THE IMPACT OF YEAST TEMPERATURE PRE-TREATMENT ON BIOETHANOL FROM CORN MASH FERMENTATION

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Abstract

For commercial corn mash to ethanol production it is known that increasing temperature can maximize ethanol yield, although care must be taken to avoid causing heat shock resulting in the death of the yeast culture. Despite the potentially negative effects of high temperature, short sub-lethal stress has been reported to procure a benefit to yeast cells. However, the effect of such yeast pre-treatment on bioethanol fermentations has not previously been investigated.

In order to understand more about the effects of sub-lethal heat treatment on yeast health and performance during corn mash fermentation, the tolerance of four industrial ethanologenic yeast strains to heat stress was determined. Consequently a miniature fermentation system was developed and two of the yeast strains, displaying different responses to temperature, were subjected to a variety of heat pre-treatments and analysed for their subsequent fermentation characteristics. It was noticed that although pre-treatment of yeast cells with heat did not lead to increased levels of the anti-stress compound trehalose, typically cultures exhibited improved sugar utilisation and viability post-fermentation. In addition, for strain LAL7 this also had the effect of increasing ethanol output, while for strain Thermosacc, ethanol yield was not significantly affected. Interestingly initial experimentation had indicated that Thermosacc was more thermotolerant than LAL7 and consequently it is suggested that although ethanol yield may be increased by heat pre-treatment, it may also be determined by the individual response of different strains to stress, or combinations of stress factors.

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Chapter I: Introduction

1.1 Biofuels

Biofuels have gained increasing interest over recent years because of their impact on food price, climate change, and depleting fossil fuel sources, as well as the potential to reduce reliance on fuel imports (Antizar-Ladislao and Turrion-Gomez, 2008). Adopting biofuels has the potential to play an important role in decarbonisation and simultaneously reducing greenhouse gas emissions (Walker, 2010). Essentially, biofuels are energy sources derived from recently dead biological materials and they can broadly be defined as solid, liquid or gaseous fuels which may be produced from any biological carbon sources. The growth of the biofuel sector has increased dramatically in recent years mainly based on the conversion of feedstocks such as wheat, corn and barley, and non-foodstocks such as biowaste derived from agriculture residues and woody biomass to produce alcohols and diesel.

Despite the current dominance of bioalcohols, there are many different types of biofuel, including biogas, syngas, green diesel, vegetable oil and solid biofuels. Each has its own benefit and potential cost structures and consequently it is very difficult to compare them directly. However, in 2008 Scharlemann and Laurance argued that although many biofuels can lower greenhouse-gas emissions (GHG), they often have a greater 'total environmental cost' than gasoline based fuels (Scharlemann and Laurance, 2008). The authors pointed out that many previous analyses of different biofuel crops placed a focus on reducing GHG and energy use, which is a very narrow evaluation. They suggested that researchers must consider the full

environmental effects in order to precisely determine how biofuels impact on the environment. For example, biofuels produced from sugar cane can decrease greenhouse-gas emission, however, this benefit will be lost if the tropical forests being razed to make sugarcane fields leads to an increase in GHG emissions (Scharlemann and Laurance, 2008). Moreover, the loss of tropical forests can lead to further consequences such as the loss of hydrological functioning, biodiversity conservation and soil protection (Laurance., 1999; Bala *et al.*, 2007).

Among the biologically produced alcohols, ethanol is the most common product, whilst other alcohols such as propanol and butanol are less common. Bioethanol, which is produced by microbial fermentation process, is known to be an ideal alternative source to fossil fuels for many reasons including its ease of production and lack of toxicity (Lu *et al.*, 2011). The yeast *Saccharomyces cerevisiae* is commonly used for ethanol production in a wide range of industrial fermentations and is currently the favored organism for biofuel production. However, this organism is by no means perfect since it is a nonmotile, unicellular organism which must rely on its physiological response mechanisms to cope with the changes in environment encountered within industrial fermentations (Gibson *et al.*, 2007). In addition, this species of yeast is unable to convert certain 5 carbon sugars into ethanol without genetic modifications and consequently, biofuel fermentation parameters are typically tailored to meet the needs of the yeast strain.

1.2 First generation biofuels and feedstocks

First generation biofuels include the fuels mentioned above: bioethanol, biodiesel, green diesel, vegetable oil, biogas and solid biofuel, all of which are derived from materials (feedstocks) which can also be used as a source of food. In general, first generation feedstock can be divided into 3 main groups, derived from sugary, starchy and lignocellulosic matter. Of the different end products, bioethanol has received the most commercial interest and can be produced from a variety of material sources, for example, sugar-rich materials (sugar cane, sugar beet, sweet sorghum and cheese whey), starchy materials such as grains (corn, wheat, triticale) and root crops (potato, cassava). Lignocellulosic material related to food sources can also be utilised for the production of second generation biofuels, as described in Section 1.5.1.1. Different sources typically vary in their carbohydrate composition. For example, while sugar cane and sugar beet are typically sucrose-based, corn, wheat and other cereals are predominantly starch-based (Walker, 2010). The speed of the fermentation process in bioethanol production is partially based on the sources used; typically simple sugars such as fructose, glucose and sucrose are fermented quicker by existing enzymes in yeasts, whilst starch-based substrates require pre-hydrolysis to convert starch into simple sugars which are more readily assimilated. As alluded to above, *Saccharomyces* yeasts cannot directly convert starch into ethanol and consequently many first generation biofuels typically employ material comprised of simple sugars, such as molasses, or those which can be easily broken down using enzyme additions, such as corn.

It must also be noted that the main crops for bioethanol production differ according to readily available resources, for example wheat is used in Europe, corn (maize) in North America, and sugar cane (molasses) in South America. The other main crops which can be used to produce ethanol include barley, triticale (a hybrid of wheat Triticum and rye Secale) and the roots of the cassava plant (Walker, 2010).

1.3 Second generation biofuels and feedstocks

Since first generation biofuels rely on food supplies as the source of ethanol production, they have limitations including economical, social, environmental and ethical issues. For example, the use of food sources can influence oil and food price, cause soil erosion, increase food waste and is the centre of the ethical debate around 'food vs biofuels'. Therefore, it is increasingly desirable that non-food feedstocks are implemented to produce 'second generation biofuels'. These fuels are produced from various sustainable sources, mainly derived from lignocellulosic materials. The two main categories of this feedstock are waste materials such as corn stover, corn fibers and cobs, straw, waste paper, spent grains and 'energy crops', such as switch grass, grown specifically for ethanol production (Walker, 2010).

Lignocellulosic materials are essentially woody biomass which comprises mainly cellulose, hemicellulose and lignin. Although lignin cannot be converted into fermentable sugar, both cellulose and hemicellulose can be broken down to simple sugars by hydrolysis. However, this process is not simple and can cause significant technical problems. One such issue is that during pre-treatment some xylose units of hemicellulose are acetylated which

leads to increased levels of acetic acid which can subsequently inhibit yeast fermentation (Walker, 2010). A further issue is that the composition of waste material is likely to be inconsistent which therefore means that is difficult to standardise and optimise the sugar extraction and fermentation processes. Consequently, although second generation biofuels are likely to dominate the biofuel industry in the future, currently there are significant process steps which must be overcome before it becomes a viable process.

1.4 World ethanol production

Bioethanol is currently the most widely adopted biofuel. The fuel ethanol industry has grown in output over the past years and world ethanol production doubled from 2003 to 2007 and continues to increase (Pilgrim, 2009). According to the report of Renewable Fuels Association (2012), in 2011 the total ethanol production worldwide was 84.6 billion liters. The US and Brazil are the leading producers of ethanol; at this time the US is the top ethanol producer with 52.6 billion liters which accounts for 62.2% of global production. Brazil is the second highest producer with 21.1 billion liters and Europe is the third largest producer having overtaken ethanol production in Asia in recent years. In Asia the main producers are found in China, Thailand, Indonesia and South Korea.

The main factors driving the growth of ethanol production in the US are the environment and, significantly, government policy. In 2005 the US Renewable Fuels Standard called for increased support for ethanol production to reduce reliance on gasoline oil. The legislation directed that the US fuel supply should be increasingly supplemented with biofuel, including bioethanol and

biodiesel (Pilgrim, 2009). Similarly, in Europe, the “biofuel directive target” was established for the incorporation of renewable fuel sources in diesel and petrol at 2% in 2005 and rising to greater than 12.5% by 2020 (Pilgrim., 2009).

In South America, biofuels are a much less recent concept, particularly in Brazil which has been producing bioethanol from sugar cane for some time. To reduce dependence on foreign oil, Brazil implemented a program called the National Ethanol Program in 1970 which was supported by adopting flexible fuel vehicles (FFVs) (Pilgrim, 2009) which can directly use blended or pure biofuels (Walker, 2010).

1.5 Production of bioethanol

Irrespective of the type of bioethanol to be produced (first or second generation), there are three main stages to production: pretreatment of the plant biomass to yield carbohydrates, saccharification to carbohydrates down into simple sugars and finally fermentation to convert the carbohydrates into ethanol. While these three steps are essential for the production of ethanol from plant material, the manner in which they are performed can vary. The most basic procedure is to perform each stage in turn, however it is possible to combine some or all of the steps of the process. Each method has potential benefits and drawbacks as described below.

1.5.1 Methods for hydrolysis of plant biomass

1.5.1.1 Separate hydrolysis and fermentation (SHF)

The simplest means of producing bioethanol is to utilize a system whereby hydrolysis of plant material (saccharification) is performed prior to the fermentation step (Figure 1.1). In this system, hydrolytic enzymes are obtained commercially or derived from a separate operation unit by a specific microorganism, for example by *Trichoderma reesei* (Elkins *et al.*, 2010). The obtained enzyme mixtures are then used for saccharification prior to transfer of the medium to a separate vessel for fermentation. The advantage of this approach is that each stage can be optimized and monitored, allowing for better process control (Walker *et al.*, 2010). However, producing biofuel in this way is time consuming and can result in high capital expenditure due to the need for enzyme additions, as well as specific vessels to perform each individual task (Walker *et al.*, 2010).

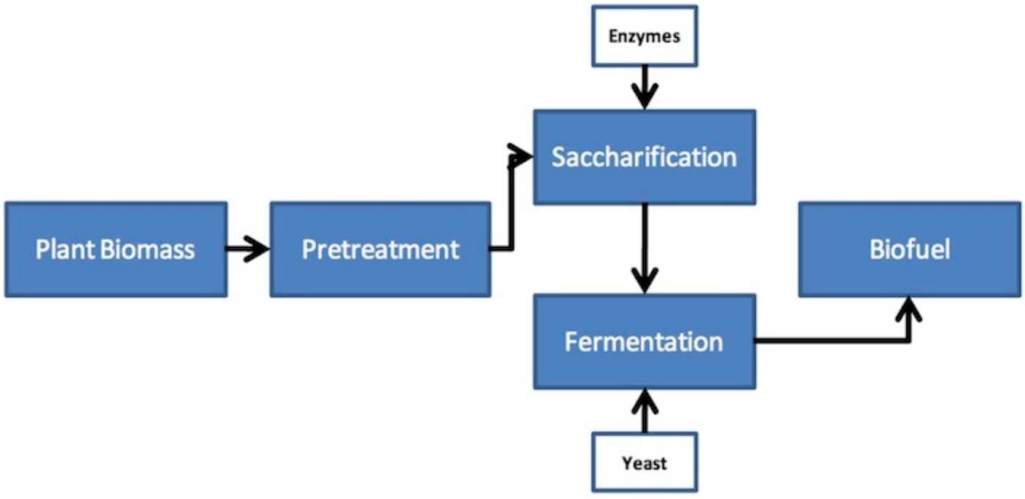


Figure 1.1: Bioethanol production using separate hydrolysis and fermentation.

1.5.1.2 Simultaneous saccharification and fermentation (SSF)

Currently the most common means of producing bioethanol is via a simultaneous saccharification and fermentation (SSF) system (Figure 1.2). In this process, pretreated plant biomass is converted into ethanol by the addition of a mixture of yeast and enzymes (Stephanopolous, 2007). The enzymes act to break down complex plant wall carbohydrates into simple sugars as described above (Section 1.5.1.1). As the enzymes begin to release glucose and other simple carbohydrates from cellulose, the yeast functions to convert it to ethanol via the fermentation pathway. Utilising an SSF system provides several advantages. Firstly there are significant savings to be made in terms of time and plant capacity. Secondly, the process can be optimized to increase efficiency of the saccharification process; typically hydrolysis enzymes are inhibited when too much glucose accumulates (Ghim *et al.*, 2010). By performing saccharification and fermentation at the same time, the yeast continuously removes glucose, thus ensuring that the enzymes remain effective. Despite the advantages over SHF, the combined SSF can be more difficult to control due to increased process variables. Furthermore, the requirement for commercially produced enzymes is still an important cost issue in SSF systems (Banat *et al.*, 1998).

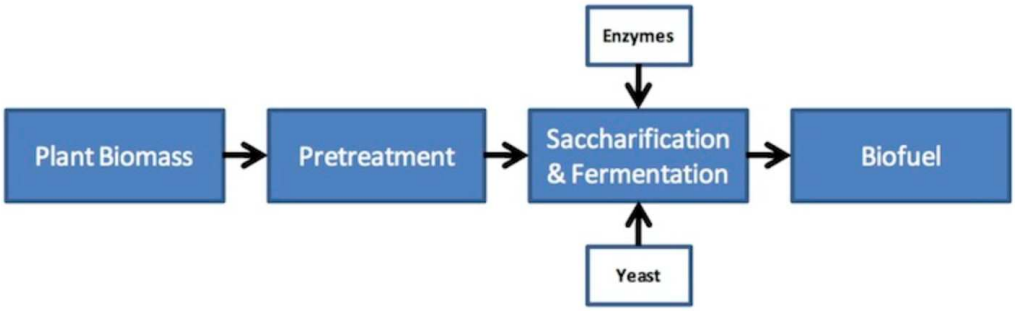


Figure 1.2: Bioethanol production using simultaneous saccharification and fermentation.

1.5.1.3 Consolidated bioprocessing (CBP)

It is possible that in the future consolidated bioprocessing (CBP) will be employed (Elkin *et al.*, 2010). In this process, the requirement of enzyme addition will be reduced or removed. However, CBP systems require the development or discovery of novel organisms which can perform saccharification and fermentation at the same time (La Grange *et al.*, 2010), for example, yeast strains which have yet to be discovered. Alternatively genetically modified organisms could be used such as *Saccharomyces* yeasts which have been manipulated to have the ability to produce cellulases, or modified bacterial strains that produce enzymes allowing for the production of ethanol (La Grange *et al.*, 2010).

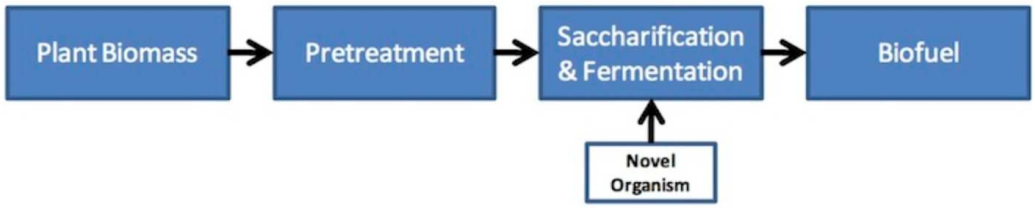


Figure 1.3: Bioethanol production using consolidated bioprocessing.

1.5.2 The process of fermentation during biofuel production

In addition to difference in the pretreatment and saccharification processes, the fermentation stage of bioethanol production can also be performed in different ways. The three main processes which can be used are batch, fed-batch and

continuous fermentations (Ingledew *et al.*, 2009). Each process has both advantages and disadvantages as described below.

1.5.2.1 Batch operation

Batch fermentation processes are the most commonly used worldwide for the production of potable ethanol as well as for fuel ethanol. In this process the yeast culture is introduced into the fermentation media and left until all of the sugar present is converted in ethanol. This type of fermentation system is typically efficient with a consistent product and a high yield of ethanol. However, vessels must be regularly cleaned and fermentation cycles can result in production being slower than with other types of fermentation system.

1.5.2.2 Fed-batch operation

Fed-batch fermentation systems are those in which nutrients are fed incrementally to the yeast culture. This can provide some benefits as it can enable a greater total amount of sugar to be added to the fermentation than in batch cultures, leading to greater yields. However, this process can be costly for monitoring and equipment, and careful control must be maintained to ensure that yeast growth is balanced with ethanol production (Walker, 2010).

1.5.2.3 Continuous fermentation operation

A viable alternative to batch fermentation systems is the use of continuous fermentations. This is a process in which nutrients are fed into the culture at a rate equal to the removal of the end product. This system has the potential to be highly productive with a continuous ethanol yield. However, although

employed successfully in many instances, the main drawback is the potential for contamination or yeast mutation over time (Walker, 2010). If either of these situations occurs then the system must be cleaned entirely and reinitiated, hence it is possible that total yield can be reduced due to 'down-time'.

1.6 The process of ethanol production from corn mash

As discussed previously, first generation bioethanol can be produced from a range of feedstocks, including corn (Section 1.2). Generally, there are 2 main types of corn ethanol production based on dry milling and wet milling (Figure 1.4). Each type of process utilises raw materials and bi-products in slightly different ways.

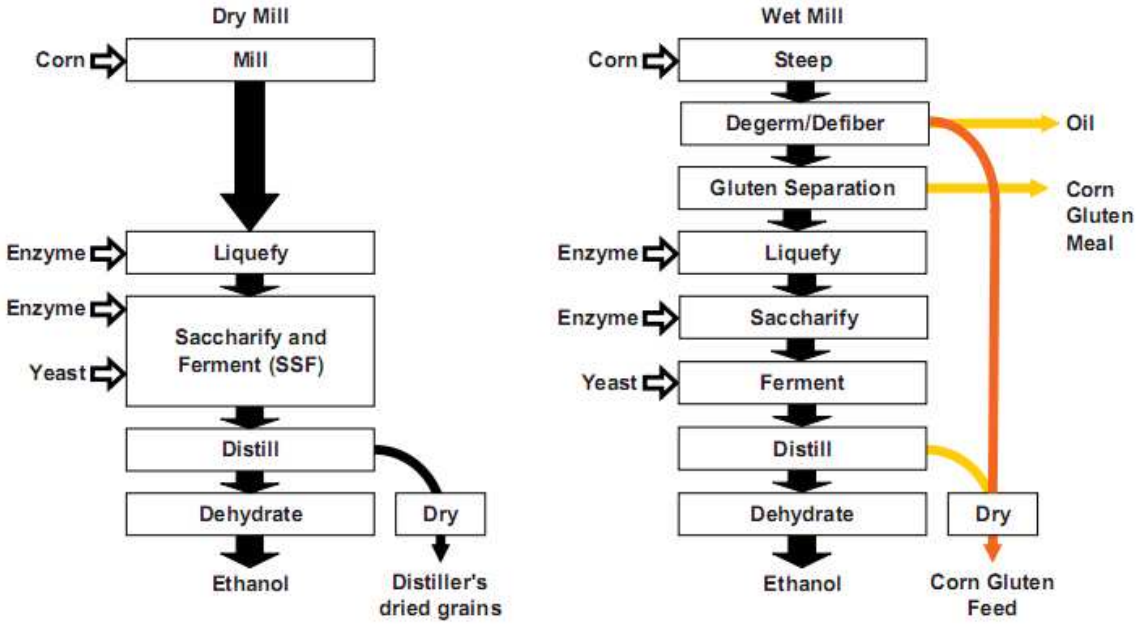


Figure 1.4: Dry milling and wet milling process for corn ethanol production.

(Bioethanol: Science and Technology of fuel alcohol, 2010)

The dry milling process can be divided into 6 steps: milling, liquefaction, saccharification, fermentation distillation and dehydration. During the milling step the corn kernels are ground into a fine powder or 'meal'. Water is then added to the meal and the temperature is increased in order to solubilize starch during the liquefaction step. The third step is saccharification in which enzymes such as alpha-amylases are added to the mash to convert starch to simple sugars, mostly glucose. After that, ammonia is added, primarily to control the pH of the corn mash, but also to act as a nutrient source for the yeast during fermentation. During the fermentation stage the yeast culture converts the starch derived sugars into ethanol, carbon dioxide and secondary metabolites. Once fermentation is complete, the product is transferred to a distillation column where ethanol is recovered. At this point the fermented medium with around 10% v/v ethanol is distilled to approx 96% v/v. The distiller dried grains (DDG) are collected at this step and can be used for animal feed or for different potential applications such as recycling for mash preparation and supplements to fermentation media. In the final step, the remaining water in ethanol will be removed by molecular sieve to produce 99% ethanol or absolute ethanol (anhydrous ethanol) (Walker, 2010).

In wet milling, the steps for corn ethanol production are mostly the same as dry milling. The major difference between the two processes is the first step, where the corn is initially treated. Unlike dry milling, the corn used in wet milling must be soaked in water or dilute acid (normally sulfuric acid) for 24 – 48 hours to separate the cereal into various components, such as starch, gluten,

oil, protein and fiber prior to starch conversion to ethanol. As seen in Figure 1, corn oil is a by-product in this step and can be extracted and sold. Another by-product is corn gluten meal which is dried and used for livestock feeding. The main product comprising starch proceeds to the saccharification and fermentation steps which are similar to those found in dry milling to produce anhydrous ethanol.

1.7 Yeast

Yeast is a microscopic unicellular fungi which can obtain energy aerobically by respiration and also anaerobically by fermentation. The yeasts are a diverse group of organisms and the number of fully characterised yeast species now stands at around 1200, although it is believed that this figure may actually describe less than one percent of the total number present on Earth (Kurtzman and Fell, 2006). In industrial biofuel production, yeast plays a very important role in the conversion of fermentable sugars to ethanol. To achieve a high ethanol conversion, it is required that yeast strains must tolerate the stressful conditions associated with fermentation and in particular ethanol which can inhibit yeast growth and the fermentation capacity of the culture (Kosaric and Vardar-Sukan, 2001). Although other species of yeast may prove to be important in future biofuel production, currently strains belonging to the species *Saccharomoyces cerevisiae* are predominantly used.

1.7.1 Yeast and fermentation

In yeast such as *S. cerevisiae*, there are two main metabolic pathways which can be referred to as respirative and fermentative metabolism. Both pathways

start with glycolysis in which glucose is converted into pyruvate. During this process, NAD+ is reduced to NADH and two molecules of ATP are produced. When oxygen is present the respiration process is typically used in preference to the fermentative process. The fermentation pathway is an anaerobic process in which energy is released from glucose with the aid of the alcohol dehydrogenase system. Glucose is converted to ethanol via the fermentation pathway according to the following reaction:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{energy}$$

1.7.2 Stress factors, the stress response and fermentation performance

1.7.2.1 Global and specific stress response mechanisms in yeast

There are many factors which have an important impact on ethanol production, including the ability of yeast to tolerate stress factors such as heat shock, oxidative stress, osmotic stress, nutrient deprivation, ethanol stress, cold shock, pH downshift and anaerobic downshift. Yeast respond to various stresses in different ways; generally in *Saccharomyces cerevisiae* there are 2 major stress response pathways. The first is known as the heat shock response which is mediated by heat shock transcription factors (HSF) (Morimoto *et al.*, 1996) and the other is called the global stress response (GSR) which is activated by a number of environment stresses including nitrogen starvation, pH change, and oxidative stress (Schmitt and McEntee, 1996). It is believed that the GSR is an evolutionary adaption that helps yeasts respond to adverse environment at conditions in a nonspecific manner whilst Heat Shock Response (HSR) helps yeast to respond to specific environment stresses

(Morimoto *et al.,* 1996). In previous work, it has been shown that the GSR is found to be involved in the up-regulation of hundreds of genes and their corresponding proteins which have an impact on a variety of cellular functions (Gasch *et al.,* 2000; Causton *et al.,* 2001).

