURE WORK

In the brewing industry it is standard practice to propagate a pure yeast culture and inoculate (pitch) it into the fermentation vessel. Yeast is then recovered from fermentation, once it is complete, and reused in subsequent fermentations (known as serial repitching) until a decline in performance occurs or the required number of successive fermentations has been conducted. Propagation is currently required to initiate the entire process again. However propagation takes time, and therefore must be scheduled, and requires resources in the form of additional equipment, energy and water inputs. It has long been proposed that Active Dried Yeast (ADY) offers an alternative method of yeast supply with the possibility of pitching into fermentation after a short rehydration period, obviating the requirement for brewery onsite propagation. Adoption of this innovation by the brewing industry has been low because of perceived issues with the fermentation performance of ADY, hygiene and strain availability. Reported studies into the use of dried yeast for brewing (Finn and Stewart, 2002; Cyr *et al.*, 2007; Powell and Fischborn, 2010) acknowledge that ADY fermentations differ from control fermentations. However, these observations centred in most cases on different attributes that comprise key fermentation performance indicators. The aim of this thesis was to address this issue by investigating a series of fermentation key performance indicators to establish why ADY performed in a different manner to control yeast. Key performance indicators including viability (Chapter 3), genomic stability (Chapter 4), membrane integrity (Chapter 5), yeast growth, attenuation, uptake of wort nutrients and aspects of flavour development (Chapters 6 and 7) were assessed.

ADY requires rehydration before use and it has been demonstrated that viability is impaired in these slurries (Finn and Stewart, 2002; van den Berg and Van Landschoot, 2003; Powell and Fischborn, 2010). In the current study, the extent of

viability loss in one lager strain (LAL1) and two ale strains (LAL2 and LAL4) of industrially prepared ADY was investigated during rehydration. Viability was dependent on the duration and temperature of rehydration. The extent of loss of viability was strain dependent. This observation demonstrates that significant damage occurs during the industrial production and utilisation of the yeast, but the mechanism and extent of this damage is unclear. The findings presented in Chapter 3 supported earlier reports indicating that rehydration temperature of ADY can affect the percent viability of a culture (Gosselin and Fels, 1998). It was proposed that loss in viability could occur as a result of genetic instability or membrane damage (Chapter 3). To test this hypothesis mitochondrial and genomic DNA integrity (Chapter 4) and changes in membrane fluidity, sterol content and membrane functional integrity (Chapter 5) were assessed. Using a combination of analyses the genomic and mitochondrial (mt) DNA was shown to be comparable to that extracted from control yeast. However, the tolerance of mtDNA to ethidium bromide mutagen challenge appeared to differ, with ADY populations demonstrating a reduced sensitivity to petite formation when compared to control populations. The reasons for this difference are not known, although the plasma membrane has been implicated in the capacity to restrict ethidium bromide assimilation into the cell (Brunner *et al.*, 1982; Coote *et al.*, 1994) and therefore one possible cause of the differences observed could be membrane integrity. The plasma membrane is a component subject to damage when yeast are submitted to osmotic stress (Simonin *et al.*, 2007b).

An analysis of plasma membrane fluidity, sterol content and integrity was conducted (Chapter 5) and it was observed that plasma membranes, and in particular fluidity, are affected by dehydration and rehydration although the extent of these differences were strain dependent. Although the exact causes and mechanisms by which these

changes occur are unclear it is suggested that the non-viable cells in ADY populations may exhibit altered fluidity, contributing to the overall differences observed. This poses the question whether these ADY non-viable yeast cells exhibit changes in fluidity as a consequence of cell death alone or in part because of the impact that dehydration and/or rehydration has on the membrane.

LAL1, the only lager strain investigated, emerged from the viability and membrane assessments as potentially less fit-for-purpose than the two ale strains LAL2 and LAL4. It exhibited a reduced viability and was more susceptible to ethanol and SDS stress. Whilst not studied within the current investigation, it is proposed that tolerance of the membrane to the combination of fermentation stresses that are considered particularly challenging to membrane integrity, such as ethanol and osmotic stresses might be reduced in certain ADY populations. It is further proposed that this should be the focus of further investigations.

Whilst the stress response of the plasma membrane was not directly assessed during fermentation, key yeast performance indicators were followed, which indicated ADY was impaired. A lag in cell division, attenuation and sugar and amino acid uptake were noted. Overall cell division was also reduced (compared to control yeast) and the viability of the yeast culture remained low. Residual amino acids pools at the end of fermentation were observed under certain fermentation conditions. Diacetyl formation occurred more rapidly and end fermentation diacetyl levels were higher for ADY. These differences in performance between ADY and control strains did not appear to be due to differences in yeast pre-growth conditions compared to typical propagation regimes.