For more specific responses, in a study focused on genomic expression programs in the response of yeast cells to environmental changes, Gasch et al (2000) reported that in order to survive in the natural environment *S. cerevisiae* strains must not only be able to respond swiftly to sudden changes in environment conditions, but also adapt to the unique features of that specific environment. By analysing genome expression, it was found that yeast cells respond to environmental changes by altering the expression of a large number of genes and in particular the environmental stress response (ESR) was shown to play an important role in protecting yeast cells (Gasch *et al.,* 2000). For example, in examining the response of yeast to an up-shift in temperature it was observed that 2 large clusters of genes were effected, amounting to approximately 900 genes. Of these genes, around 50 were induced by a variety of stresses through the stress response element (STRE) promoter sequence which is recognized by transcriptional factors Msn2p and Msn4p (Gasch *et al.,* 2000). The study revealed that although genes showed a similar response to different environments tested, the regulation of the genes was not the same. It is supposed that the regulation of these genes is dependent on the signaling systems that act in response of specific environments, so specific genes for a particular environmental condition are induced (Gasch *et al.,* 2000). It should be noted that in the cluster of genes referred to above, approximately 600 genes which were repressed in ESR are involved in various

aspects of RNA metabolism (processing, splicing, translation, initiation and elongation of RNA, tRNA processing and synthesis), protein synthesis or cell growth. In contrast, approximately 300 of the genes that were induced as part of the ESR were involved in processes such as carbohydrate metabolism, detoxification of reactive oxygen species (ROS), cell wall modification and DNA damage repair (Gasch *et al.,* 2000). The ESR can also help to differentiate expression of isozymes, which means that different enzymes for similar functions can be synthesized in different environment conditions (Gasch *et al.,* 2000). The enzymes involved in carbon metabolism, protein folding and defense against ROS are examples. Among the induced genes, some genes were observed to have multiple functions in response to environmental changes. For example, genes involved in the synthesis of trehalose and glycogen were also observed to encode for enzymes to degrade these carbohydrates in the ESR (Gasch *et al.,* 2000). Interestingly Gasch et al (2000) also observed that when changing temperature for yeast growth from elevated temperature (33, 37^{o}C or hyperosmolarity) conditions to suboptimal conditions (17 and 21^{o}C) the signaling response was not triggered. Therefore, it can be hypothesized that in suboptimal environments where the physiological systems are not optimized, the result of a shift in conditions can lead to a series of secondary instabilities within the cells which may potentially affect cell physiology. For adaption to these kinds of environmental changes, the genome of yeast cells has evolved to initiate the ESR to protect and maintain the critical features of yeast cells in response to such diverse conditions. In fermentation systems, Briggs et al (2004) suggest that process parameters must therefore be tightly controlled as stress responses

can be interlinked. For example it is important to ensure a sufficient nutrient supply, and to control the pitching (inoculation) rate, the amount of dissolved oxygen, fermentation temperature and fermentation time.

1.7.2.2 Osmotic stress and industrial yeast strains

One of the first stress factors that yeast cells must cope with is osmotic stress, caused by the high concentration of sugar at the beginning of the fermentation process. Osmotic stress occurs when an imbalance between intracellular and extracellular osmolarities occurs. This stress is believed to cause a deleterious change in the physiology of yeast (Csonka and Hanson, 1991). There are two types of osmotic stress: hypo-osmotic stress where a low external osmotic pressure (for example in deionized water) results in an influx of water into cells, and hyper-osmotic stress in which a high external osmotic pressure (for example a high concentration of soluble compounds) causes water inside the cell to be drawn out into the environment (Blomberg and Adler, 1992, Csonka and Hanson, 1991, Dihazi *et al.,* 2001, Klipp *et al.,* 2005). Consequently at the start of fermentation hyperosmotic stress occurs due to the high concentration of sugar, and once sugars begin to be depleted hypo-osmotic stress may occur. In a study of brewing yeast strains, it was reported that hyperosmotic stress can lead to the deterioration of viability, growth and fermentation performance (D'Amore, 1992). Further studies also support this finding, indicating that a loss of yeast viability may occur due to the reduction of intracellular water (Cahill *et al.,* 2000, Dumont *et al.,* 2003). Therefore, in order to protect against osmotic stress in the environment, yeast cells have developed two physiological adaption response mechanisms: osmotolerance and

osmoadaption. Osmotolerance is the innate physiological resistance of yeast cells to osmotic stress under chronic environmental hyperosmotic pressure, while osomoadaption represents the mechanisms which help cells survive by adjusting their normal physiology (Poolman and Glaasker, 1998).

1.7.2.3 Oxidative stress and industrial yeast strains

A supply of oxygen during the early stages of fermentation is required for successful alcohol production. Oxygen is required for yeast cells to grow and divide, generating biomass, and also ensures that yeasts are in the optimum physiological condition for effective fermentation (Hammond, 2000; Husle, 2003). Oxygen is required for lipid synthesis, which plays an important role in maintaining plasma membrane integrity and function for cell replication (Hammond, 2000; Briggs *et al.,* 2004). Oxygen is also required for biosynthesis of both sterols and unsaturated fatty acids that help yeast to maintain growth and perform endocytosis, the process by which yeast cells absorb molecules by engulfing them (Lorenz & Park, 1991). However, excessive yeast growth can occur if yeasts are exposed to excess oxygen in the fermentation vessel (Briggs *et al.*, 2004).

During aerobic respiration other derivative forms of oxygen, termed reactive oxygen species (ROS) are also produced. The reactive oxygen species can be named as hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-). They are believed to cause damage to cell components, contributing to cell ageing and physiological deterioration (Beckman & Ames, 1998). More specifically, ROS can inactivate cellular enzymes (Cabiscol *et at*., 2000), cause lipid peroxidation (Girotti, 1998) and lead to nucleic acid

damage (Salmon *et al.*, 2004; Ribeiro *et al.*, 2006). Moreover ROS can also damage mitochondrial DNA, leading to the generation of respiratory deficient "petites" mutants (O'Rourke *et al.*, 2002a, b; Doudican *et al.*, 2005; Gibson *et al.*, 2006).

1.7.2.4 Ethanol stress and industrial yeast strains

The primary purpose of fermentation from an industrial perspective is to produce ethanol from fermentable sugar. During fermentation in batch cultures, the ethanol concentration naturally increases, with the result that yeast cells are exposed to increasingly toxic levels of ethanol. In high-gravity bioethanol fermentations the initial sugar concentration is typically greater than 30g per 100ml (up to 39g/100ml), leading to a final ethanol concentration of approximately 15-18% (v/v) (Puligundla *et al.*, 2011).

The effects of ethanol stress on yeast physiology are diverse, however they are known to include growth inhibition and reduced cell cycle rate (Schmidt *et al.*, 2006), cell size reduction (Canetta *et al.*, 2006), and reduced respiration and glucose uptake rates (Fernandes *et al.*, 1997). Furthermore ethanol can lead to increased membrane permeability (Marza *et al.*, 2002; Schmidt *et al.*, 2006), enzyme inactivation, lipid modification and loss of proton motive force across the plasma membrane (Petrov & Okorokov, 1990; Mizoguchi & Hara, 1997). In general the effect of this is to cause a general reduction in both yeast health (viability) and fermentation rate (Nagodawithana & Steinkraus, 1976; Cahill *et al.*, 2000; Stewart, 2001).

1.7.2.5 Temperature stress and industrial yeast strains

During fermentation, *S. cerevisiae* yeast cells produce heat during the process of converting glucose to ethanol. As a consequence, the fermentation temperature is usually controlled to ensure that yeast cells are not killed by excessive heat (Ingledew, 2009). In addition, fermentation temperatures are usually set below those considered to be optimum for yeast in the plant (34-35^{o}C) for several reasons. Yeast growth and ethanol production can be affected in both positive and negative ways by temperature. According to Serra et al (2005) if the temperature is increased from below optimum to optimum the yeast growth rate will increase. This is because biochemical reactions occur faster at higher temperatures and therefore the time and cost for fermentation can be reduced. However, high temperatures can also cause stress to yeast, leading to reduced cell viability which can have a disastrous effect on fermentation performance. Once the temperature exceeds the optimum temperature for the strain, the yeast growth rate decreases rapidly since the cellular membrane becomes damaged and essential enzymes for cellular metabolism are denatured (Serra *et al.*, 2005). Many previous reports have also shown that high temperature can cause stuck fermentations as yeast stress tolerance decreases as temperature increases (Laluce *et al.*, 1991; Thomas *et al.*, 1993).

In response to elevated temperature, yeast cells exhibit a rapid molecular response which is known as the Heat Shock Response (HSR) as described briefly in Section 1.7.2.1. It is known that even sub lethal heat shock treatments can induce the synthesis of specific proteins commonly named as heat shock proteins (Hsps). In yeast, many Hsps perform molecular

chaperoning functions which prevent protein aggregation while other Hsps can help yeast increase thermotolerance (Parsell and Linquist, 1994). For example, in *S. cerevisiae*, Hsp104 plays a role in protecting yeast against heat denaturation and high temperature during respiratory growth (Lingquist and Kim, 1996), whilst Hsp83 acts as a chaperone to prevent potential damage from protein misfolding caused by heat (Walker, 1998). Hsp gene expression is also known to be involved in the increased transcription of genes containing promoters of the heat shock element (HSE). The increase in gene transcription occurs due to the activation of heat shock transcription factor (HSF) in the presence of heat in order to allow yeast to grow at high temperatures (Ruis and Schuller, 1995). It must be noted that HSEs only respond to heat shock and not to other stresses, and as such is a different kind of response to the Global Stress Response (GSR) pathway although many products of these pathways are similar.

Another response of yeast to high temperature stress is the accumulation of protective compounds such as glycerol (Omori *et al.,* 1996), and enzymes such as mitochondrial superoxide dismutase and catalase (Costa *et al.,* 1993). However, arguably the most important protective compound for thermotolerance is trehalose (Van Laere, 1989; Wiemken, 1990; Neves and Francois, 1992). Trehalose is produced in response to the GSR pathway and also in direct response to heat shock, and helps to protect yeast by stabilizing cell membranes, as well as increasing the temperature stability of yeast cellular proteins. Typically, trehalose functions to replace water molecules in cellular membranes and forms a hydration shell around proteins (Iwahashi *et al.,* 1995). Moreover, Elliot and coworkers (1996) have found that in *S.*

cerevisiae, trehalose acts synergistically together with Hsp104 to confer thermoprotection (Elliot *et al.,* 1996).

It is not only high temperature that can cause stress to yeast, but low temperatures are also known to affect yeast cell physiology and fermentation ability. Generally, cold shock occurs in *S. cerevisiae* when a downshift to temperatures of 20^{o}C or lower are encountered (Kondo and Inouye, 1991; Kondo *et al.,* 1992; Kowalski, 1995). Cold shock can lead to a reduction in the fluidity of a yeast cells membrane (Shinitsky, 1984). Furthermore, the membrane may be modified from a liquid crystalline form to a gel state (Thieringer *et al.,* 1998) which leads to various function changes in yeast cells. For example, the transportation pathways of metabolites and proteins do not function as normal (Gibson *et al.,* 2007). Another effect of cold is that hydrophobic interactions between the carbon skeleton of polypeptides and the side chains of amino acids are reduced leading to protein denaturation (Gounot and Russell, 1999). In response to cold shock, yeast cells are able to adapt as a result of changes in gene expression (Sahara *et al.,* 2002; Schade *et al.,* 2004). It has been reported that yeast respond to varying degrees of cold shock by differential regulation of specific genes (Zhang *et al.,* 2001; Sahara *et al.,* 2002; Zhang *et al.,* 2003; Schade *et al.,* 2004). Typically, there are two phases of the cold shock response. The early phase involves changes to membrane fluidity as well as preventing destabilization of RNA secondary structures for efficient protein translation. The late phase involves the up-regulation of genes involved in global stress response (GSR) such as genes encoding heat shock proteins or trehalose and glycogen up-down regulation. In addition there are specific cold shock genes including *TIP1* (Lee *et al.,* 1991), *NRS1* (Kondo and

Inouye, 1992; Kondo *et al.,* 1992) and the LOT genes (*LOT1, LOT2, LOT3*) (Zhang *et al.,* 2001). Trehalose also plays a role in the tolerance of yeast to cold shock (Kandor *et al.,* 2002), with accumulation of trehalose occurring at temperatures of 10^{o}C and lower to help protect yeast from loss of viability (Kandor *et al.,* 2002; Kandor et al, 2004).

It can be seen that although both cold and heat stress produce their own characteristic changes to yeast gene expression and physiology, some of the effects of these stresses are shared. For example, trehalose is produced in response to both stresses and is known to be important in stabilizing membrane structures (Mansure *et al.,* 1994) and enzyme function under a variety of conditions (Sola-Penna & Meyer-Fernandes, 1998). It has also been suggested that trehalose may participate in repair of proteins (Simola *et al.,* 2000) and can act as a carbon source during starvation (Fales, 1951; Eaton, 1960; Chester, 1963; Panek, 1963). As such the level of intracellular trehalose is related to a variety of stress factors and has a significant effect on stress tolerance and adaption (Hottiger *et al.,* 1987a,b; D'Amore *et al.,* 1991). There are many genes involved in trehalose synthesis including *TPS1, TPS2, TSL1, TPS3* as well as trehalose degradation such as *NTH1, NTH2, ATH1* (Zahringer *et al.,* 2000). These genes are regulated by STRE elements (Winderickx *et al.,* 1996) and are up-regulated in response to various stresses including heat stress and oxidative stress (Parrou *et al.,* 1997). While the synthesis of trehalose plays an important role in protecting cells, the removal of trehalose has also been the subject of investigations, since degradation is essential for the resumption of normal cellular activity. Indeed, trehalose is known to inhibit the activity of enzymes such as glutathione reductase, an enzyme which helps

yeasts reduce oxidative damage within the cell and maintain cellular homeostasis (Sebollela *et al.*, 2004).

1.8 Methods to improve bioethanol yeast fermentation performance

1.8.1 Genetic manipulation to improve stress resistance and ethanol production

There are significant efforts worldwide to develop new strains with increased stress tolerance and enhanced fermentation capacity for biofuel production. However, although the biochemical and physiological basis of yeast fermentation have been well characterised, as we have seen the genetic basis of yeast stress tolerance is complicated to the extent that it is difficult to manipulate yeast at the molecular level to increase yeast stress tolerance (Zhao and Bai, 2009). However, there have been several reports demonstrating successful improvement of yeast stress tolerance by single gene manipulation (Zhao and Bai, 2009). Jung and Park (2005) showed that by using antisense RNA-mediated inhibition of *ATH1* transcription trehalose degradation was decreased. As a consequence, the ethanol tolerance and fermentability of *Saccharomyces cerevisiae* was improved (Jung and Park, 2005). Similarly Gorsich et al (2006) successfully overexpressed the *ZWF1* gene which encodes glucose-6-phosphate dehydrogenase to allow *S. cerevisiae* to grow in the presence of high levels of furfural, a well known inhibitor present in lignocellulosic fermentations (Gorsich *et al.*, 2006).

An alternative approach is to use global transcription machinery engineering (gTME) which involves the manipulation of multiple genes by inserting randomly mutated copies of global transcriptional factor, followed by selection of favorable mutants by growth in ethanol. The global transcriptional

factor controls a large set of genes (Alper *et al.,* 2006) which can influence phenotypes such as stress tolerance. Alper et al (2006) successfully employed this method to create an engineered strain, *spt15-300*, with multiple mutations over hundreds of genes. The result was that the mutant strain was able to produce 69% more ethanol when compared to the parental strain (Alper *et al.,* 2006).

Genome shuffling has also been used, based on classical breeding techniques to generate novel strains. This method has been used to produce ethanol resistant and thermal tolerant strains (Shi *et al.,* 2009), and inhibitor resistant yeasts (Lu *et al.,* 2011) all of which produce higher ethanol yields. An alternative approach is to exploit the natural adaptive response of yeast to stressful environments. Typically, random mutations in the yeast genome are induced by environment stresses and acccumulate in surviving individuals. By employing selection protocols, adaptive evolution *S. cerevisiae* strains with increased stress tolerance have been generated (Cakar *et al.,* 2005; Zhao and Bai, 2009).

1.8.2 Improvement of strain ethanol production by adaption of process parameters

A different strategy to that based on modifying or developing new strains for biofuel fermentation is to simply improve the capacity of existing strains to produce alcohol. Although new strains are likely to provide viable alternatives in the future, small changes to process conditions can lead to improved yeast fermentation performance. In associated industries, such as brewing, a significant amount of research has focused on yeast viability and vitality, and

methods to ensure that a particular strain can perform to its optimum level. A similar strategy in the bioethanol industry would also lead to improvements such as faster, more consistent fermentation with a higher yield. However, bioethanol fermentations hold a unique challenge in that they are required to push the limits of the strain to produce as high a proportion of ethanol as possible. Given that multiple interlinked fermentation stress factors play a significant role in determining ethanol production (Section 1.7.2), preparing yeast to withstand stress holds some potential benefits. This can be approached by several means, such as supplying yeast nutrition or enhancing yeast physiology during the preparation of the yeast inoculum as well as prior or during fermentation.

Within the ADY industry, the production process typically includes a series of pre-conditioning steps aimed at protecting cells or at enhancing the physiological state of yeast prior to drying (Powell and Fischborn, 2011). One such measure includes nutrient limitation for a short period of time to arrest division and to ensure that all cells within the population are present as discrete individuals. Dividing cells are believed to be susceptible to stress factors associated with the production of ADY, possibly due to the absence of natural defense mechanisms (Gasch & Werner-Washburne, 2002; Werner-Washburne *et al*, 1993), and incomplete development of the cell wall or metabolic differences in virgin cells (Powell *et al*, 2003). Manipulation of growth conditions, treatment of cream yeast, or subjecting a culture to mild stress factors are also common practice (Powell and Fischborn, 2011). In particular, it is known that applying osmotic stress (Eleutherio *et al.*, 1997) and in particular mild heat (Hottiger *et al.*, 1987) can increase the

concentration of compatible solutes and other protective compounds such as trehalose (Elbein *et al.,* 2003; Crowe *et al.,* 2001). While starvation is not likely to be a viable option for the preparation of yeast immediately prior to bioethanol fermentation, due to potentially negative effects on yeast vitality, applying mild stress to yeast cultures prior to fermentation may function to procure a benefit to yeast prior to bioethanol fermentations.

1.9 Aims and Objectives

The success of both first and second generation biofuel production is dictated by the final ethanol yield, and even a small increase can have a significant impact on process costs, overall efficiency and profit. Consequently, it is of primary importance that the yeast culture employed is able to function to its maximum capacity. Although this is largely dictated by the intrinsic characteristics of the strain, it is also influenced by the yeast response to the changing fermentation environment. As such, ensuring the condition of yeast prior to fermentation and thus its ability to withstand and perform under fermentation conditions can have a significant impact on ethanol yield.

Although there are many methods which can be used to improve the resistance of industrial yeast strains to stress, in this study the heat shock response was exploited in an attempt to improve the stress resistance of yeast prior to fermentation. According to Hottiger et al (1987a, b), heat shock can result in rapid accumulation of trehalose which plays an important role in protecting yeast, as discussed previously. Interestingly, Greenfield Ethanol, Canada, noticed that pitching yeast into an industrial scale fermentation vessel at an elevated temperature appeared to result in a higher ethanol yield than typically

expected (Stephan Brey, Unpublished Data). The aim of this study was to explore this phenomenon by investigating the impact of yeast temperature preconditioning on yeast physiology and ethanol yield during corn mash fermentation. In order to understand the affects of temperature on yeast, different commercially available yeast strains used for alcohol production were analysed for their capacity to tolerate heat, their physiological response to heat and for the effects of heat treatment on parameters associated with the production of bioethanol from corn mash.

Chapter 2. Materials and Methods.

2.1 Yeast strains

Four bioethanol yeast strains were used in this study. The strains employed are summarised in Table 1. All yeast belonged to the species *Saccharomyces cerevisiae*.

Table 2: Name and source of yeast used in this study.

Yeast strains	Sources
Saccharomyces cerevisiae Superstart™ Instant Dry Yeast LAL7	Lallemand Inc (Montreal, Canada)
Saccharomyces cerevisiae Thermosacc®Dry Yeast	Lallemand Inc (Montreal, Canada)
Saccharomyces cerevisiae 2592	National collection of yeast (Norwich, UK)
Saccharomyces cerevisiae Ethanol Red	Fermentis (Marcq en Baroeul, France)

2.2 Growth and Storage

2.2.1 Media composition

All media components were obtained from Oxoid (USA) unless otherwise stated. The media were prepared using reverse osmosis (RO) water and then autoclaved immediately at 121^{o}C and 15 psi for 15 minutes.

2.2.1.1 YPD (Yeast extract Peptone D-glucose)

Yeast was grown and maintained on YPD medium composed of 10g/L yeast extract, 20g/L neutralized bacteriological peptone, 20g/L D-glucose (Fisher Scientific, UK). After preparation, media were autoclaved immediately at 121°C and 15psi for 15 minutes.

For YPD agar plates, 20g/L technical agar no. 3 was added into medium prior to autoclaving. After autoclaving, approximately 20ml of YPD agar was poured into sterile Petri dishes and allowed to solidify.

2.2.1.2 Corn mash

Corn mash media was kindly donated by Greenfield Ethanol, shipped directly from the Chatham plant (Ontario, Canada). Corn mash was provided semi-sterilized having been passed through a jet cooker at 120^{o}C followed by liquefaction at 80^{o}C. Once obtained, corn mash was stored at -80^{o}C in a freezer and defrosted overnight at room temperature prior to use.

2.2.2 Rehydration of Active and Instant Dry Yeasts

Both Superstart^{TM} instant dry yeast (IDY) and Thermosacc® active dry yeast (ADY) were supplied in dried form by Lallemand Inc (Montreal, Canada). For convenience, both forms of dried yeast are referred to as ADY within this thesis. All samples were obtained in packets sealed under vacuum and stored

at 4°C prior to use. Rehydration was conducted based on the manufacturer's guidelines. Typically, 1 g of dried yeast was sprinkled onto 10 ml sterilized tap water in a 30 ml Universal tube. ADY was then incubated at 30^{o}C for 15 min in static incubator (Certomat, Sartorius, USA) and mixed gently to separate any clumps. ADY was incubated for a further 45 min to allow cells to rehydrate fully.

2.2.3 Storage of yeast strains

YPD slopes were prepared by aliquoting 10ml of YPD and 20g/L molten agar into sterilized glass Universal bottles placed at a 45^{o}C angle and allowing to solidify at room temperature. Yeast strains were inoculated and grown on YPD slopes for 48 hours at 25^{o}C and then stored at 4^{o}C for up to one month.

For cryogenic storage, the stock cultures of yeast were pre-grown in 10 mL YPD at 25°C for 2 days and then transferred into 1.2ml cryovials (Nalgene Nunc International, UK) which contained 50% v/v YPD and glycerol. The tubes were stored in a freezer at -80^{o}C.

2.2.4 Reactivation of Yeast from Cryogenic Storage

After defrosting cryovial tubes which contained yeast, a loop of yeast culture was transferred into 10ml of YPD in a sterilized glass universal bottle and then incubated for 2 days in an orbital shaker (Certomat BS-1, Sartorius, USA) at 25^{o}C and 120 rpm.

2.3 Cell enumeration and viability estimation

Cell cultures were diluted to an appropriate concentration and were counted by haemocytometer (Weber Scientific International Limited, UK) using a light microscope (Olympus, UK) at 40X magnification. Methylene blue stain was used for estimating yeast cell viability. For preparation, methylene blue powder (Sigma-Aldrich, UK) was dissolved in 2% (w/v) sodium citrate solution at a final concentration of 0.01%. A diluted yeast cell suspension was stained with Methylene blue at a ratio of 1:1 and left for 5 min at room temperature. Approximately 10µl aliquots of samples were loaded onto the haemocytometer to count the number of cell dead and cell alive. The stained (dark blue) cells were counted as dead while unstained cells were counted as live. The cell density in the original culture was calculated using equation 1. The viability was calculated by the percentage of number of live cell over the total cells counted as shown in equation 2.

Equation 1: Calculation of cell density

Cells per ml = Number of cells in grid (25 squares) x dilution factor x 10^4

Equation 2: Calculation of viability

% Viability = Number of viable cells/Number of total cells x 100

2.4 Analysis of growth characteristics

In order to analyse the growth characteristic of each yeast strain, kinetic growth curves of each strains were generated. Yeast cells which were pre-grown on YPD medium were enumerated using a haemocytometer (Section 2.3) and $1x10^6$ cells were transferred into 200µl wells of a 96-wells plate. The

wells were then filled with fresh YPD up to 200µl and incubated at the temperature desired in a TECAN automated micro-plate reader: Infinite® 200 PRO series (TECAN, UK) which determined the OD of samples at 600 nm wavelength at intervals of 1 hours. Data were analyzed and collected by Magellan^{TM} Data Analysis Software (TECAN, UK). In order to ensure statistical accuracy, all yeast strains were sampled in triplicate. A 200 µl aliquot of un-inoculated YPD was also analysed in each plate as a negative control sample.

2.5 Spot plate analysis for determination of strain temperature sensitivity

The spot plate technique was used to examine the effect of temperature on the growth characteristic of different yeast strains on YPD agar (Section 2.2.1.1). Starter yeast cultures were obtained by inoculating yeast strains from agar slopes into 10ml of YPD medium and incubating in an orbital shaker (Certomat BS-1, Sartorius, USA) at 25^{o}C, 120 rpm for 2 days. A 100µl aliquot of starter culture was then transferred into a 250ml pre-sterilized shake flask containing 100 ml of fresh YPD. These cultures were incubated in an orbital shaker at 25^{o}C, 120rpm for 3 days.

Cells were harvested after propagation and transferred into pre-sterilized 50ml centrifuge tubes. Tubes were then centrifuged at 4^{o}C, 4000 rpm for 5 minutes. The supernatant was discarded and pellets were resuspended in sterilised RO water. Cell suspensions were then prepared based on optical density (OD) determined using a spectrophotometer (MODEL, COMPANY). Samples were diluted until to obtain an OD of 1 at a wavelength of 600nm. Four subsequent 1:10 dilutions were carried out to prepare serially diluted samples. A 10µl volume of each dilution was spotted onto YPD agar plates in triplicate and

each plate was prepared to contain 4 strains (Figure 2). All plates were then incubated in static incubators (Certomat, Sartorius, USA) at either 10, 15, 20, 25, 30, 35, 40 and 45^{o}C for 7 days. Data was recorded by photographing spot plates using a gel imaging system (Gel Doc, Bio-Rad Laboratories, U.S.A) and VisionWorksLS program.

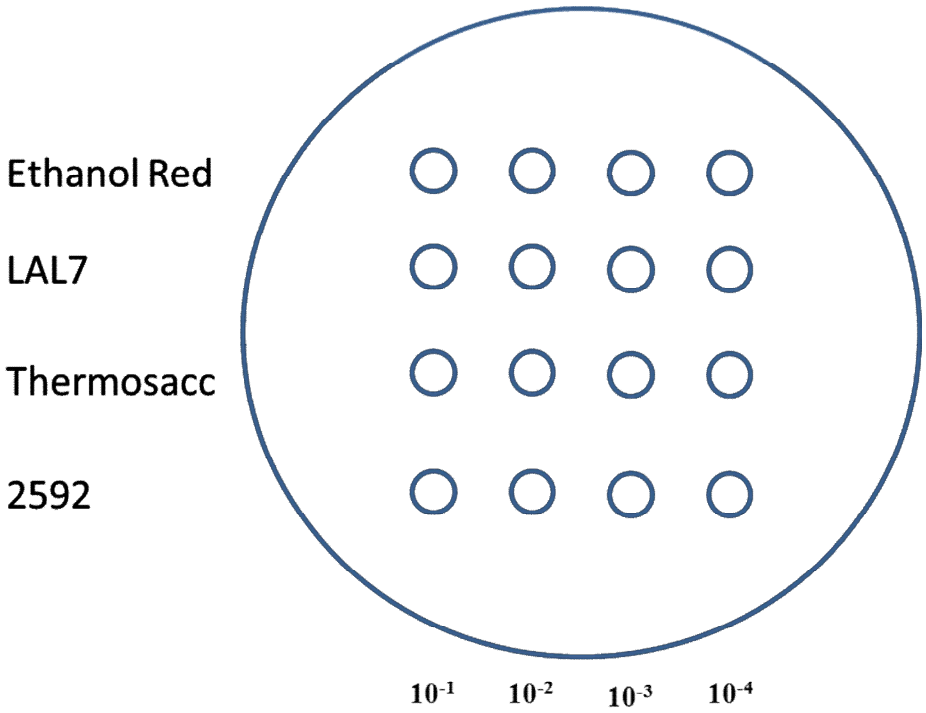


Figure 2.1: Determination of temperature sensitivity using spot plate analysis. Four yeast strains were pre-grown in YPD for 48 hours at 25^{o}C. The cultures were serially diluted to make four cells suspensions at concentration from 10^{-1} to 10^{-4} cells per mL (from left to right).

2.6 Analysis of cell size - measure the cell size before growth

Cell size was determined before and after growth at different temperatures including 25, 30, 34, 36, 38 and 40^{o}C as described in Section 2.5.1.2. 1 ml aliquots of each yeast culture were transferred to sterile Eppendorf tubes (Sigma, UK). A drop of 5µl aliquot cultures was then transferred onto a glass slide and cover slip. Yeast cell samples were observed under a Brightfield microscope (Leica, UK) with a camera attachment and the diameter of approximately 50-70 independent cells was determined using ImageJ software (National Institute of Health, USA).

2.7 Determination of intercellular trehalose and glycogen

Intracellular glycogen and trehalose concentrations were determined using the method of Parrou and Francois (1997). In order to determine the concentration of glycogen and trehalose, these compounds were first reduced by digestion to their constituent component; glucose. Glycogen and trehalose are enzymatically digested by α-amyloglucosidase and trehalase respectively. Cell samples were diluted to 1x10^{9} cells/ml and washed two times in sterile deionised water. 250µl of 0.25M $NaCO_3$ was added to each tube and samples were incubated for 2 hours in a 95°C waterbath (Northern Media, UK) with occasional agitation. The pH of the solution was then buffered to 5.2 by adding 600µl of 0.2M sodium acetate (pH 5.2) and 150µl acetic acid (1M) to each tube. 500µl aliquots were removed and 10µl, containing 3 mUnits trehalase (Sigma-Aldrich, UK) suspended in deionised water, was added (trehalose assay). 10µl of 10mg/ml α-amyloglucosidase (Sigma-Aldrich, UK) was added to the remaining 500µl (glycogen assay). Trehalose samples were

digested for a period of at least 8 hours in a 37ºC waterbath (Northern Media, UK). Glycogen samples were incubated in a 57ºC waterbath for an identical period of time.

The concentration of glucose produced from glycogen and trehalose was determined by means of a quantitative spectrophotometric method utilising the glucose assay kit (Megazyme, Ireland), according to the method of Parrou and Francois (1997). This method involves a coupled enzymatic reaction. In the presence of oxygen and glucose, glucose oxidase produces hydrogen peroxide, which reacts with p-Hydroxybenzoic acid and 4 Aminoantipyrene in the presence of Peroxidase to form a pink coloured Quinoneimine dye. The intensity of the pink colour was determined spectrophotometrically by determining the absorbance at 510nm wavelength against a reagent blank to obtain ΔA_{sample} and $\Delta A_{D\text{-glucose standard}}$. The D-glucose level was calculated as shown in equation 3.

Equation 3: The amount of glycogen and trehalose presented in samples was calculated from absorbance reading.

$$\text{D-Glucose } (\mu g/0.1 \text{ mL}) = \frac{\Delta Asample\ X\ 100}{\Delta A\ D-glucose\ standard\ (100\mu g)}$$

Assays were performed in triplicate to ensure statistical validity. Glycogen and trehalose concentrations are expressed as µg equivalent glucose per 10^8 cells, or as µg/0.1 mL of a $1x10^9$ cells/ml sample.

2.8 Fermentation of corn mash to ethanol

2.8.1 Corn Mash

Corn mash samples were obtained from Greenfield Ethanol (Canada) and stored at -80^{o}C until required. Prior to use samples were defrosted overnight at room temperature. Corn mash consisted of approximately 18% sugars. HPLC analysis (Section 2.8.2) was performed to provide a simple indication of the range of sugars available, rather than for complete characterisation. This analysis indicated that the main component of corn mash were complex sugars (DP2, DP3 and DP4+), while initial fermentable disaccharides and glucose were only present in small concentrations (Figure 2.2). Low concentrations of glycerol were also detected.

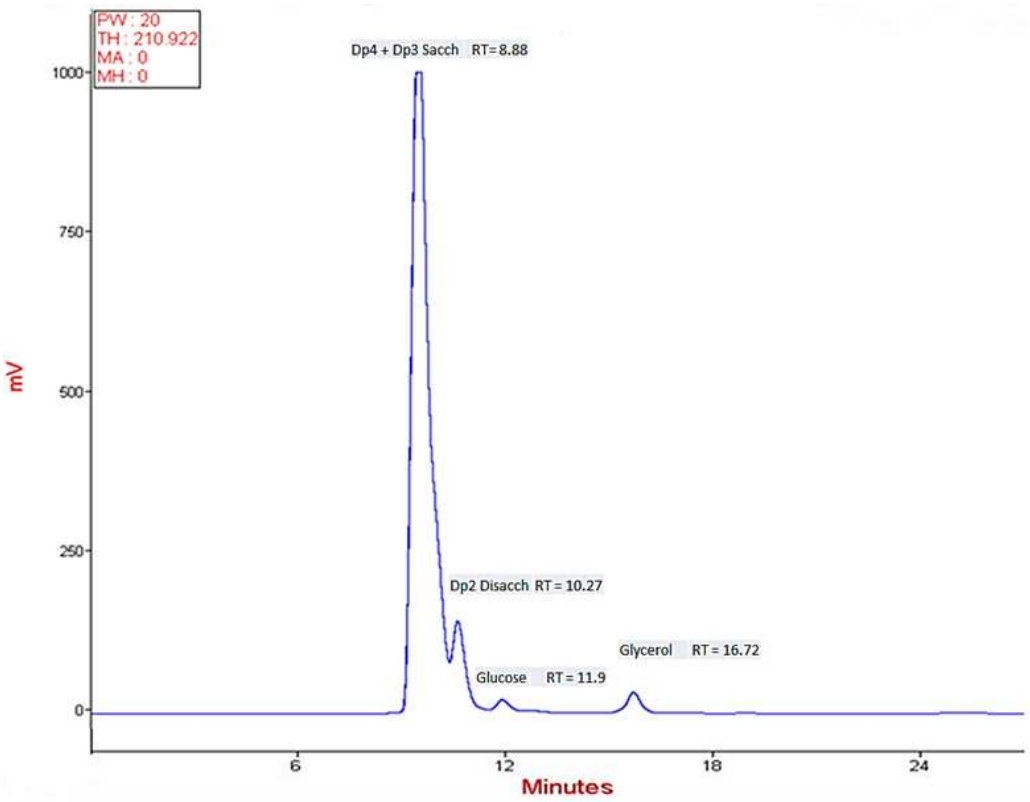


Figure 2.2: Analysis corn mash composition by high pressure liquid chromatography (HPLC). An injection of 10µl volume of corn mash from 1ml prepared sample in vial tube was analyzed for 28 minutes.

2.8.2 Sugar content analysis of corn mash by HPLC

HPLC analysis was performed to provide an indication of the relative concentrations of sugars within the corn mash composition, not to directly quantify sugars and glycerol.

Approximately 30ml of corn mash was transferred into 50ml conical flasks and centrifuged at 4^{o}C, 3500 rpm for 5 min. The supernatant was taken using a 20ml sterilized syringe (Sigma-Aldrich, UK) and filtered through Whatman® GD/X syringe filter 0.45µm pore size, glass fiber membrane (Sigma-Aldrich, UK). 1ml of the filtered samples was collected in a 2.2ml HPLC tube (Chromacol, UK). The tubes were then sealed using polyethylene caps (Sigma-Aldrich, UK). All samples were analysed in triplicate.

Sugar composition was determined using a Jasco AS-2055 Intelligent autosampler (Jasco, Japan) and a ROA column, 5µl, 4.6 x 250 mm (Phenomenex, UK) by chromatographic separation. The mobile phase was 0.005M H_2SO_4 (Sigma-Aldrich, UK) with flow rate of 0.5 mL per min. A Jasco RI-2031 Intelligent refractive index detector (Jasco, Japan) was used for detection. Azur software (version 4.6.0.0, DATALYS, France) was used to obtain data. 10µl volume of sample was injected and the analysis was completed within 28 minutes. Different retention times were displayed associated with specific compounds present in the corn mash.

2.8.3 Small-scale laboratory fermentations

2.8.3.1 Fermentation in mini fermentation vessels (FVs)

Fermentations were performed in glass serum bottle using a method adapted from Quain et al (1985). Prior to fermentation, 150-ml serum bottles (Sigma, UK) containing 20mm magnetic followers were autoclaved at 121^{o}C, 15psi for 15 minutes. A 100ml aliquot of corn mash (approximately, 105g) (Section 2.2.1.2) was aliquoted into each fermenter. Before dispensing into bottles, the corn mash was checked to ensure that it was not below pH=5. The pH was determined using a Hanna PH212 pH meter (Hanna, Norway) with calibration using pH 4.0 and pH 7.0 phosphate buffers (Fisher Scientific, UK). A dose of glucoamylase enzyme, 136µl of Liquozyme SC DS (Novozyme, UK) was added into 100ml corn mash at time 0h of fermentation and another 90µl dose was added after 7 hours. A volume of 600µl urea solution 4M (Sigma-Aldrich, UK) was added into bottles to provide a nitrogen source for each yeast culture.

Pitching was performed using yeast cell cultures taken after laboratory propagation (Section 2.5.1.1) for wet yeasts and after rehydration (section 2.2.2) for dry yeasts. Pitching is described in more detail below (Section 2.8.4)

Miniature fermenters were sealed using suba seals (Fisher, UK) and metal crimps (Fisher, UK) using a handheld crimper. A Bunsen valve gas outlet port was constructed by inserting a sterile needle connected to a Durham tube (10mm diameter) via a section of silicone tubing (10mm diameter). The tubing exhibited a narrow cut in its structure to allow carbon dioxide release without ingesting air. Plastic cable ties were used to fasten the tubing to the needle and Durham tube (Figure 3). Fermentations were then incubated in a constant temperature incubator at 30^{o}C. A flat-bed 15-space submersible

magnetic stirrer at 500 rpm was used to ensure homogeneity of the suspensions during fermentations. All samples were conducted in triplicate.

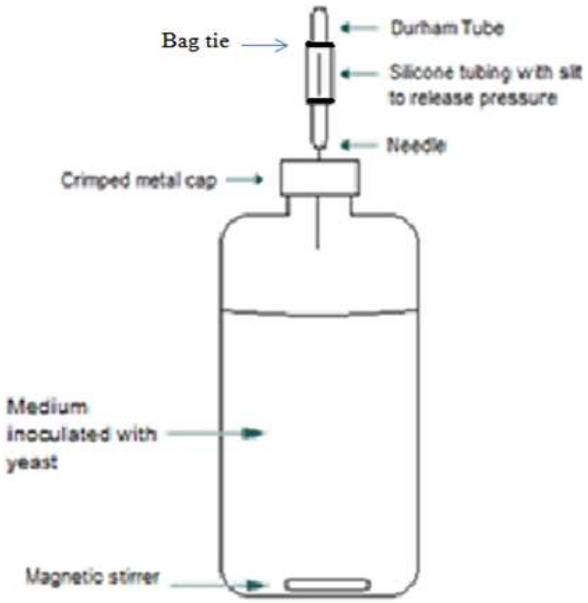


Figure 2.3 Mini fermentation vessels (FV) used for anaerobic fermentations. After pitching the cultures into the mini FVs, the bottles were sealed using suba seals and metal crimp seals using a handheld crimper. A gas outlet port was constructed using a sterile needle and Durham tube connected via a section of silicone tubing with a narrow cut in its structure.

2.8.3.2 Fermentation of corn mash to ethanol

After preliminary experiments using the fermentation vessels described above (Section 2.8.3.1), an alternative laboratory scale fermentation system was designed to examine the fermentation performance of different yeast strains. This was done as the previous system was deemed to be insufficiently agitated since yeast and corn mash were not always dispersed evenly, but accumulated at the side of the vessels despite the use of magnetic stir bars. Specifically,

100ml of corn mash (Section 2.2.1.2) was added to 250ml Erlenmeyer flasks. The flasks were sealed using foam bungs and autoclaved at 121^{o}C, 15psi for 15 minutes. The same procedure was applied (including the amount of glucoamylase enzyme and urea solution) as for the miniature FVs fermentation (Section 2.2.1.2). The flasks were then covered with a double layer of parafilm (Nescofilm, Osaka, Japan) completely covering the bung to prevent any gas ingestion. A bunsen valve was also inserted through the parafilm and foam bung using a sterile needle to prevent pressure build up (Figure 2.4). Flasks were incubated in an orbital shaker (Certomat, BS-1, Sartorius, USA) at 30^{o}C, 130 rpm. All experimental flasks were prepared and analysed in triplicate

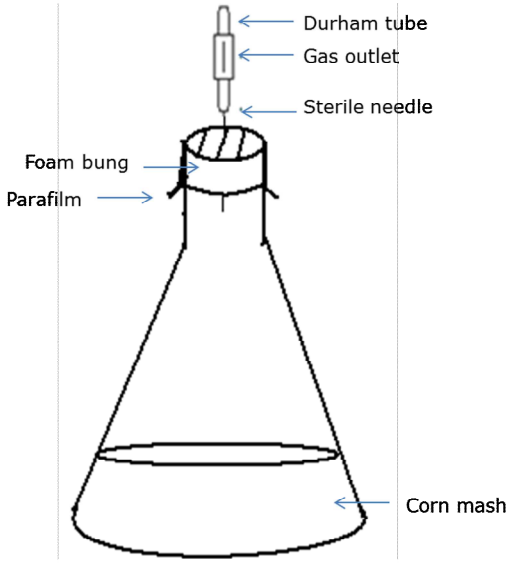


Figure 2.4 Flask fermentation design used for anaerobic fermentations. After pitching the cultures the flasks were sealed using a foam bung covered with two layers of parafilm. A gas outlet port was constructed using a sterile needle and Durham tube connected via a section of silicone tubing with a narrow cut in its structure for gas release.

2.8.4 Pitching small scale fermentations

Before fermentation, all yeast cultures (including propagated wet yeasts and rehydrated dry yeasts) were checked for cell number and viability using the protocol described in Section 2.3. This was performed to ensure that an accurate pitching rate could be achieved and also as a means of evaluating any changes in viability at the end of fermentation. The pitching rate typically employed was $1x10^7$ cells/ml per milliliter of corn mash, or in 100ml of corn mash media a total of $1x10^9$ cells. In this study, an additional pitching rate of $2x10^7$ cells/ml was also examined in order to determine how altering pitching rate affected the fermentation.

2.8.5 Assessment of fermentation progression

Fermentation progression was monitored by measuring sugar ultilization in terms of weight loss over time (Jakobsen and Lie, 1982; Ayrapaa, 1973). Fermentation vessels (mini FVs and flasks) were weighed at approximately 6 hour intervals until no further changes were observed. The sampling points typically used were 0h, 7h, 24h, 44h, 55h, 65h and 72h. Typically, no further change was observed around 30 to 72 hours after inoculation, depending on the pitching rate.

2.8.6 Determination of ethanol production

After fermentation was complete, approximately 40ml of corn mash culture was transferred into 50ml conical flasks. The flasks were then centrifuged at 4^oC, 3500 rpm for 5 minutes. A 15ml sample of supernatant was taken using a sterilise syringe and filtered through Whatman® GD/X syringe filter 0.45µm

pore size, glass fiber membrane (Sigma-Aldrich, UK). 1ml of the filtered sample was collected in a 2.2 ml HPLC tube (Sigma, UK). All samples were analysed in triplicate to ensure statistical accuracy. Given that each fermentation was also run in triplicate, in total 9 samples were examined for each strain.

Corn mash samples were then analyzed by using Jasco AS-2055 Intelligent autosampler (Jasco, Japan) and a ROA column, 5µl, 4.6 x 250 mm (Phenomenex, Macclesfield, UK) by chromatographic separation. The mobile phase was 0.005M H_2SO_4 (Sigma-Aldrich, UK) with flow rate of 0.5 mL per min. A Jasco RI-2031 Intelligent refractive index detector (Jasco, Japan) was used for detection. Azur software (version 4.6.0.0, DATALYS, France) was used to obtain data. 10µl volume of sample was injected and the analysis was completed within 28 minutes. Different retention times were displayed associated with specific compounds present in the corn mash. Ethanol concentration was determined by comparing unknown samples to a series of ethanol standards, prepared in 2.2 mL vial tubes (Fisher Scientific, UK): 1%, 2%, 4%, 6%, 8% and 10% (v/v). A standard curve of % (v/v) ethanol against the peak area was plotted and used for determination of ethanol at the end point of fermentation.

2.8.7 Yeast growth and yeast viability analysis post-fermentation

After fermentation was completed, 40ml of corn mash containing yeast was filtered through a sterilized muslin bag into 50ml conical flasks. The bag was carefully squeezed in order to extract the supernatant containing yeast cells from corn mash. This procedure was developed as analysis of yeast viability in

the presence of corn mash yielded inconsistent data. In order to determine the number of cells dead or live, yeast suspensions were diluted if required before staining with Methylene blue (Section 2.3) at ratio 1:1. The same procedure was performed to calculate the cell number and cell viability as described previously (Section 2.3).

2.9 Heat-treatment of ethanol yeast strains

ADY strains were rehydrated as described previously (Section 2.2.2). Subsequently three 50ml shake flasks were prepared containing 5ml of the respective ADY (either Thermosacc®Dry Yeast or Superstart^{TM} Instant Dry Yeast). Each yeast was heat treated by incubating in a static incubator (Certomat, Sartorius, USA) at either 35^{o}C or 40^{o}C. During heat treatment samples were obtained after 0.5h, 1h and 2h and yeast suspensions were analysed for cell count and cell viability using the procedure described in section 2.3.

Heat treated cells were typically assessed using the methodology described above, including fermentations (Section 2.8.3), fermentation analysis (2.8.5) and analysis of ethanol (Section 2.8.6) and intracellular glycogen and trehalose (Section 2.7).

2.10 Statistical Analysis

The mean and standard deviation of a data set was calculated by using the AVERAGE and STDEV equation in Microsoft® Excel 2011 (Microsoft Corporation, USA).

Chapter 3: The effect of temperature on the growth characteristics of ethanol producing yeast strains

3.1 Introduction

It is known that there are various stresses which occur prior to and during the fermentation processes including temperature, osmotic, pH, nutrient deprivation, and stresses associated with the accumulation of ethanol and carbon dioxide (Hirasawa *et al*., 2006, Gibson *et al*., 2007). In order to maintain fermentation performance as well as survive, bioethanol yeast strains must cope with these environmental changes by relying on their physiological response mechanisms (Graves *et al*., 2007, Gibson *et al*., 2007).

Temperature is one of the most important physical parameters which has a direct influence on yeast growth and fermentation performance (Walker, 1998). Although many yeasts exploited for alcohol production are mesophilic organisms which are capable of growth between 0^{o}C and 48^{o}C, the preferred temperature for *Saccharomyces* yeasts is between 25 to 35^{o}C (Walker, 1998). As described previously (Chapter 1), temperature has a direct affect on the growth and productivity of yeast. It is known that yeast growth rate and metabolism increases when temperature is raised from sub-optimal to optimal temperatures and decreases when temperature is increased (Serra *et al*., 2005). However, the optimum temperature for yeast growth is a narrow range and analysis of the metabolic response of *S. cerevisiae* to continuous heat stress has demonstrated that when the temperature is increased to 43^{o}C, yeast cells began to lose their viability (Mensonides *et al*., 2002).

There have been many studies focused on improving heat resistance in yeast in attempt to maintain performance while increasing metabolic activity

(Hottiger *et al*., 1987a, b; Piper, 1995; Lu *et al*., 2011). Previous reports have revealed that trehalose, a disaccharide composed of two molecules of D-glucose which functions as a reserve carbohydrate (Thevelein, 1984), is a particularly important stress protectant. Trehalose can protect cells against various stress factors including osmotic stress (MacKenzie *et al*., 1988; Majara *et al*., 1996a), oxidative stress (Mansure *et al*., 1994, Parrou *et al*., 1997), ethanol stress (Eleutherio *et al*., 1993; Mansure *et al*., 1994; Majara *et al*., 1996b) as well as heat stress (Hottiger *et al*., 1987a, b; D'Amore *et al*., 1991; Piper, 1995; Majara *et al*., 1996a, b; Conlin and Nelson, 2007) and is known to accumulate in *Saccharomyces cerevisiae* in response to heat shock (Hottiger *et al*., 1987a).

Since temperature stress and trehalose concentrations can have such an important impact on the growth characteristics of yeast strains with subsequent affects on fermentation performance, in this chapter four ethanologenic yeast strains were used to examine the influence of temperature on their physiological characteristics. More specifically, each yeast strain was examined for cell size at different temperatures, the temperature limits at which cells could grow and survive and the capacity to accumulate trehalose at various temperatures.

3.2 Results

Bioethanol *S.cerevisiae* yeast strains LAL7 and Thermosacc were obtained from Lallemand Inc (Canada) whilst Ethanol Red was obtained from Fermentis (France), and 2592 from the National Collection of Yeast Cultures (UK). The yeasts were all supplied in active dried form and were rehydrated

prior to use as described in Chapter 2. Each strain was then assessed for its physiological characteristics in response to temperature.