Aspects of ADY performance were dependent on the fermentation assay applied. In larger scale fermentations the end fermentation amino acid pool was depleted. This

may have been a result of increased growth, although this was still impaired compared to control fermentations. Although the first fermentation (G1) conducted by ADY appeared to differ from control fermentations this different phenotype was not maintained during serial repitching. Indeed most aspects of ADY fermentation performance matched the control during the second fermentation (G2), despite viable cell yield remaining impaired compared to control yeast. This may indicate the remaining viable cells from the ADY populations were performing more efficiently in fermentation. It is proposed that ADY could be utilised to replace freshly propagated yeast, but direct addition to fermenters may require an improvement of performance during the first fermentation or the adjustment of fermentation parameters to accommodate the intricacies of the yeast.

### **8.1 FUTURE WORK**

This thesis has focused on the dynamics of the yeast cell during rehydration and fermentation, as this is one of the key aspects which have made some brewers reluctant to utilise the technology. Another, the level of contamination in the product, has not been directly assessed here. While the presence of bacteria or wild yeast may impact on the fermentation data presented, fermentation characteristics directly associated with the presence of bacteria (such as off flavours), or with wild yeast (such as super-attenuation), were not observed. Furthermore, ADY product specifications suggest that levels of contamination compare favourably with propagated yeast (Quain, 2006), whilst the absence of hops in the propagation medium may result in increased sensitivity during fermentation, leading to rapid death of any bacterial cells present (van den Berg and Van Landschoot, 2003). Consequently it is unlikely that microbial contamination issues influenced the data presented here. Despite this, further studies into the precise levels of contamination in ADY, persistence through fermentations and effect on the quality of beer

produced may provide an interesting insight into the fermentation performance of dried yeast strains and provide greater reassurance regarding this issue for brewers.

During the current study, only a few ADY strains were assessed and only one lager strain was included, the only strain assessed during fermentation. It therefore cannot be concluded that the data presented in this thesis would be representative of all ADY strains. Indeed differences between the three strains have been highlighted throughout this thesis, whilst differences between batches of ADY were also noted. It is proposed that this work be replicated in other lager and ale ADY strains to establish which changes in phenotype are strain specific and which are more generic.

It could be argued that it might be feasible to select strains for ADY production that would inherently tolerate dehydration and rehydration, or more rapidly recover to exhibit more typical fermentation performance attributes. Indeed work is already being conducted identifying sub groups of *S. pastorianus* (Saaz and Frohberg) which demonstrate greater stress resistance (Layfield *et al.*, 2011; Powell, Personal communication). Should the membrane be identified as the key site of cellular damage during dehydration or rehydration, strains should be sought which are known to be particularly sturdy with regard to this component. The identification of strains more suited to drying may mean that some established brewing, strains selected on the basis of their suitability to early fermentation and yeast handling procedures employed by the industry, are not suited to ADY processing.

In conjunction with this search for different strains, production specifications should be altered to ready the yeast for the rigors of drying. Steps are already taken to increase the stress resistance of yeast by encouraging the accumulation of the protectant trehalose and key membrane component ergosterol. Cells are also dried when they are in stationary phase as they exhibit greater stress resistance at this

point, but it is likely that greater protection could be achieved. Optimisation of trace elements in the growth process, for example iron, copper and zinc may further protect the plasma membrane as they play a role in maintaining fluidity (Garcia *et al.*, 2005). It has also been shown that wine yeast rehydrated in the presence of sterols have an improved fermentation capacity (Soubeyrand *et al.*, 2005), this could be trialled with regard to brewing yeast.

An attempt should also be made to identify the yeast cells within populations which are prone to cell death. Heterogeneity with respect to cell age, size and generational differences may also reflect sub-populations unable to tolerate certain conditions. For ADY this could be manifested in cell death or indeed a reduced rate of progression through the cell cycle or capacity to exhibit typical fermentation performance. Knowledge of these groups may enable further production changes to limit cell death.

## **8.2 FINAL REMARKS**

The potential for ADY in the brewing industry is clear, however, the work completed in this thesis suggests that it is not yet ready for deployment as a replacement of brewery propagation yeast. Immediate use may require acceptance of varying traits or use of a different yeast strain. However, in the long term, strain and production optimisation may alleviate the issues raised in this thesis permitting proprietary strains to be utilised as ADY.

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