3.2.1 The effect of temperature on yeast growth on solid medium

In order to assess the effect of temperature on cell growth, the spot plate assay was used. This method has previously been employed for analysis of stress and inhibitor tolerance (Miyazaki *et al*., 2004; Lewis *et al*., 2010; Grosshans *et al*., 2006; Avone *et al*., 2010) and relies on the ability of yeast strains to produce colonies on nutrient agar plate under specific conditions. The growth and formation of colonies on YPD plates was therefore used as the basis for comparison between yeast strains at different temperatures. In this study a series of dilutions were carried out from 10^1 to 10^4, and samples of each were spotted onto plates and incubated at 10, 15, 20, 25, 30, 35, 40 and 45^{o}C until colonies appeared, as described previously (Section 2.4.1.2).

After 2 days of incubation, the results indicated that all yeast strains were able to be grown at a wide range of temperatures ranging from 20^{o}C to 40^{o}C (Figure 3.1). The amount of growth of each of the four yeast strains was quite similar between temperatures of 25^{o}C and 40^{o}C. However, it was noticed that at 20^{o}C the colonies of all yeast strains only appeared at dilutions of 10^{-1} and 10^{-2} and not at higher dilutions. Moreover, at 45^{o}C there was only minor growth of Thermosacc, whilst *S.cerevisiae* Ethanol Red, LAL7 and 2592 were not able to be cultivated at these temperatures. Furthermore, no colonies were observed for any of the four yeast strains at 10 or 15^{o}C.

After 5 days of incubation, full growth was observed at all dilutions when cells were incubated at temperatures of between 15^{o}C and 40^{o}C (Figure 3.2). It

was interesting to observe that at 40^{o}C strain 2592 produced the greatest amount of growth, whilst Ethanol Red and Thermosacc were quite similar, at a lower level (Figure 3.2). Yeast strain LAL7 showed the least amount of growth from any of the four strains at 40^{o}C. However, at the extreme temperatures (10 and 45^{o}C) there continued to be differences. Observation of yeast at 10^{o}C indicated that colonies from each of the four strains were only present at innoculation rates of 10^{-1} and 10^{-2}, and growth at 10^{-3} and 10^{-4} was not observed. At 45^{o}C there was no change in the result previously observed; although Thermosacc yeast showed clearer growth than after 2 days incubation, there was still no sign of growth for the other strains. Furthermore, growth for Thermosacc continued to be poor, indicating that even this yeast struggled to produce biomass at this temperature. After 7 days there was an identical pattern of results, indicating that only Thermosacc was able to grow at this temperature (Data not shown). For growth at low temperatures (10 and 15^{o}C), Thermosacc, Ethanol Red and LAL7 showed similar growth patterns, although Thermosacc may arguably have produced slightly more biomass than the other 2 strains (Figure 3.3). At 15^{o}C strain 2592 produced a similar level of biomass, however it was clear that this strain was not as tolerant to 10^{o}C as the other strains at 15 ^{o}C and was not able to grow (Figure 3.3).

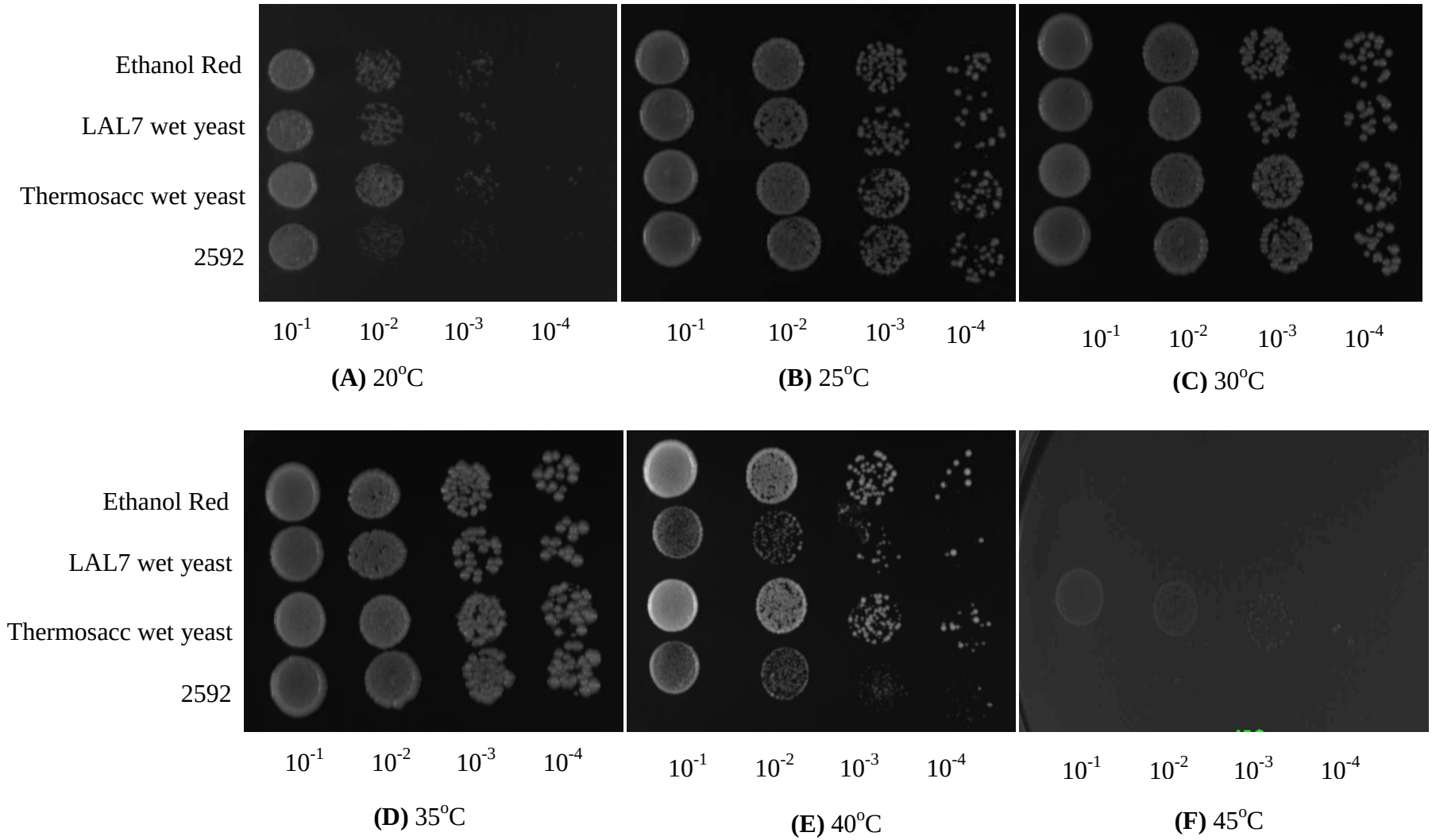


Figure 3.1. Growth of yeast strains for 2 days using spot plates at different temperatures. Sequential dilutions were spotted onto YPD plates at a volume of 10µl. Plates were then incubated in a static incubator at the desired temperature.

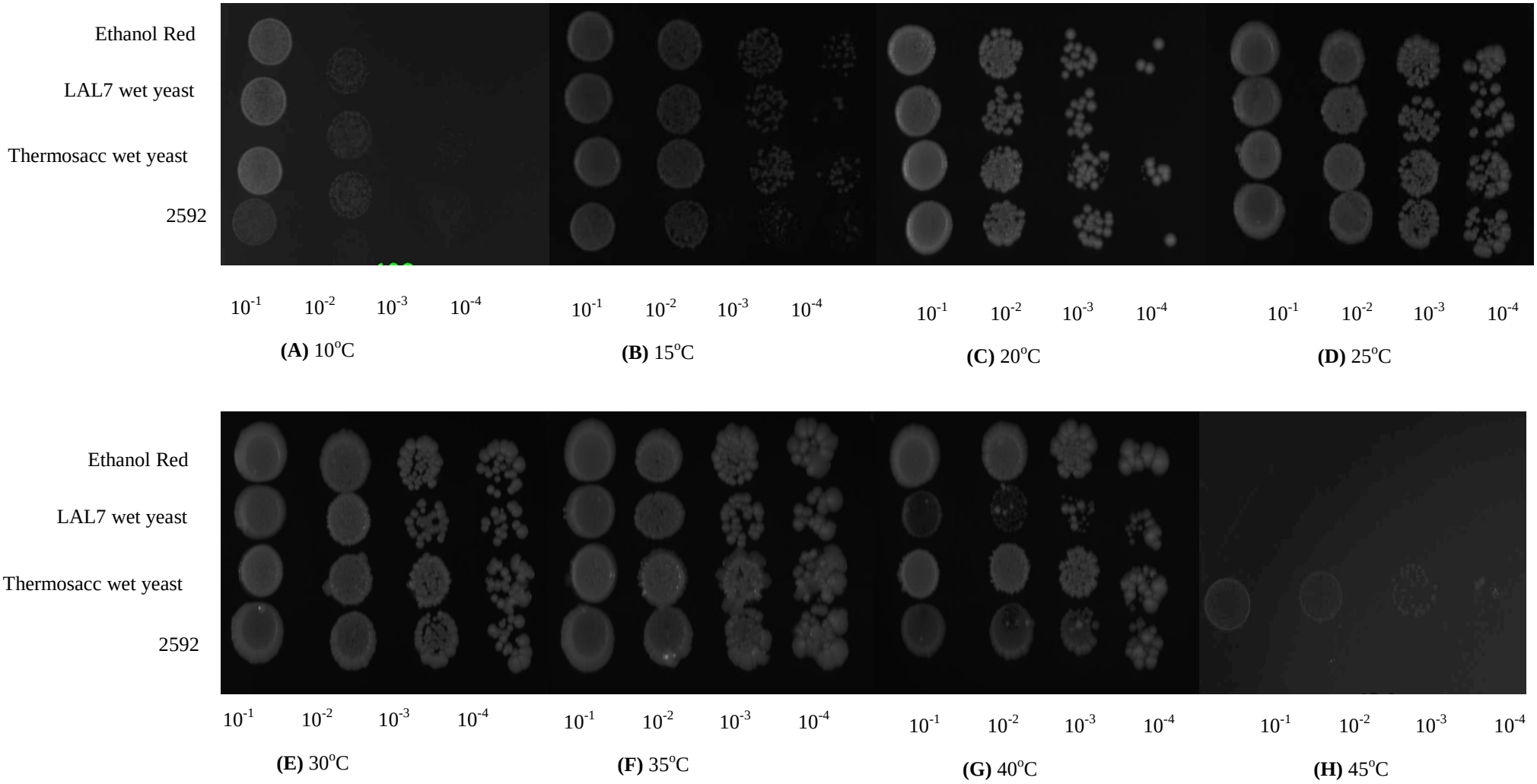


Figure 3.2. Growth of yeast strains for 5 days using spot plates at different temperatures. Sequential dilutions were spotted onto YPD plates at a volume of 10µl. Plates were then incubated in a static incubator at the desired temperature.

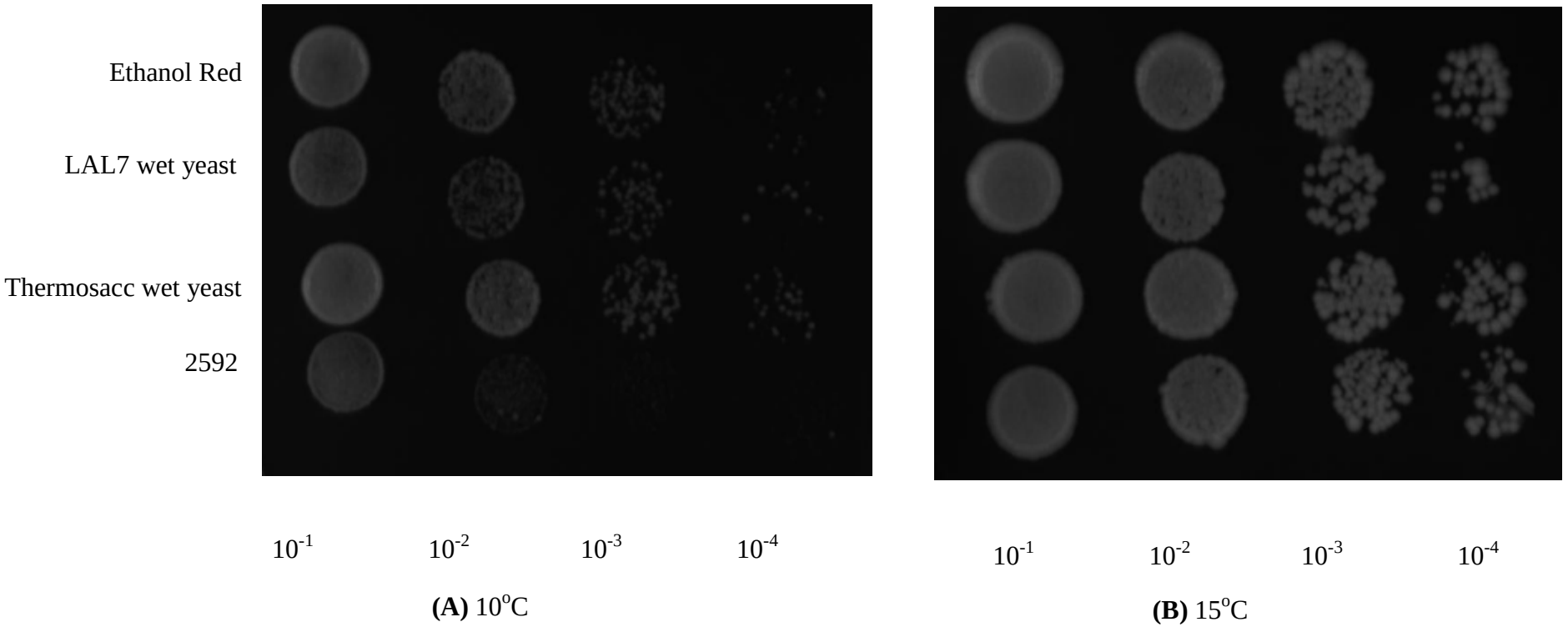


Figure 3.3. Growth of yeast strains for 7 days using spot plates at different temperatures. Sequential dilutions were spotted onto YPD plates at a volume of 10µl. Plates were then incubated in a static incubator at the desired temperature. For analysis at other temperatures, identical results to 5 days were observed.

3.2.2 The effect of temperature on yeast growth in liquid medium

Although the data obtained from growth on solid medium provided an indication of strain differences, this technique does not provide an accurate indication of growth dynamics, including lag phase and time to reach stationary phase. Consequently to achieve a more complete appreciation of the effect of high temperature on cell growth, each strain was cultivated in liquid media within 96 well plates at 25, 30, 34, 36, 38 and 40°C as described in Chapter 2. Since most strains did not grow on spot plates at 45°C, this temperature was omitted in this and subsequent experiments.

The results indicated that all yeast strains were able to grow at temperatures between 25 and 40^{o}C, supporting the previous data obtained from spot plate analysis (Section 3.2.1). However, the current analysis indicated that different yeast strains grew best at different temperatures. The growth of each strain at 25, 30, 34, 36, 38 and 40^{o}C are shown in Figures 3.4-3.9, respectively and the effect of growth at high temperature is summarized in Table 3. Growth comparisons were performed by comparing strain optical density (OD) during lag, exponential and stationary phase. In general, a similar, short, lag phase and exponential phase were observed on all yeast strains during their growth at different temperature tested. Interestingly, strain LAL7 frequently showed a longer lag phase and a lower optical density at stationary phase, perhaps indicating poorer growth or biomass production when compared to the other strains tested in this assay.

At higher temperatures all yeast strains began to adopt different growth patterns, with an obviously longer lag phase. Although this was true for all

strains, LAL7 typically showed the longest lag phase, especially at 36^{o}C and 40^{o}C (Figure 3.5 and Figure 3.6). At temperatures of 36°C and above, all strains began to exhibit an altered stationary phase with either a lower cell density being achieved or a reduction in OD at this time, indicating possible cell death almost as soon as lag phase had completed. This was particularly evident for LAL7 and Ethanol Red at 38°C (Figure 3.8) which appeared to be sensitive to higher temperatures. In contrast, Thermosacc and 2592 showed greater resilience, and serious effects on growth were not observed until 40°C, characterised by restricted growth and reduced biomass production (Figure 3.9).

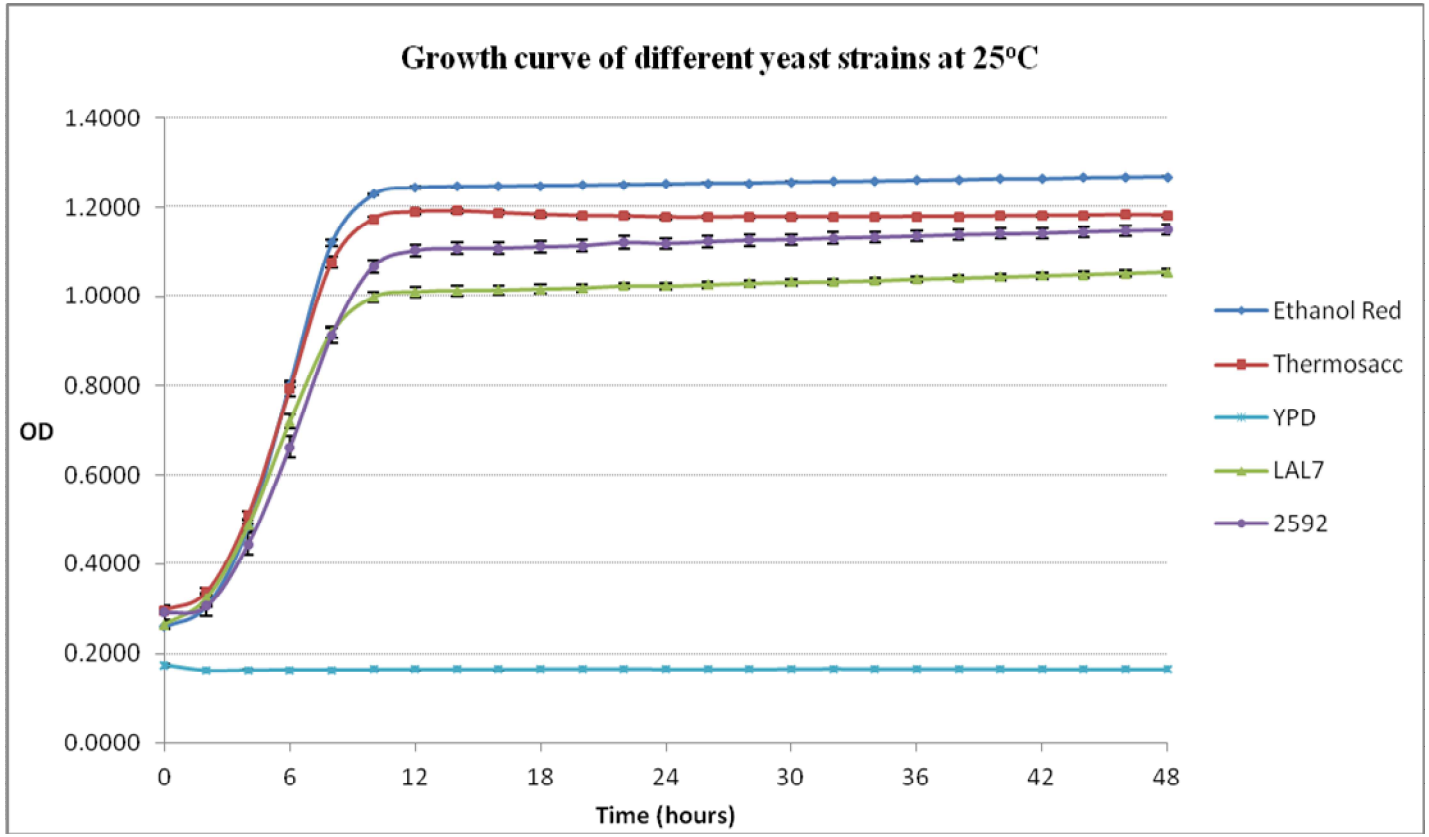


Figure 3.4. Growth curve analysis of Ethanol Red, Thermosacc, LAL7 and 2592 at 25°C. In each instance $1x10^6$ cells per mL were inoculated in triplicate into a 96-well plate. Cell growth was determined by automated measurements of optical density at 600nm each hour. Error bars indicate the standard deviation of triplicate samples.

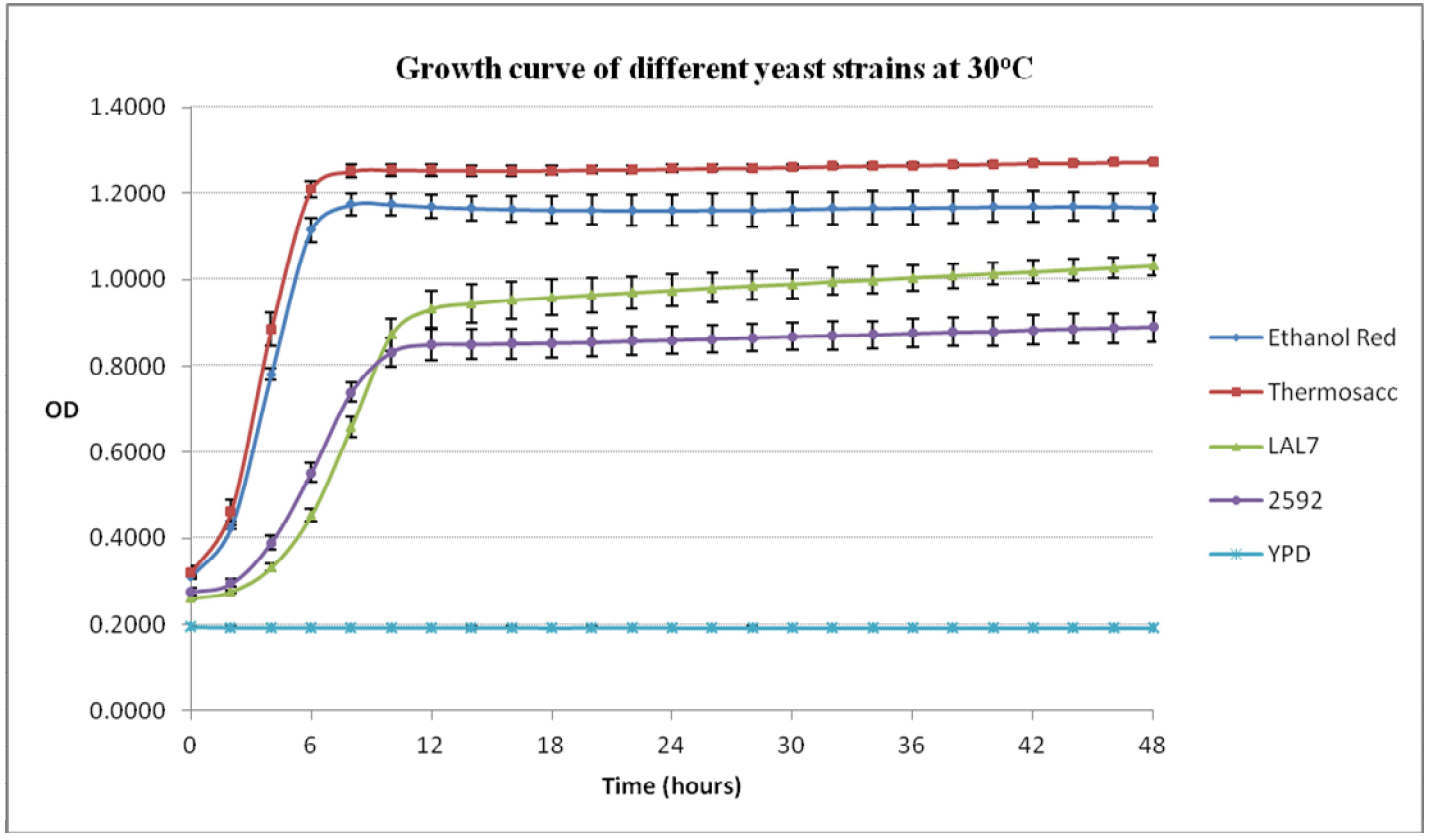


Figure 3.5. Growth curve analysis of Ethanol Red, Thermosacc, LAL7 and 2592 at 30°C. In each instance $1x10^6$ cells per mL were inoculated in triplicate into a 96-well plate. Cell growth was determined by automated measurements of optical density at 600nm each hour. Error bars indicate the standard deviation of triplicate samples.

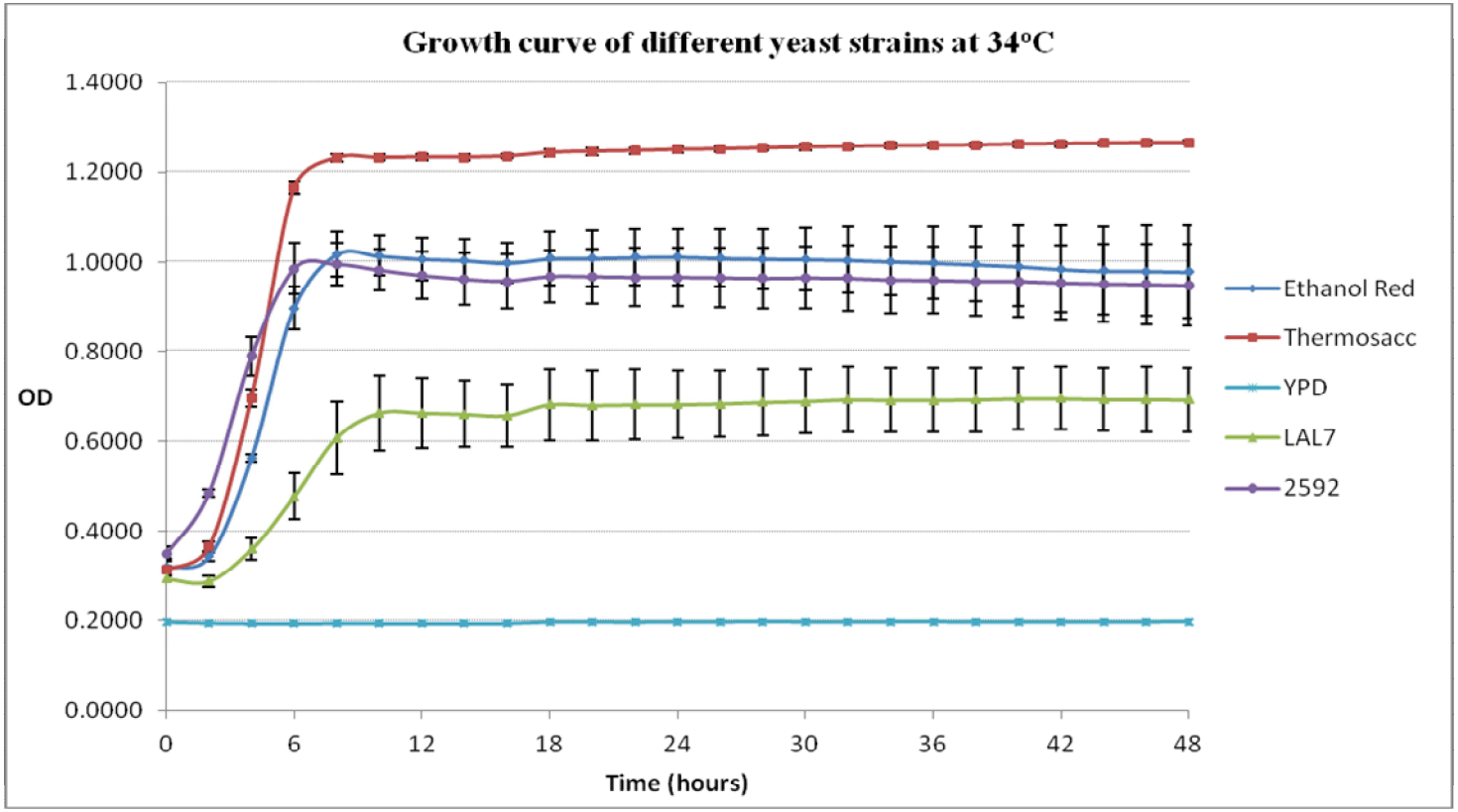


Figure 3.6. Growth curve analysis of Ethanol Red, Thermosacc, LAL7 and 2592 at 34°C. In each instance $1x10^6$ cells per mL were inoculated in triplicate into a 96-well plate. Cell growth was determined by automated measurements of optical density at 600nm each hour. Error bars indicate the standard deviation of triplicate samples.

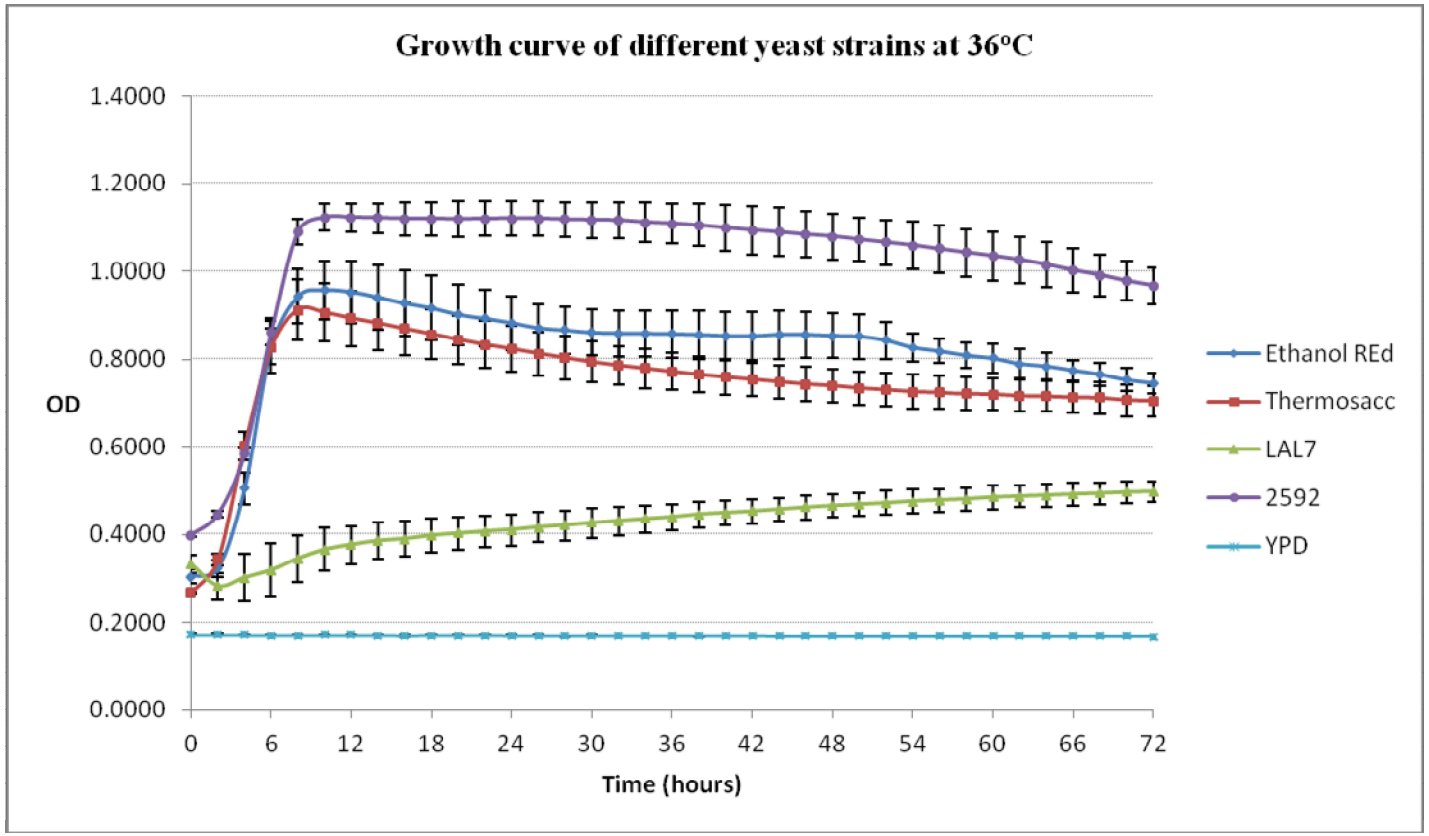


Figure 3.7. Growth curve analysis of Ethanol Red, Thermosacc, LAL7 and 2592 at 36°C. In each instance $1x10^6$ cells per mL were inoculated in triplicate into a 96-well plate. Cell growth was determined by automated measurements of optical density at 600nm each hour. Error bars indicate the standard deviation of triplicate samples.

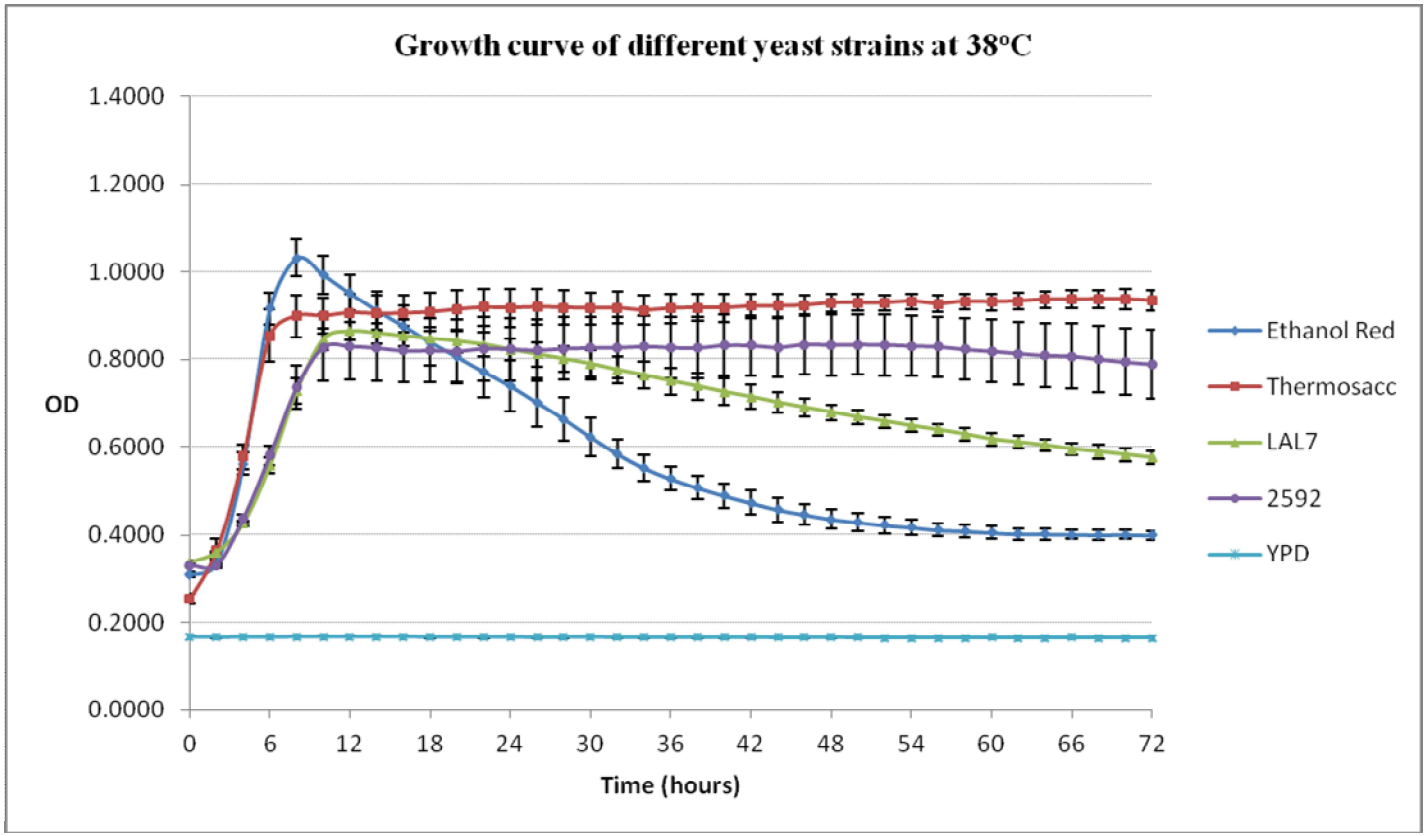


Figure 3.8. Growth curve analysis of Ethanol Red, Thermosacc, LAL7 and 2592 at 38°C. In each instance $1x10^6$ cells per mL were inoculated in triplicate into a 96-well plate. Cell growth was determined by automated measurements of optical density at 600nm each hour. Error bars indicate the standard deviation of triplicate samples.

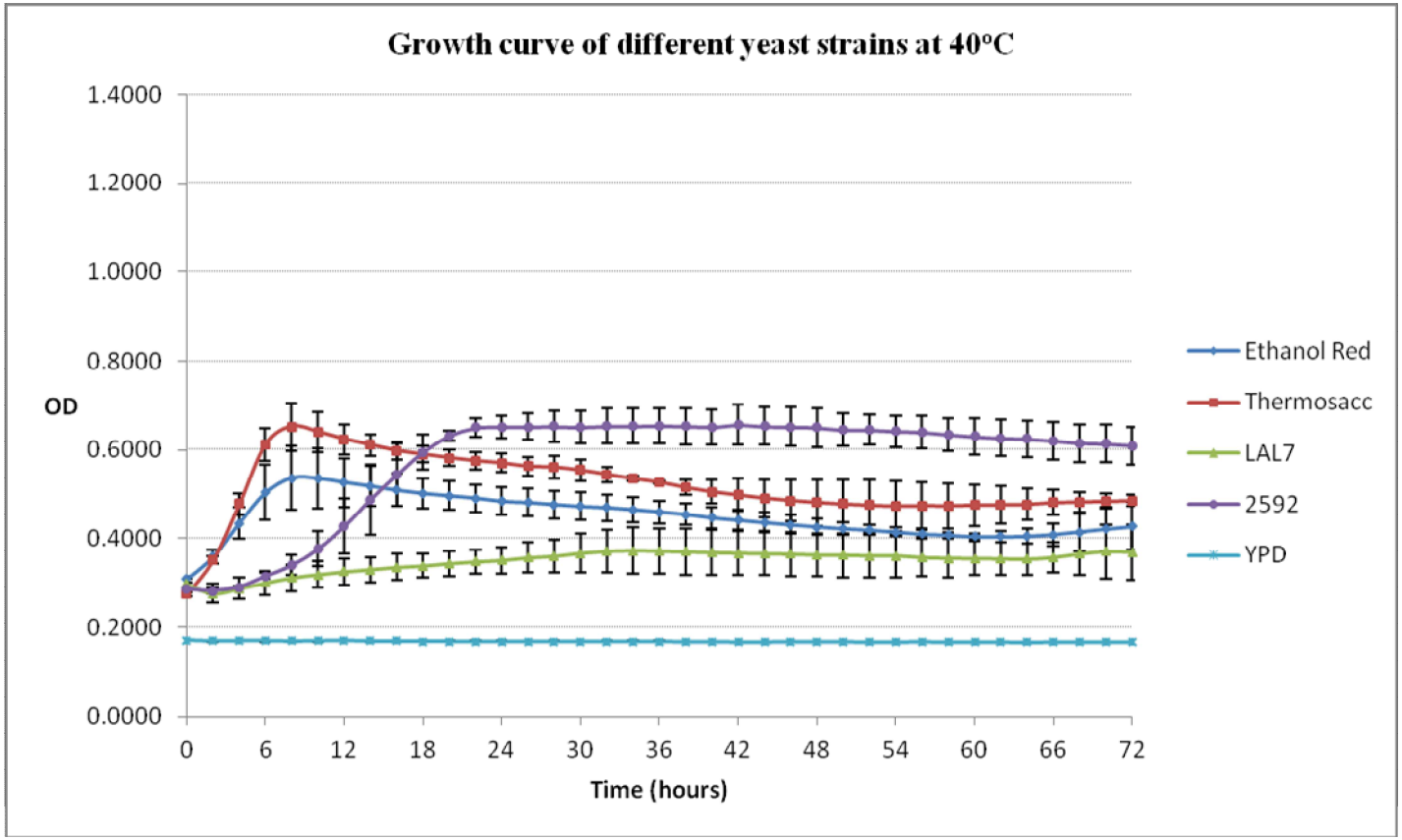


Figure 3.9. Growth curve analysis of Ethanol Red, Thermosacc, LAL7 and 2592 at 40°C. In each instance $1x10^6$ cells per mL were inoculated in triplicate into a 96-well plate. Cell growth was determined by automated measurements of optical density at 600nm each hour. Error bars indicate the standard deviation of triplicate samples.

Table 3.1: Summary of the growth as determined by final OD of yeast strains in liquid YPD medium at different temperatures. The abundance of growth is represented by: (++++) as best growth among 4 yeast strains at the temperature incubated, (+) as weakest growth among 4 yeast strains at the temperature incubated, (-) as no growth. YPD indicates an un-inoculated control sample.

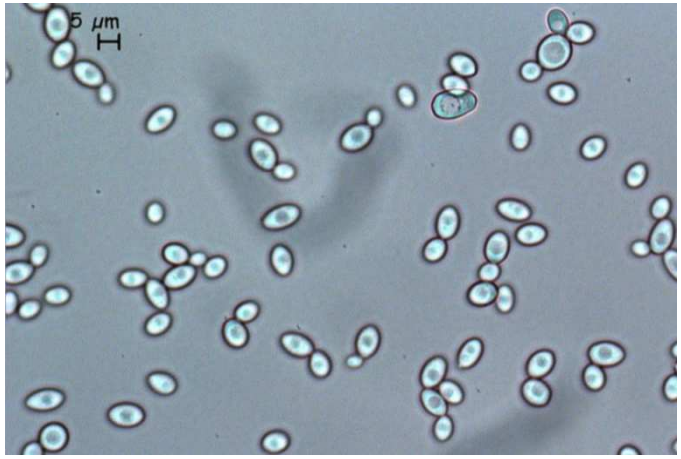
	Ethanol Red	Thermosacc	LAL7	2592	YPD
25^{o}C	++++	+++	+	++	-
30^{o}C	+++	++++	++	+	-
34^{o}C	+++	++++	+	++	-
36^{o}C	+++	++	+	++++	-
38^{o}C	+	++++	++	+++	-
40^{o}C	++	+++	+	++++	-

3.2.3 Analysis of cell size before and after growth at different temperatures

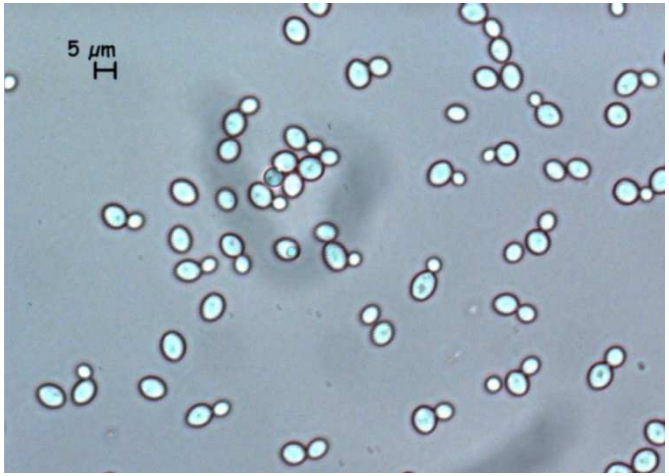
In addition to analysing cell growth at elevated temperatures, cell size was also assessed. This was performed partly due to the dependence of the previous experimentation on optical density readings, which could be influenced by cell size (Section 3.2.2.), and partly due to previous reports that temperature can have an effect on cell physiology (Csonka and Hanson, 1991; Kondo and Inouye, 1991; Kondo *et al.*, 1992; Kowalski, 1995; Canetta *et al*., 2006).

Analysis of cell size indicated that, in general, the length and width of yeast cells of each of the four strains decreased along with an increase in temperature. It was found that at all temperatures, except 40^{o}C, LAL7 had the largest cell size (182.74 µm^{3}), whilst 2592 had the second biggest size among the strains tested in this study (Figure 3.10; Table 3.2). This cell size is within the range specified by Lodder (1970), who reported that cells of the species *S. cerevisiae* typically range from 2.5-11µm in diameter. Before experimentation at different temperatures, cells from the Ethanol Red strain (68.11 ± 0.05 µm^{3}) were larger than those of Thermosacc, (60.95 ± 0.12 µm^{3}) (Figure 3.10; Table 3.2), however, a similar cell size was recorded after they were grown at 25, 30 and 35^{o}C (Figures 3.11, 3.12 and 3.13). At 40^{o}C, the yeast cell size of Ethanol Red was slightly larger than Thermosacc. Interestingly, at 40^{o}C the yeast cell size of strain LAL7 reduced the most whilst the size of the other strains examined only decreased slightly (Figure 3.14), with Ethanol Red having the biggest cells under these conditions.

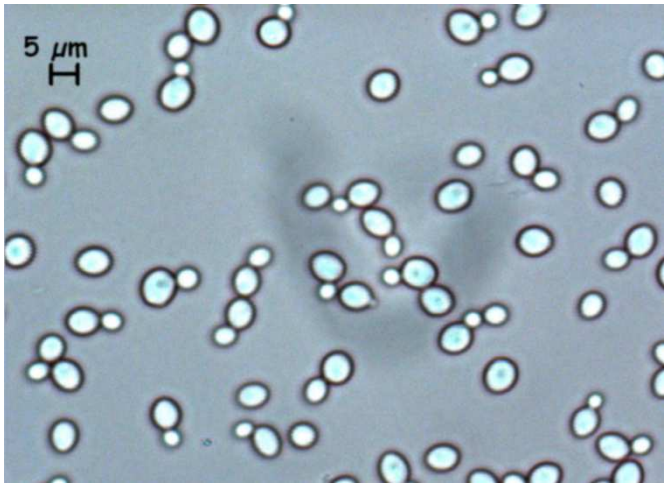
Moreover, the results also showed that all yeast strains had changes in cell morphology. Specifically, it is easy to see that the yeast cells of each of the four strains typically had an oval shape initially (Figure 3.10). However, the shape of cells tended to become more spherical as the temperature was increased. In addition, it was observed that cell-cell interactions also varied according to growth temperature. Cells of each of the four strains did not show any tendency to form clumps at 25, 30 and 35^{o}C, but were observed to produce aggregates at 40^{o}C. In particular, LAL7 was observed to produce floc-like clumps/chains, possibly due to incomplete separation of mother and daughter cells, although in some instances it appeared that the cell wall had become damaged. Some cells were found to be lysed while others appeared to be shrunken in appearance and in poor condition.



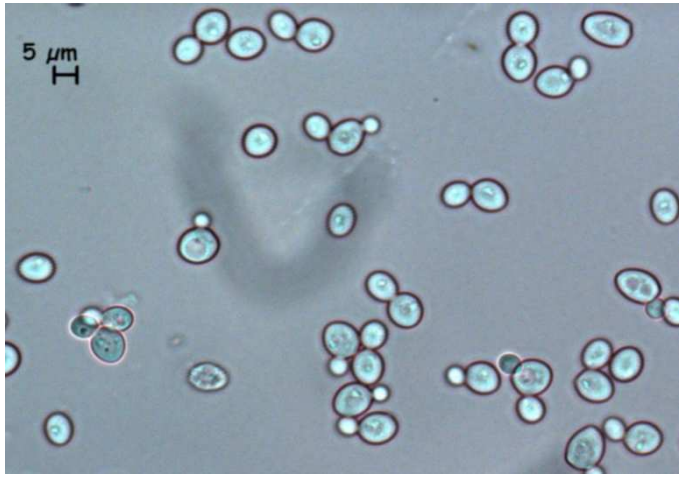
(A) 2592



(B) Ethanol Red

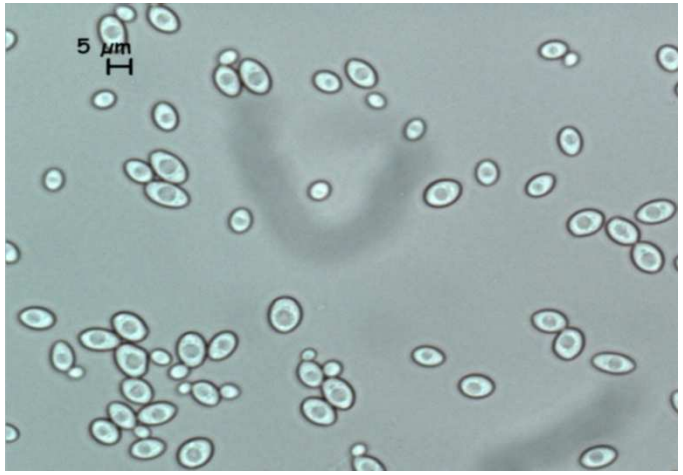


(C) Thermosacc

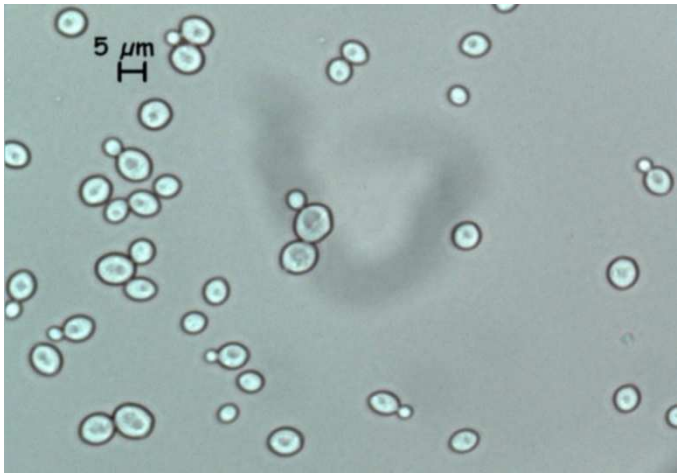


(D) LAL7

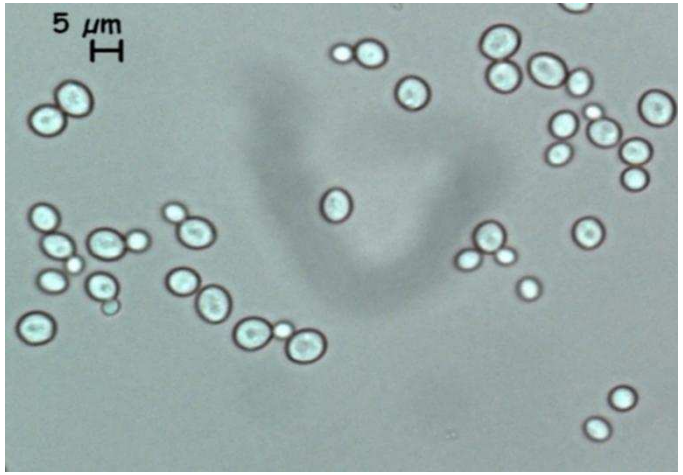
Figure 3.10. Cells of Ethanol Red, Thermosacc, LAL7 and 2592 prior to growth at different temperatures. In each instance the size bar represents 5µm. The length and width of cells was measured and used to calculate the cell volume.



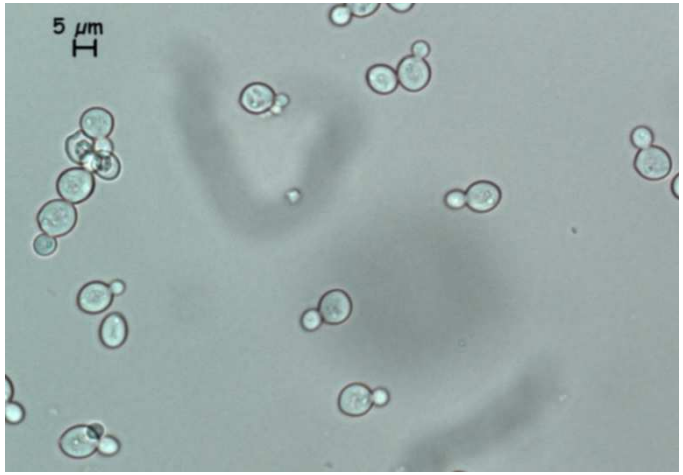
(A) 2592



(B) Ethanol Red

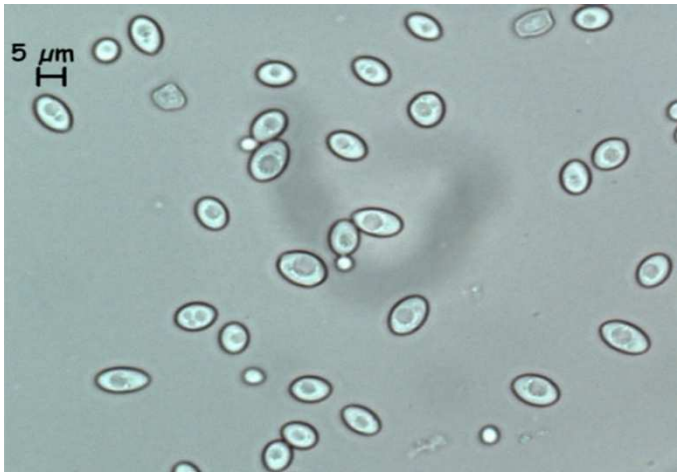


(C) Thermosacc

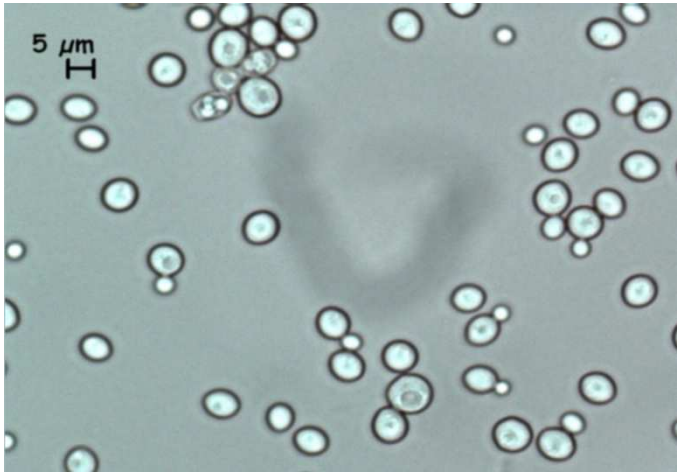


(D) LAL7

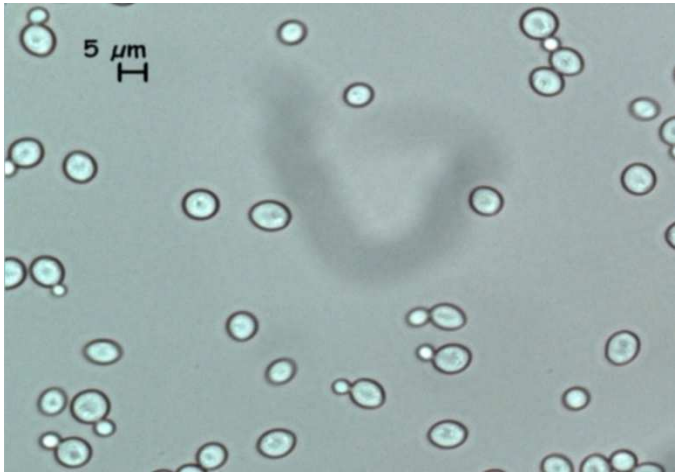
Figure 3.11. Cells of Ethanol Red, Thermosacc, LAL7 and 2592 after growth for 3 days at 25^{o}C. In each instance the size bar represents 5µm. The length and width of cells was measured and used to calculate the cell volume.



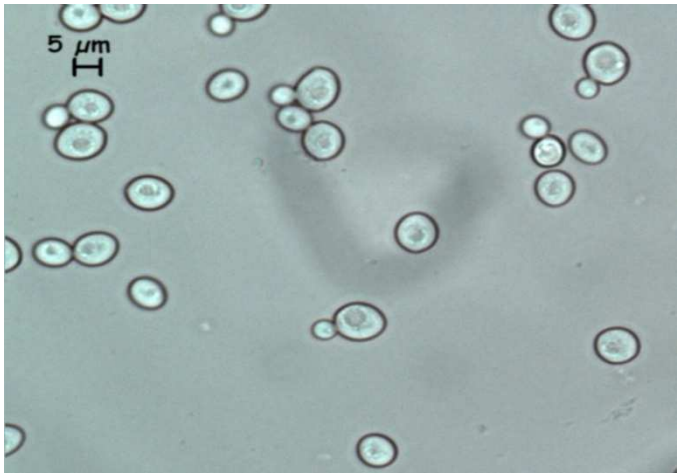
(A) 2592



(B) Ethanol Red

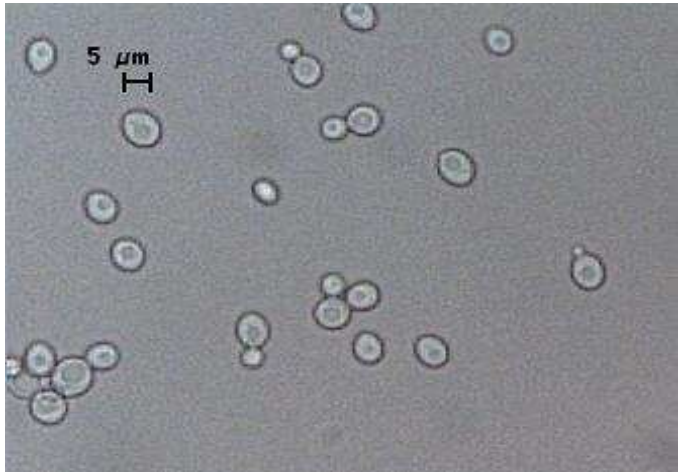


(C) Thermosacc

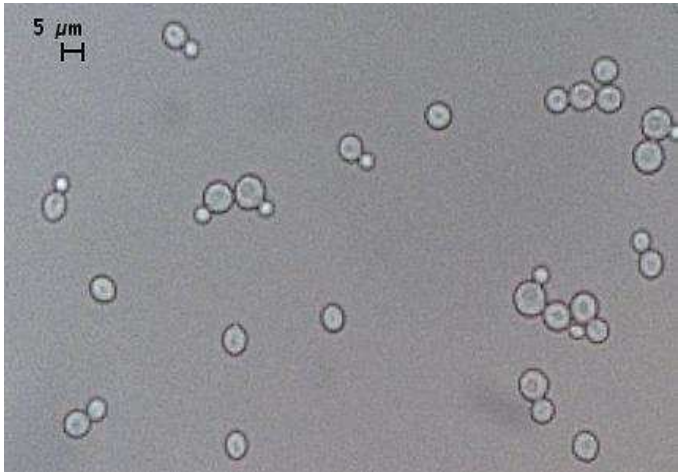


(D) LAL7

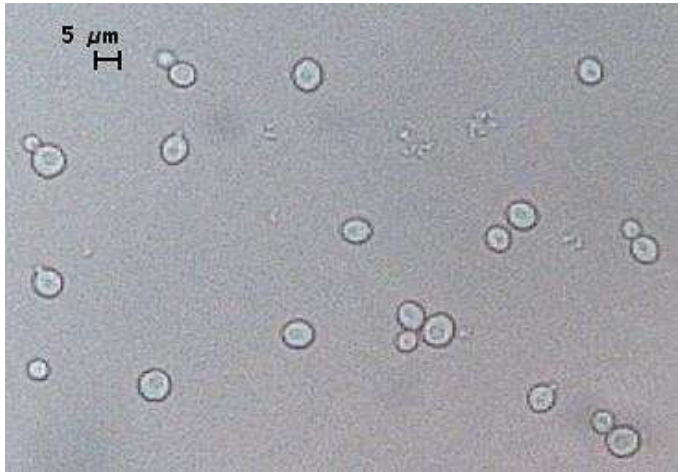
Figure 3.12. Cells of Ethanol Red, Thermosacc, LAL7 and 2592 after growth for 3 days at 30^{o}C. In each instance the size bar represents 5µm. The length and width of cells was measured and used to calculate the cell volume.



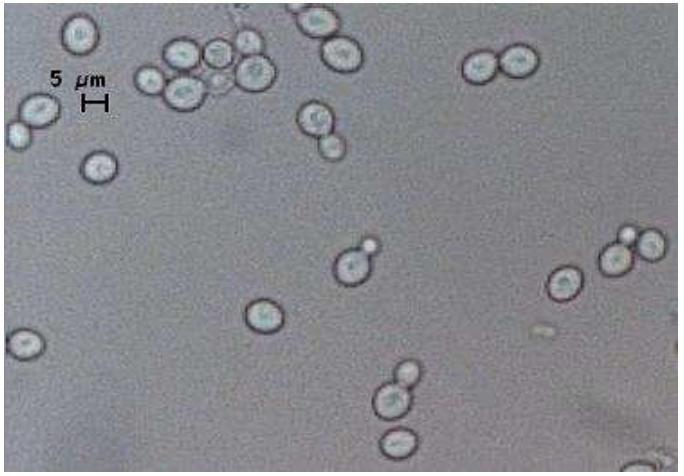
(A) 2592



(B) Ethanol Red

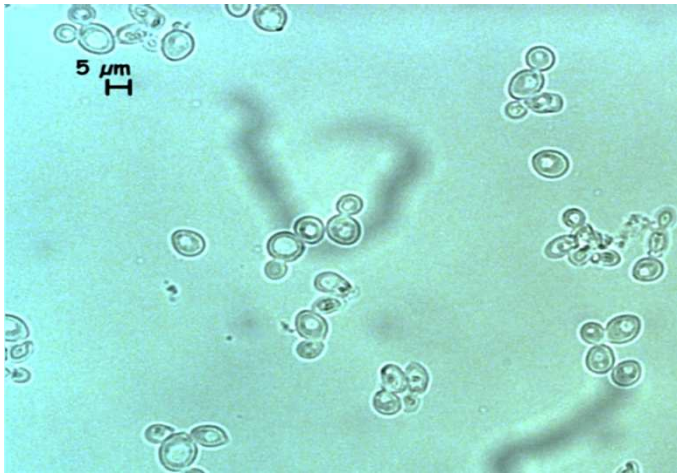


(C) Thermosacc

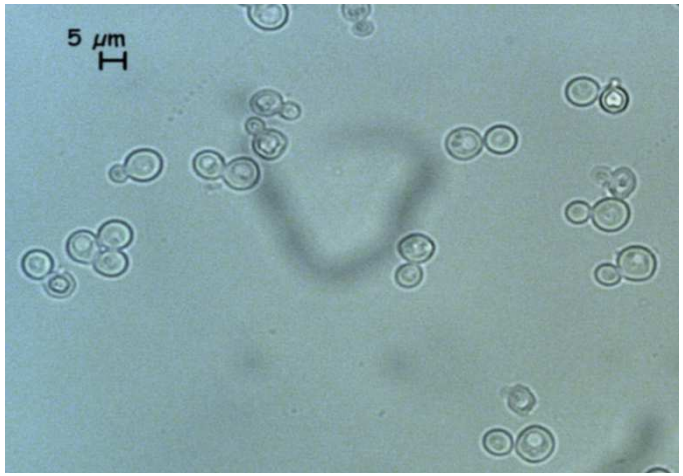


(D) LAL7

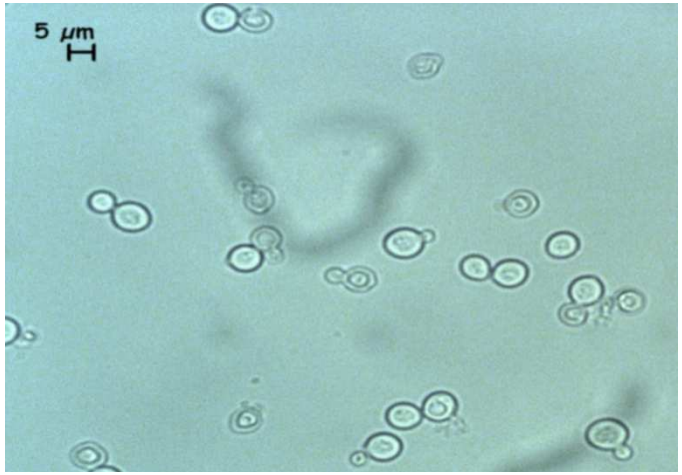
Figure 3.13. Cells of Ethanol Red, Thermosacc, LAL7 and 2592 after growth for 3 days at 35^{o}C. In each instance the size bar represents 5µm. The length and width of cells was measured and used to calculate the cell volume.



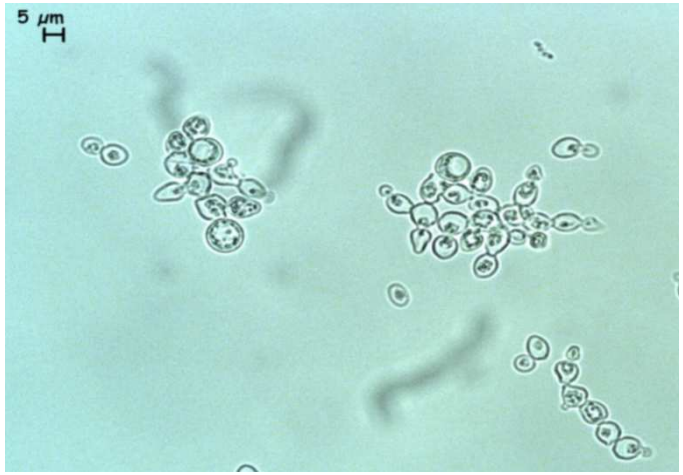
(A) 2592



(B) Ethanol Red



(C) Thermosacc



(D) LAL7

Figure 3.14. Cells of Ethanol Red, Thermosacc, LAL7 and 2592 after growth for 3 days at 40^{o}C. In each instance the size bar represents 5µm. The length and width of cells was measured and used to calculate the cell volume.

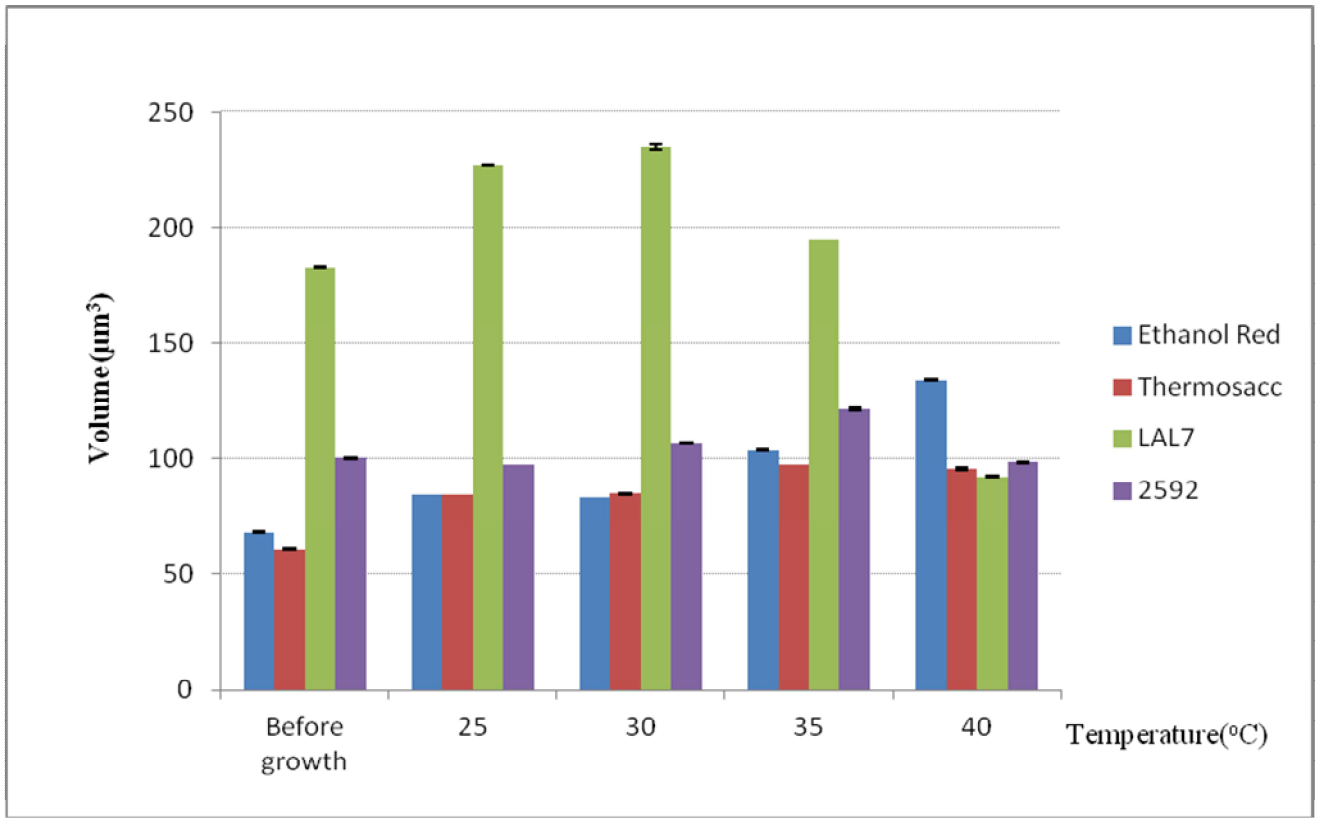


Figure 3.15. Yeast cell volume (µm^3) of Ethanol Red, Thermosacc, LAL7 and 2592 after growth on YPD for 3 days at 25, 30, 35 and 40^{o}C. Cell volume was calculated based on length and width measurements of

Temperature	Ethanol Red	Thermosacc	LAL7	2592
Length (µm)				
Control	6.51 ± 0.57	5.80 ± 0.69	8.11 ± 0.86	8.01 ± 1.28
25°C	5.76 ± 0.77	5.70 ± 0.67	7.76 ± 0.71	7.82 ± 1.11
30°C	6.00 ± 0.43	5.92 ± 0.66	8.12 ± 1.01	7.20 ± 1.17
35°C	6.42 ± 0.78	6.42 ± 0.71	8.47 ± 0.72	7.29 ± 1.24
40°C	6.53 ± 0.99	6.27 ± 0.75	6.68 ± 0.98	6.46 ± 0.79
Width (µm)				
Control	4.47 ± 0.39	4.48 ± 0.57	6.56 ± 0.84	4.89 ± 0.60
25°C	5.29 ± 0.68	5.32 ± 0.69	7.47 ± 0.81	4.88 ± 0.68
30°C	5.15 ± 0.78	5.23 ± 0.72	7.43 ± 1.56	5.32 ± 0.71
35°C	5.55 ± 0.82	5.39 ± 0.67	6.63 ± 0.73	5.64 ± 0.82
40°C	6.26 ± 0.78	5.40 ± 0.96	5.14 ± 0.74	5.40 ± 0.94
Volume (μm^3)				
Control	68.11 ± 0.05	60.95 ± 0.12	182.74 ± 0.32	100.29 ± 0.24
25°C	84.40 ± 0.19	84.47 ± 0.17	226.73 ± 0.24	97.51 ± 0.27
30°C	83.32 ± 0.14	84.79 ± 0.18	234.71 ± 1.29	106.70 ± 0.31
35°C	103.54 ± 0.27	97.66 ± 0.17	194.94 ± 0.20	121.42 ± 0.44
40°C	133.99 ± 0.32	95.73 ± 0.36	92.41 ± 0.28	98.63 ± 0.37

Table 3.2: Summary of cell size and volume of yeast strains grown in liquid YPD medium at different temperatures ± standard deviation.

3.2.4 Accumulation of trehalose and glycogen at different temperatures 25, 30, 35 and 40^{o}C

One of the most well documented effects of heat shock on yeast cells is the incorporation of trehalose, which is known to function as a cell protectant (Section 1.7.2.5). Glycogen on the other hand is a molecule which acts as a carbohydrate storage compound (Section 1.7.2.5). Consequently, levels of glycogen can typically be interpreted to indicate how healthy a culture is, while trehalose can be used to assess the capacity of yeast to respond to a changing (stressed) environment.

Analysis of trehalose indicated that under favourable laboratory conditions (25°C), there was no significant difference between the Ethanol Red and Thermosacc yeast strains as well as between 2592 and LAL7, ($P=0.293 > 0.05$) and ($P=0.248 > 0.05$), respectively (Figure 3.16), although Ethanol Red (120 ± 4.65 µg/1x10^{8} cells) and Thermosacc (117 ± 2.06 µg/1x10^{8} cells) may have contained slightly higher concentrations than LAL7 (92 ± 10 µg/1x10^{8} cells) and 2592 (95 ± 6.5 µg/1x10^{8} cells). For each strain, growth at higher temperatures resulted in a change to the amount of trehalose within cells. For LAL7 there was a decreasing trend in the amount of trehalose present with extremely low levels observed at 40^{o}C (Figure 3.16). For the other yeast strains a different pattern was observed with a peak in trehalose when grown at 35^{o}C followed by a reduction at 40^{o}C. Interestingly there was also a difference between strains with 2592 showing the highest concentration at 35^{o}C (204.19 ± 7.3µg/1x10^{8} cells) which was significantly higher than Thermosacc (175 ± 9.5 µg/1x10^{8} cells) which was in turn significantly higher

than Ethanol Red. Despite the variability at this temperature, there did not appear to be a difference between the levels of trehalose in 2592, Thermosacc or Ethanol Red at either 30 or 40^{o}C (Figure 3.16).

Analysis of intracellular glycogen indicated a different pattern of results. Each strain was observed to display an increase in glycogen when grown at 30^{o}C compared to 25^{o}C, except for Thermosacc which appeared to contain similar concentrations at these temperatures. Interestingly LAL7, which did not accumulate trehalose, instead appeared to produce a significant amount of glycogen, perhaps indicating that this strain may not have been as stressed as first appeared. Analysis of glycogen at 35^{o}C indicated that concentrations were observed to decrease for each strain (Figure 3.17), in contrast to the increase detected for trehalose over the same range of temperatures. This decrease was particularly marked for 2592 (decreasing from (46.52 ± 4.78 to 10.4 ± xx 1.4/1x10^{8} cells) and LAL7 (decreasing from (61.12 ± 2.25 to 20.18 ± 2.85 µg/1x10^{8} cells), although less so for Ethanol Red (decreasing from (51.9 ± 5.73 to 36.88 ± 3.08 µg/1x10^{8} cells).

Analysis of the results overall indicated that in general the concentration of trehalose was inversely related to that of glycogen. While the trehalose level was observed to increase in each strain, the glycogen level decreased. Typically strains exhibited the highest trehalose levels at 35^{o}C indicating that these strains were responding to heat shock, while glycogen was highest at 30^{o}C, suggesting that this temperature was the most favourable for the strains analysed, allowing them to build glycogen energy reserves (Figures 3.16 and 3.17).

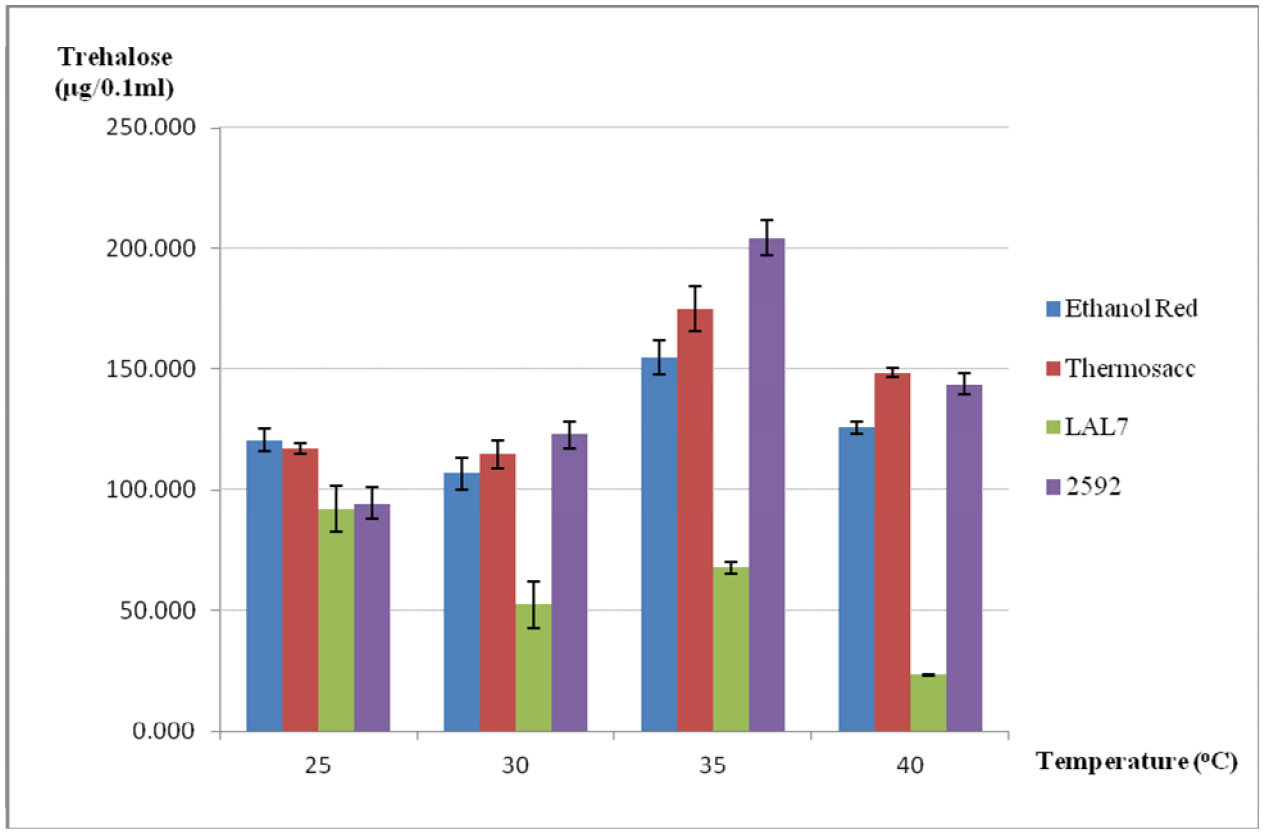


Figure 3.16. Trehalose content of Ethanol Red, Thermosacc, LAL7 and 2592 at different temperatures. Yeast strains were grown in liquid media for 3 days at 25, 30, 35 and 40°C. Glycogen is expressed in the units of glucose (µg) in 0.1mL sample ($1x10^8$ cells) with error bars reflecting the standard deviation of triplicate samples.

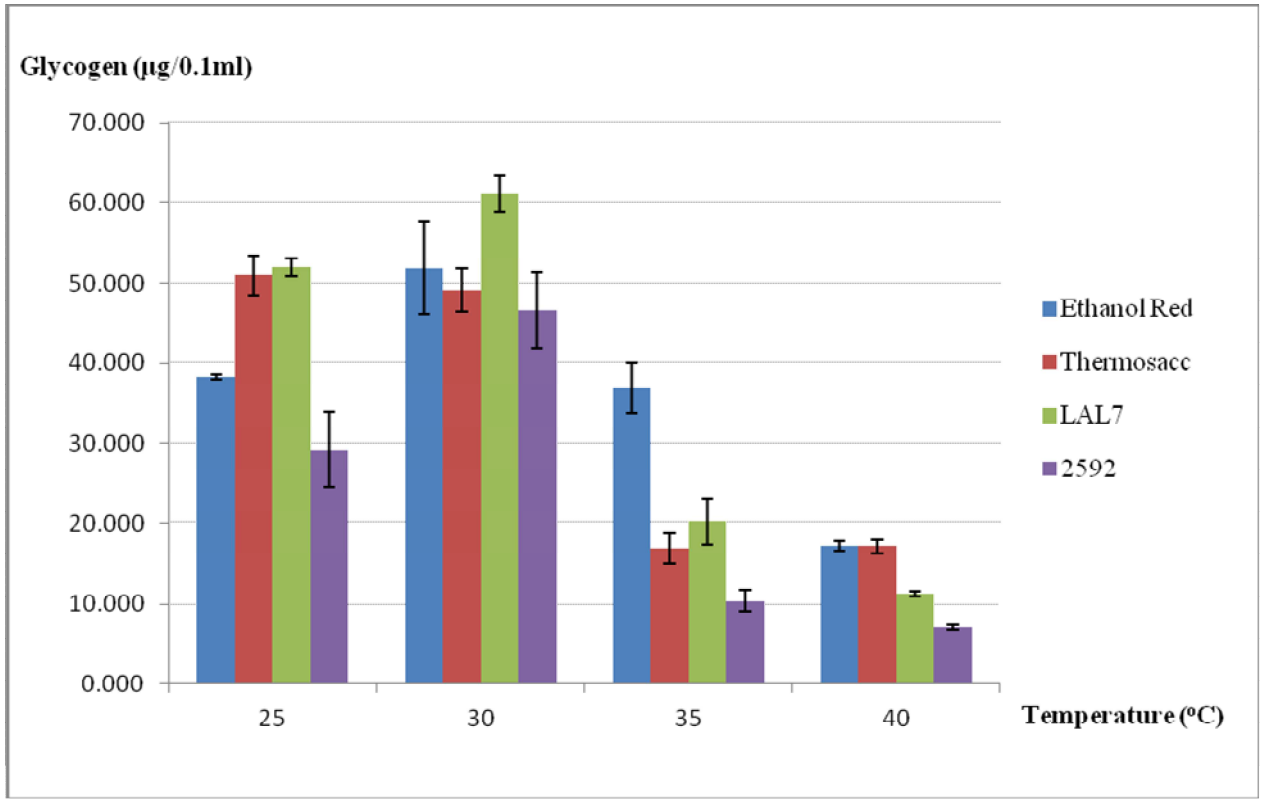


Figure 3.17. Glycogen content of Ethanol Red, Thermosacc, LAL7 and 2592 at different temperatures. Yeast strains were grown in liquid media for 3 days at 25, 30, 35 and 40^{o}C. Glycogen is expressed as units of glucose (µg) in 0.1mL sample ($1x10^{8}$ cells) with error bars reflecting the standard deviation of triplicate samples.

3.3 Discussion

The aim of this chapter was to determine the effect of temperature on the growth characteristics of four bioethanol yeast strains. Analysis of yeast growth using spot plates was performed to obtain an overview of the range of temperatures under which each strain was capable of dividing. As expected, the temperature was shown to have an impact on growth, and a negative effect was observed when temperatures outside of the range of 25-35^{o}C were applied. At higher temperatures (40-45^{o}C) and lower temperatures (10-20^{o}C) it was observed that yeast cells required more than 4 days for colony formation, if they were able to produce colonies at all. While all strains grew slowly under cold conditions, at particularly higher temperatures (45^{o}C), no strains except Thermosacc showed any sign of growth, indicating that these yeasts were killed and could not protect themselves against heat stress. Performing a similar set of experiments using liquid media also yielded comparable results, with Thermosacc proving to be robust at higher temperatures. Interestingly, although Thermosacc produced the shortest lag phase at higher temperatures, strain 2592 was observed to show a better tolerance to stress, with optical density remaining stable during stationary phase. In contrast to Thermosacc and 2592, LAL7 seemed to have the weakest resistance to high temperature conditions according to both analysis on spot plates and growth in liquid medium and did not appear to favour temperatures greater than 34°C.

As described previously (Chapter 1), trehalose is known to be an important protective compound to various stress factors, including heat (Thevelein, 1984; Hottiger *et al*., 1987a, 1987b; Bandara *et al*., 2009). The results from the

previous assays suggested that a high level of trehalose may be observed in Thermosac and 2592 compared to Ethanol Red and, particularly, to LAL7. In order to examine this, analysis of the concentrations of both trehalose and glycogen were conducted. Trehalose was analysed due its known role in heat resistance, while glycogen was assessed as a measure of cell health. As expected, the results obtained supported the presumption above. At temperatures of above 30^{o}C, the trehalose content was found to be significantly higher in Thermosacc and 2592 than in Ethanol Red and LAL7. Although strain 2592 had the highest level of trehalose at 35^{o}C, at 40^{o}C Thermosacc contained significantly more than any of the other strains. This suggests that Thermosacc may be able to respond better to higher temperature than 2592 (and consequently LAL7 and Ethanol Red), allowing the cell to procure greater heat resistance. In this assay, Ethanol Red also showed a high trehalose level, however it was not as high as 2592 and Thermosacc. Therefore, it can be seen that overall the response to heat resistance in terms of trehalose accumulation was lowest for LAL7.

Interestingly LAL7 appeared to be quite different to the other strains examined in several ways. This yeast accumulated more glycogen than the other strains, indicating that carbon flux may play a significant role in survival at high temperatures. Furthermore, this strain was significantly larger in size than the other yeasts examined. Further visual analysis of yeast via microscopy also revealed interesting effects of temperature on each yeast strain. As the temperature was increased over 30^{o}C the cell size of the four yeast strains began to be reduced. It is known that the mean size of cells is not constant, but varies dependent on a number of factors such as the stage of growth (Hartwell

and Unger, 1977), environmental conditions (Robinow and Johnson, 1991; Quain, 1988) and cell age (Powell et al., 2000). Interestingly, it has previously been reported that cell size may become increased in response to temperature (Watson, 1987), however this was not observed in the ethanol strains examined here, except for LAL7. The fact that the other three strains were able to tolerate heat better than LAL7 and produced smaller cells indicates that size may actually play a role in heat tolerance or stress resistance in general. Indeed, it has been suggested (Ingledew, 2009) that strains which naturally form smaller cells are more successful at fermenting particularly high gravity substrates. Although Ingledew (2009) did not offer an explanation for this, it is possible that the large size of LAL7 may contribute to its poor resistance to heat, however, further analysis would be required to explore this hypothesis. It should be noted that although many yeast cells from each strain appeared to be shrunken in structure or lysed at 40^{o}C, Ethanol Red cells actually showed an increase in cell volume at this temperature. Again, the reasons for this are unknown, however it is possible that this is may have arisen due to the incorporation of compatible solutes such as glycerol, known to be induced as part of the global stress response pathway in yeast (Gasch *et al*., 2000). This may have led to osmotic imbalance leading to water influx and subsequent cellular expansion.

These data reported here suggest that heat resistance in *Saccharomyces* yeasts may be strain specific, however under certain circumstances it is suggested that growth and lag phase time at higher temperatures may impact both on biofuel fermentation and on yeast pre-conditioning, which are discussed in the following chapters.

Chapter 4: Fermentation of corn mash to ethanol

4.1 Introduction

In order to produce ethanol from corn mash, corn must be broken down into its constituent sugars and fermented using yeast strains as described previously (Chapter 1). To obtain the sufficient biomass to initiate industrial ethanol fermentations, yeast can be cultivated in liquid medium. However, a more readily available solution is to purchase yeast from specialist suppliers as Active Dried Yeast (ADY). Logistically this approach makes sense for ethanol production, as preparation of liquid yeast on site requires capital expenditure and close process control, while ADY can be transported easily and stored for up to 2 years if required prior to use (Quain, 2006). Typically ADY are treated in various ways during production to facilitate or enhance fermentation performance (Powell and Fischborn, 2010). However, the process of drying ADY is known to be detrimental to yeast health and poor viability can arise as a result of stress, including damage from heat which occurs during the drying process (Beker & Rapoport, 1987; Bayrock & Ingledew, 1998; Poirier et al., 1999; Oshita et al., 2002). Although it was not a primary goal of the project, given the dependence of the bioethanol industry on ADY, it was important to assess differences not only between the fermentation performance of yeast strains, but in the form of the yeast used for pitching. Consequently, in this chapter the intrinsic ability of each strain to ferment was tested by using yeast cultures in ‘wet’ form. Subsequently two strains were selected and evaluated further in both wet and ADY form to determine differences between the two yeast types in terms of fermentation performance.

In order to achieve this, novel miniature fermentation systems to evaluate the fermentation performance of yeast strains in the conversion of corn mash to ethanol were investigated. To this end two methods were designed, based on weight loss, to determine fermentation progression. The first was a miniature stirred FV system and the second was based on a similar principle, but with the use of miniature shaken fermenters.

4.2 Results

In order to assess the fermentation performance of each yeast strain, initially a miniature fermentation vessel system based on Quain et al (1985) was adapted for use with corn mash. This included the use of 150ml bottle containing corn mash mixed via magnetic stirrers (Section 2.8.3.2). An alternative method was also developed which utilised 250ml shake flasks to agitate corn mash (Section 2.8.3.3). Each system incorporated a gas outlet valve with gas outlet ports to allow for the escape of CO_2. Consequently fermentation progression was determined by monitoring weight loss over time Section 2.8.5).

A novel yeast sampling method was also developed to allow analysis of yeast cells, including cell number and viability. By using traditional methods it was not possible to analyse yeast cell number or viability due to the nature of the corn mash, which is an extremely viscous and particulate substance. The consistency of the corn mash rendered visualisation of cells using microscopy very difficult. Furthermore, the corn mash was observed to rapidly clog filters and membranes which prevented yeast cells from being isolated in this way. Consequently, a method was developed whereby yeast and corn mash were separated using a sterile course muslin bag as described in Section 2.8.7.

4.2.1 Fermentation progression analysis using miniature stirred fermenters

The aim of this assay was to examine fermentation performance and ethanol production of yeasts using a stirred mini-FV fermentation method. In this experiment, both wet and ADY yeasts were inoculated at a rate of $2x10^7$ cells/ml corn mash in triplicate. It should be noted that oxygenation was not controlled prior to fermentation, although yeast was cultivated aerobically (for wet yeast) and was rehydrated in the presence of air (for ADY). The results obtained show that the fermentations were finished in approximately 24 hours after pitching either using active dry yeast or wet yeast (Figures 4.1 and 4.2 respectively).

It can be seen that the final attenuation at the end of fermentation was variable, indicating different capacities of wet yeasts to assimilate sugars. Typically, LAL7 strain gave the best weight loss both in wet yeast form (Figure 4.1) and in active dry yeast form (Figure 4.2). Although fermentation using Thermosacc was not as good as LAL7, it gave better result than Ethanol Red and 2592 which showed a slower time to reach attenuation (Figure 4.1).

Comparing the performance of Thermosacc and LAL7 in wet and dry format indicated that fermentations were typically quicker for wet yeasts, reaching attenuation within 14hours. However, for Thermosacc, a higher attenuation (99.48% ± 0.02%) was achieved when using wet yeast when compared to ADY (99.41% ± 0.01%) (Figure 4.2). Similarly, for LAL7 the same pattern of results was observed (99.05% ± 0.02% compared to 98.9% ± 0.01%).

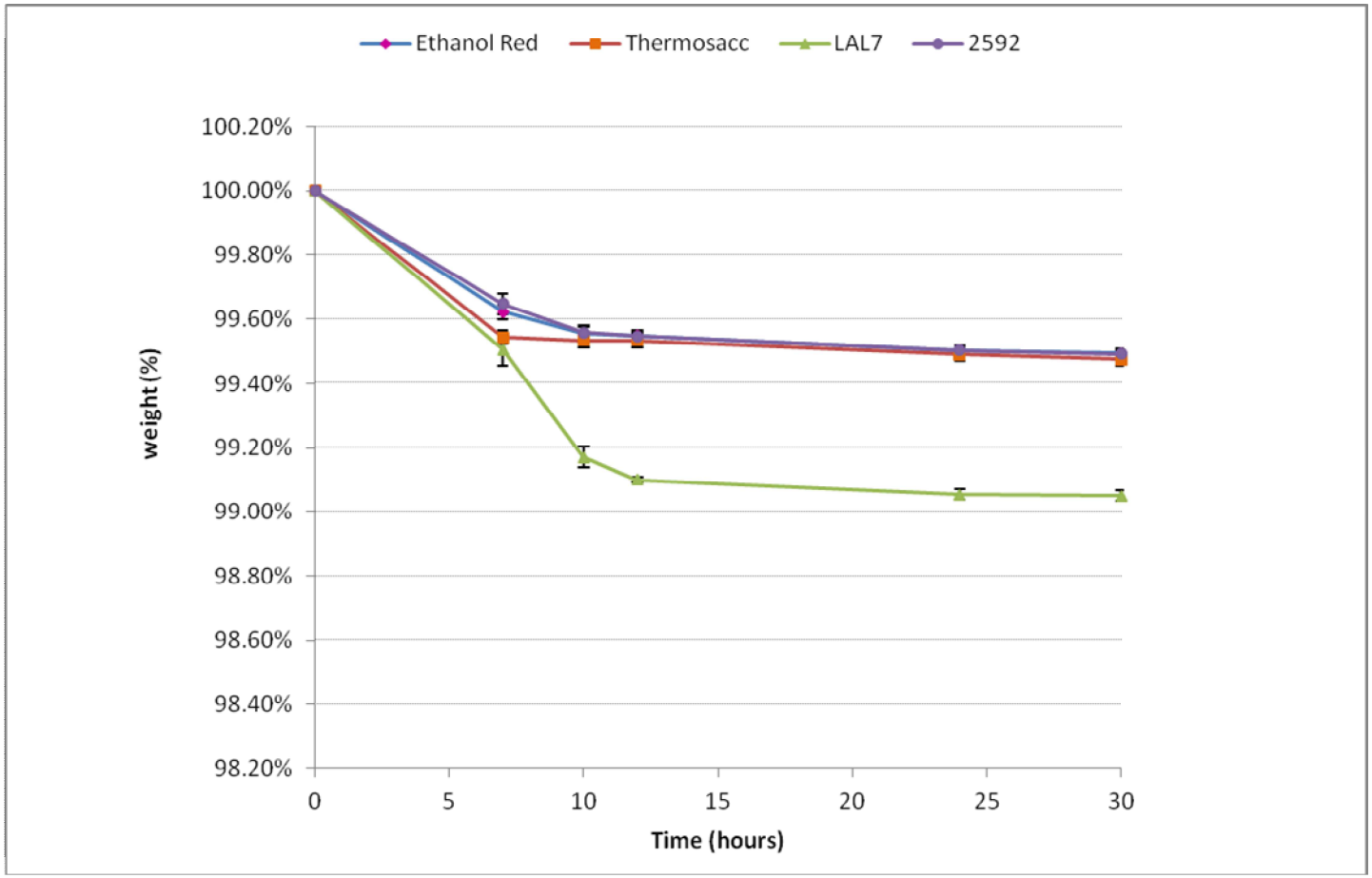


Figure 4.1. Fermentation progression of 4 wet yeast strains at 30°C using a stirred FV fermentation method. An inoculation rate of $2x10^7$ cells per ml of mash was used to pitch 100ml of corn mash. Error bars indicate standard deviation of triplicate samples.

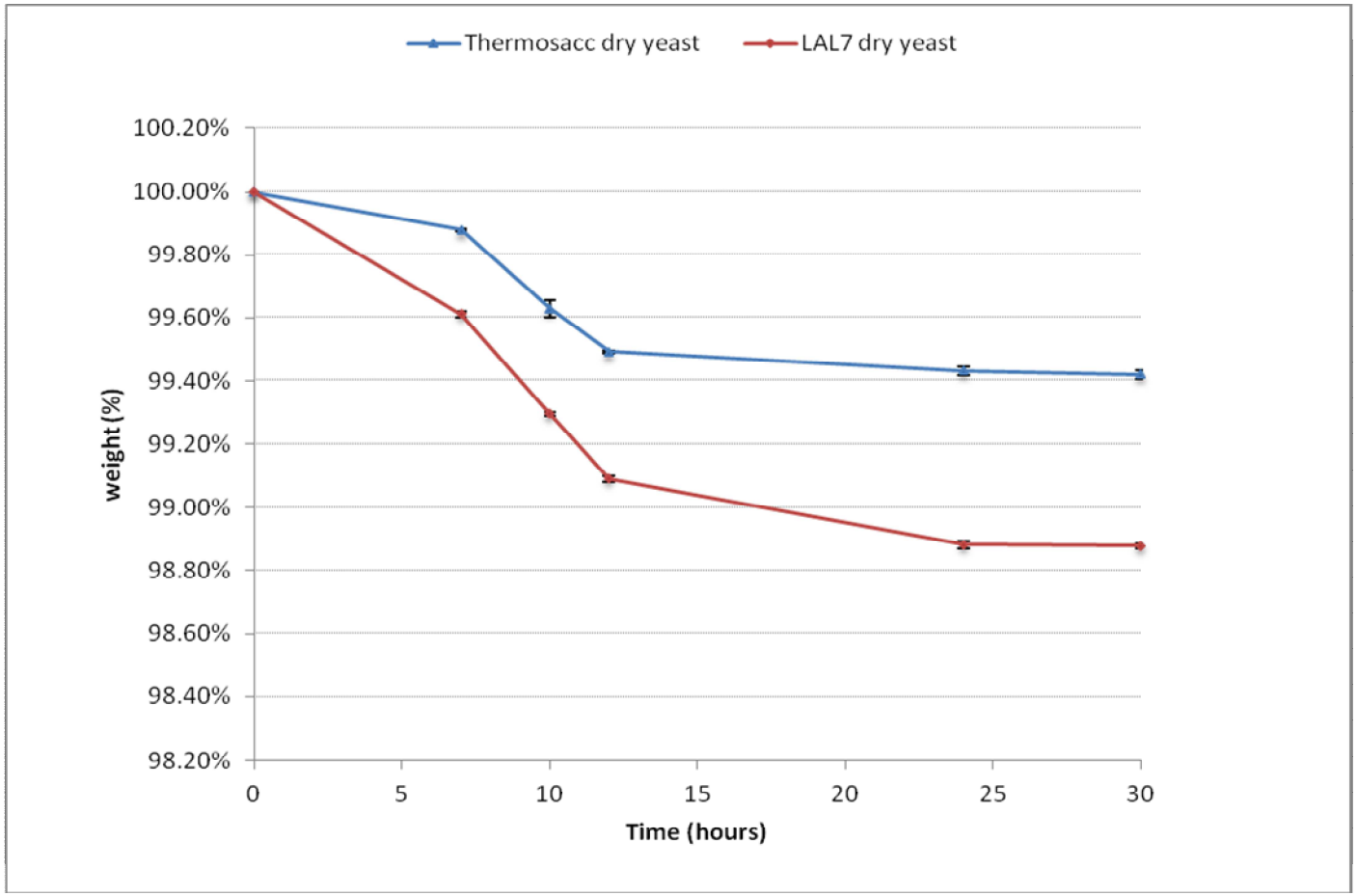


Figure 4.2. Fermentation progression of 2 active dry yeast strains at 30°C using a stirred FV fermentation method. An inoculation rate at $2x10^7$ cells per ml of mash was used to pitch 100ml of corn mash. Error bars indicate standard deviation of triplicate samples.

Analysis of the final ethanol concentration at the end of fermentation provided corresponding data, the final ethanol yield in each instance was in the range of 1.9-3.7% depending on the yeast strain and the yeast form used (Figure 4.3). For the wet yeast strains, LAL7, which gave the lowest final attenuation, also produced the best ethanol yield (3.25 ± 0.06 %) among the yeasts tested (Figure 4.3). There was no significant difference between the ethanol yields of the other 3 strains analysed ($P > 0.05$), supporting the previous data from sugar utilisation (Figure 4.1). Analysis of ADY cultures indicated a similar pattern of results, with LAL7 producing the highest amount of ethanol concentration, 3.7 ± 0.2 % compared to Thermosacc which produced 2.2 ± 0.1%.

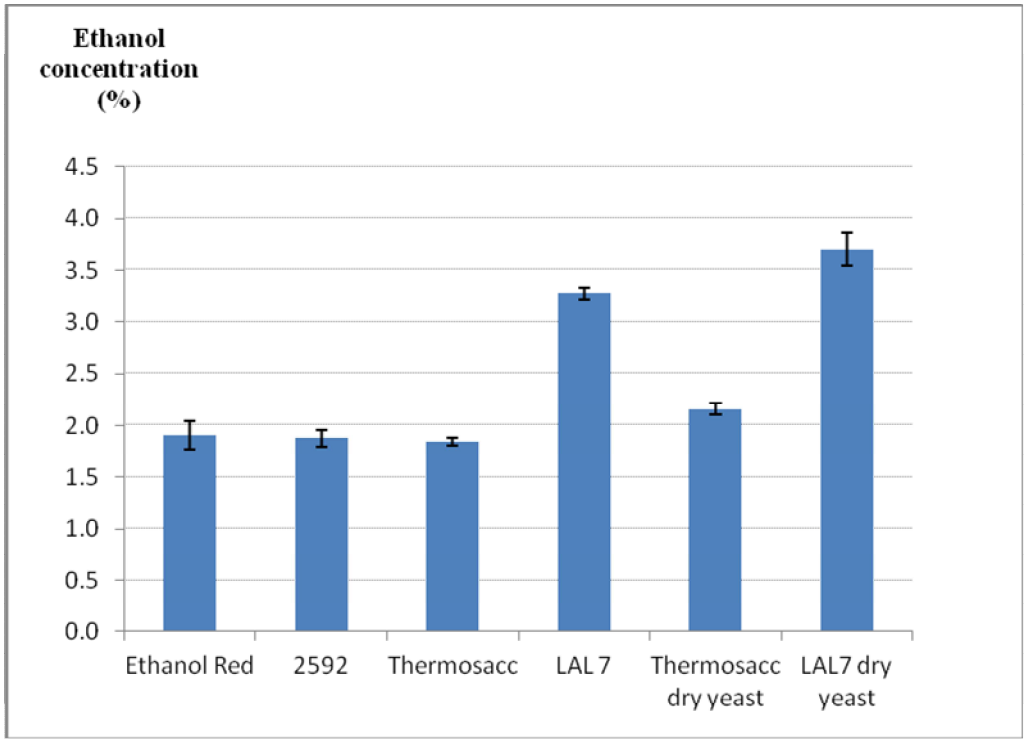


Figure 4.3. Ethanol concentration produced by different yeast strains using stirred fermentations at 30^{o}C. Error bars indicate standard deviation of triplicate samples.

It should be noted that analysis of cell cultures at the end of fermentations indicated that there were extremely low viabilities, irrespective of the form of yeast used (Table 4.1). Although fermentations were fast and completed within a day, it can be seen that in each instance the yeast viability at the end of fermentation was approximately 12% or lower, except for LAL7 dry yeast which exhibited a viability of 30% (Table 4.l). Furthermore, cell biomass was low in each instance, indicating that very little growth had occurred at the start of fermentation. This was particularly true for LAL7, which exhibited a final cell number of just $1.64x10^{7}$. This decrease in viability was unexpected due to the modest yield in ethanol, and the causes are unknown. However, it was noticed that due to the thick nature of the corn mash, stirring was required to be at an extremely high level and was not consistent between vessels. Furthermore, this could have influenced oxygen availability to the yeast population at the start of fermentation, perhaps acting as a source of cell weakness.

Yeast strain	% Viability		Total Cell Number Post Fermentation
	Pre fermentation	Post fermentation	
Ethanol Red	99.51%	6.57%	$1.16x10^{8}$
2592	99.32%	6.83%	$9.36x10^{7}$
Thermosacc	99.35%	8.62%	$1.25x10^{8}$
LAL7	99.50%	12.02%	$1.64x10^{7}$
Thermosacc dry yeast	77.14%	11.81%	$1.15x10^{8}$
LAL7 dry yeast	74.45%	30.06%	8.32x107

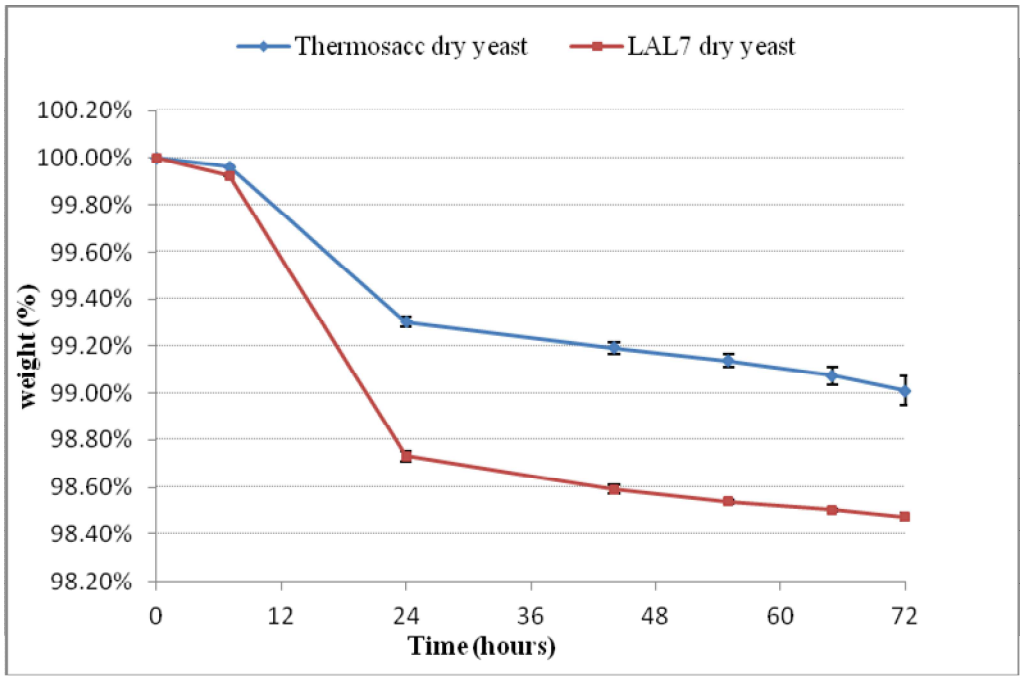
Table 4.1. Percent viability before and after fermentation for each yeast strain. Viability was assessed by methylene blue staining and analysis of populations comprising >400 cells in each instance.

4.2.2 Fermentation progression analysis using miniature shaken fermenters

Due to the results obtained previously, which indicated an extremely poor viability at the end of fermentation, an alternative fermentation method was developed. This involved using a shake flask system with a larger head space to facilitate agitation and oxygen transfer at the start of fermentation (Section 2.8.3.2). Furthermore, the previous pitching rate of $2x10^7$ cells/ml was reduced to $1x10^7$ cells/ml and ADY yeast were used exclusively to match the current practice at Greenfield Ethanol and to avoid nutrient depletion occurring before yeast had adapted to their new environment.

In accordance with previous results, Figure 4.3 showed that fermentations using ADY strain LAL7 produced more weight loss (assimilated more sugar) than Thermosacc at the end of fermentation, with 98.47% remaining compared to 99.01%, respectively. In addition the time to finish fermentation at this pitching rate was more than 72 hours, although a significant amount of sugar was assimilated within the first 24 hours. In contrast to the sugar utilisation profiles, the result from Figure 4.5 indicated that the yeast strain Thermosacc was better than LAL7 yeast strain in yielding ethanol, 5.2 ± 0.2% compared with 3.1 ± 0.1% respectively. Furthermore, a comparison of ethanol yield between shaken and stirred fermentations indicated that for Thermosacc a significantly greater amount of ethanol was produced (5.2 ± 0.2% compared with 2.2 ± 0.1% respectively), while for LAL7 a similar concentration was produced (3.7 ± 0.2% compared with 3.1 ± 0.1%).

Analysis of viability at the end of shaken fermentations indicated that a high yeast viability for both strains was recorded (Table 4.2). In comparison to the stirred fermentation method, Thermosacc and LAL7 showed a greater number of live cell at the end of fermentation, 93.99% compared with 30. 06% for LAL7 and 81.52% compared with 11.81% for Thermosacc (Tables 4.1 and 4.2). In addition, biomass production was greater, indicating 2-3 population duplications during fermentation for each yeast (Table 4.2).



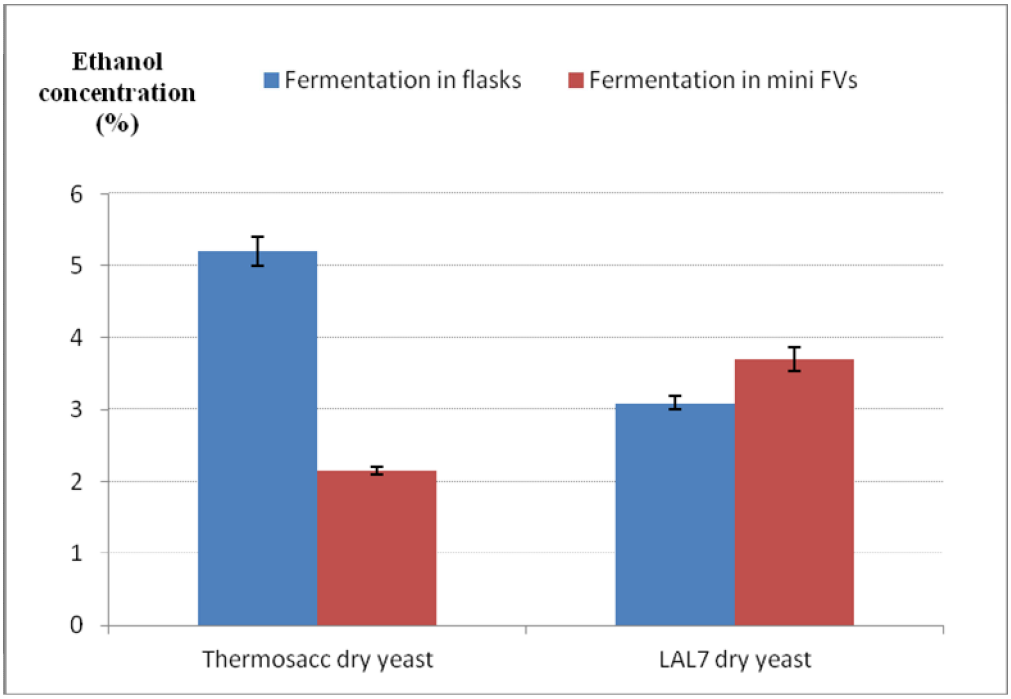


Figure 4.5. Comparison of ethanol concentration produced by two active dry yeast strains by using shaken flask fermentation and mini stirred FV fermentation method. Error bars indicate standard deviation of triplicate samples.

Yeast strain	% Viability		Total Cell Number Post Fermentation
	Pre fermentation	Post fermentation	
Thermosacc ADY	77.78%	93.99%	$2.93x10^8$
LAL7 ADY	74.34%	81.52%	$1.1X10^8$

Table 4.2 Yeast cell count and viability analysis before and after fermentation at 30°C using stirred FV's. Pitching rates were standardised at $1x10^7$ in each instance.

4.3 Discussion

Analysis of wet and ADY yeasts using a stirred fermentation vessel system yielded unusual results. Although fermentations were completed quickly, yeast health was noted as being extremely poor and a low ethanol yield was achieved. However the use of shaken fermenters as a means of agitation produced more confident data. When comparing the fermentation performance of Thermosacc and LAL7, two different inoculation rates of yeast were used for each vessel type: $2x10^7$ cell/ml for stirred fermenters and $1x10^7$ cells/ml for shaken fermenters. According to the study of Narendranath and Power (2004), yeast inoculation rate does not have an affect on the final ethanol yield, however it is recommended that in the ethanol industry an inoculation rate of at least $1x10^6$ cells/ml per percent dry solid should be used for contamination-free mashes. The mash in this study was 28.5% solids which

implies that an inoculation rate of $2.85x10^7$ cells/ml should be carried out. Unfortunately, when a pitching rate of $2x10^7$ was used with the stirred mini FV fermentation method it was found that although fermentation was completed fast after 24 hours the yeast viability of four strains were very low (under 30%). However ethanol yield was almost the same with the result obtained with inoculation rate 2×10^7 cells per ml per mash for each strain in this study. The reason for this can be explained is that when pitching at a high rate, fermentation progresses quickly due to the high biomass present. As a consequence, nutrients as well as the oxygen content in corn mash becomes depleted and the carbon dioxide concentration increases rapidly. It is suggested that the rapid onset of these stress factors may have caused poor yeast growth rate and viability at the end of fermentation (Kunkee and Ough, 1966; Jones and Greenfield, 1982). Moreover, the depletion of oxygen content within a few hours after inoculating yeast leads to an anaerobic environment (Boulton and Quain, 2001). While it would be surprising if this influenced yeast health directly, this environment is known to effect cellular functions, including modifications to yeast cell wall (Abramova et al., 2001; James et al., 2003), yeast cell volume (Lumsden et al., 1987), yeast cell division (Norton and Krauss, 1972) and yeast cell metabolism (Hammond, 1993; Lewis and Young, 1995). The result obtained from mini stirred fermentations may indicate the impact of these factors may be reflected in the poor viability at the end of fermentation.

However despite this result, it is interesting to note that the viability became increased in both ADY LAL7 and Thermosacc during shaken fermentations at a lower pitching rate. The reason for this may be explained by the amount of

oxygen available in flasks and the improved transfer due to the method of agitation. As the density of yeast cells/ml of mash was not as high as in the stirred fermenters it is possible that increased cell division may have enabled cells to adapt to the environment and allow them to cope with stresses gradually. This lower inoculation rate is also a likely explanation for the slower fermentation time required for completion (72 hours), even though ethanol yield was improved.

The results in this chapter showed a correlation between fermentation progression with ethanol yield. Specifically, a similarity in the fermentation progression profiles of the three wet yeast strains Ethanol Red, 2592 and Thermosacc showed a similar result in terms of ethanol concentration during stirred fermentations. LAL7 wet yeast gave a better result in weight loss than Thermosacc dry yeast and an improved ethanol yield. However, Thermosacc ADY did not perform as well as LAL7 ADY in terms of sugar uptake, although ethanol yield was higher. This is difficult to explain since the biomass yield was also higher for Thermosacc, however it may be a result of the lower viability of LAL7. This suggested that health was poorer in this culture, possibly resulting in increased energy expenditure for cell repair. Between the 2 fermentation methods used in this study, the results of ADY yeast in yeast fermentation progression and yeast viability demonstrated ethanol production was dependent on the method used and the inoculation rate. As discussed above, if yeast was not faced with a rapid increase in stress intensity it is possible that yeast cells would be able to survive better and produce a better fermentation performance. The yeast Thermosacc in ADY

form gave the best ethanol yield according to ethanol concentration obtained when inoculating at lower pitching rates in shaken fermentations.

Through the various experiments carried out in this current chapter, it can be concluded that shaken fermentations and inoculation rate at $1x10^7$ cells/ml of mash gave better ethanol yield than stirred fermentations with an inoculation rate $2x10^7$ cells/ml. Stirred fermentations seemed to produce greater stress for yeast, although the reasons for this are largely unknown, it is possible that poor oxygen transfer may have led to poor yeast health. Therefore, in the next chapter when examining the effect of temperature and temperature pre-treatment on yeast during fermentation the shaken fermentation method is preferred.

Chapter 5: The effect of temperature pre-conditioning on corn mash fermentation characteristics and yeast health

5.1 Introduction

As discussed in Chapter 3, temperature has a significant impact on yeast, including effects on yeast growth, yeast cell size and the accumulation of trehalose and glycogen. However, the results described previously (Chapter 3) indicated that these effects were not uniform between yeasts and there appeared to be a difference in the heat tolerance of different strains. In particular, Thermosacc appeared to be potentially the most heat resistant and LAL7 the least heat tolerant and consequently these strains were selected for further analysis. Interestingly, Greenfield Ethanol (Canada) noticed that pitching Thermosacc yeast at higher than normal temperatures resulted in an increased yield during bioethanol fermentations (Stephan Brey, Unpublished Data). Previous reports have suggested that a short dose of sub-lethal heat shock can result in a benefit to cells in terms of stress resistance. For example, it has been demonstrated that heat-treating yeast results in the activation of many genes involved in trehalose synthesis such as *TPS1*, *TPS2*, *TPS3*, *TSL1*, known to be important in tolerance to adverse conditions (Zahringer *et al*., 2000; Chapter 1). Furthermore, this has been shown to be beneficial in the ADY industry where heat treatment is used to increase stress resistance during production (Powell and Fischborn, 2010). It is possible that a similar treatment prior to fermentation may cause cells to produce protective compounds against various stresses associated with fermentation, leading to greater yeast vitality and hence improved fermentation performance.

In order to understand more about the effects of sub-lethal heat treatment on yeast health and performance during corn mash fermentation, yeast strains Thermosacc and LAL7 were subjected to heat treatment prior to pitching into shaken fermentation vessels. All fermentations were then monitored for fermentation progression by measuring weight loss due to CO_2 evolution, time to finish fermentation, final ethanol concentration and yeast viability. Finally, the trehalose and glycogen content of yeast pre-heating and post heating were also assessed in order to determine the effects of sub-lethal short-term temperature shock on intracellular carbohydrates.

5.2 Results

Bioethanol *S.cerevisiae* yeast strains LAL7 and Thermosacc were obtained from Lallemand Inc (Canada) in active dried yeast form. Yeasts were rehydrated following the rehydration method in Chapter 2 and then incubated in a static incubator for 0.5, 1 and 2 hours at either 35 or 40^{o}C. Each culture was also assessed for its intracellular trehalose and glycogen content, fermentation progression and yeast viability as described in Chapter 2.

5.2.1 The effect of heat pre-treatment on yeast trehalose and glycogen

In order to determine the initial effect of heat treatment on intracellular trehalose and glycogen, cell populations were subjected to temperatures of either 35 or 40°C for 0.5h, 1h and 2h. After each time period, the levels of intracellular carbohydrates were assessed by digesting both glycogen and trehalose to glucose, and using an enzyme assay kit to determine the concentration of glucose as described previously (Section 2.7). Glycogen and trehalose are expressed in µg glucose/1x10^{8} cells.

The results indicated that active dried yeast (ADY) strain LAL7 had a significantly higher glycogen content than ADY Thermosacc both before and after heat treatment ($P < 0.05$), supporting the data obtained in Chapter 3. The glycogen level of LAL7 ADY was almost double compared to Thermosacc ADY in all conditions tested (at 35 and 40^{o}C for 0.5, 1 and 2 hours heating) (Figure 5.1). Before heat treatment, the concentration of glycogen was 187.37 ± 10.36 µg/1x10^{8} cells for LAL7 and 75.78 ± 2.51 µg/1x10^{8} cells for Thermosacc and after heat treatment it continued to be within the same range for both strains; there was no significant change in glycogen level of LAL7 after different heat treatment conditions. For the Thermosacc strain, there was also no significant difference between glycogen after heat treatment, except for 2 hours treatment at 35^{o}C. Under these conditions the glycogen level of Thermosacc increased to 116.49 ± 6.97 µg/1x10^{8} cells. Although this could have been a direct result of heat, it may also have been due to metabolism of carbohydrates as yeast growth may have been initiated by this stage of treatment. However, further analysis is required to investigate this further.

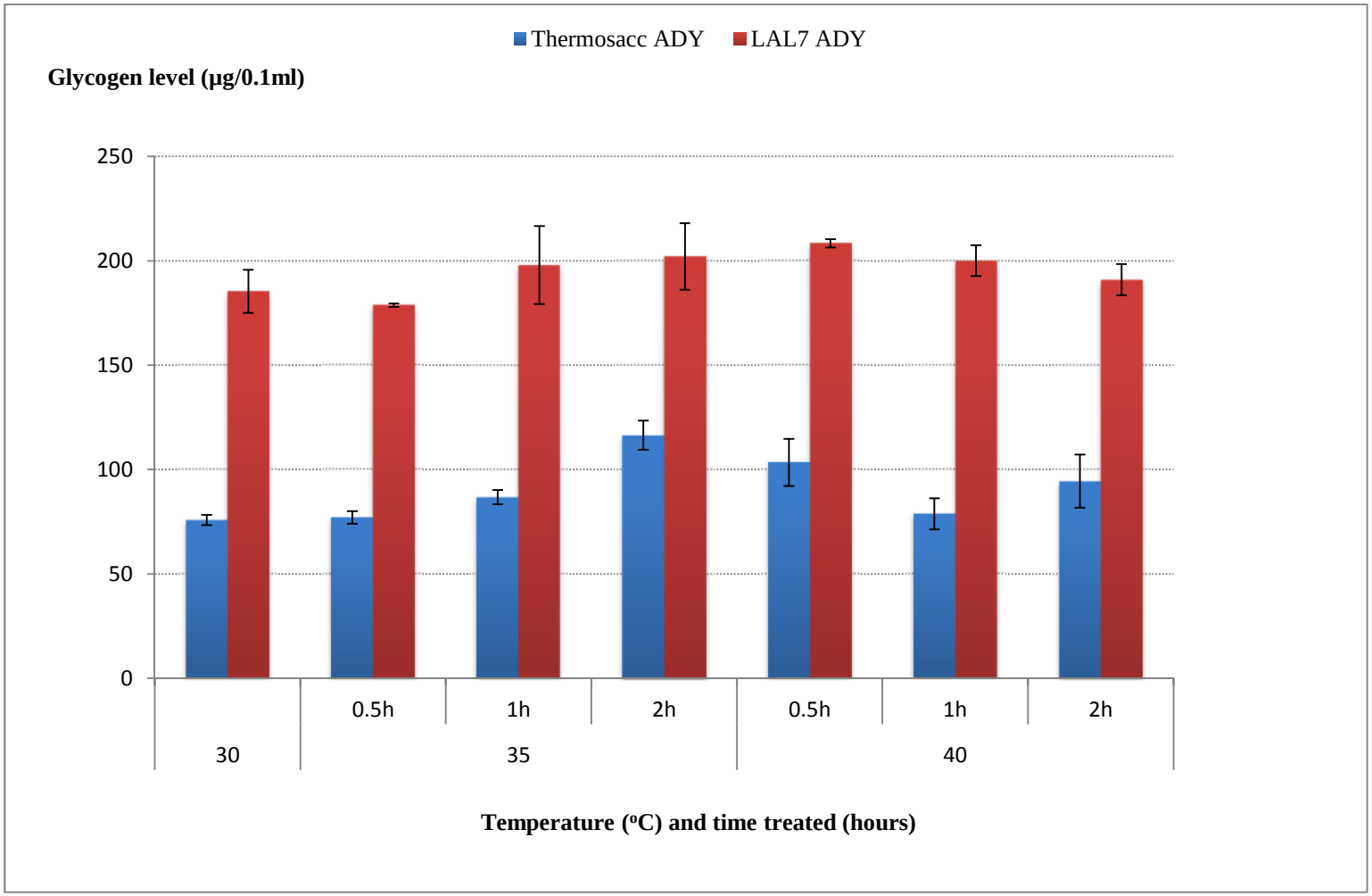


Figure 5.1: Glycogen level of two active dried yeast strains LAL7 and Thermosacc before (30°C) and after heat treatment (35 and 40°C) for 0.5, 1 and 2 hours. ADY LAL7 and ADY Thermosacc were rehydrated as described in section 2.2.2 and incubated at 35 and 40°C for a period of time. Error bars indicate the standard deviation of triplicate samples.

As discovered previously in Chapter 3, the levels of intracellular glycogen in heat treated cells were not as high as for intracellular trehalose. However, the results indicated that there were only small changes in the level of trehalose within cells following heat treatment. When treated with heat at 35 or 40^{o}C the trehalose level of Thermosacc increased slightly compared with the control 30^{o}C sample, but this increase was not significant between time points (Figure 5.2) ($P > 0.05$). Irrespective, in our study the highest temperature and longest incubation time produced the highest level of trehalose for Thermosacc ADY. This was recorded at 99.3 ± 3.7 µg/1x10^{8} cells and, although heat treatment at 40^{o}C for 0.5 and 1 hour showed similar results, this was significantly higher than the control sample (30^{o}C sample) ($P = 0.0042 < 0.05$).

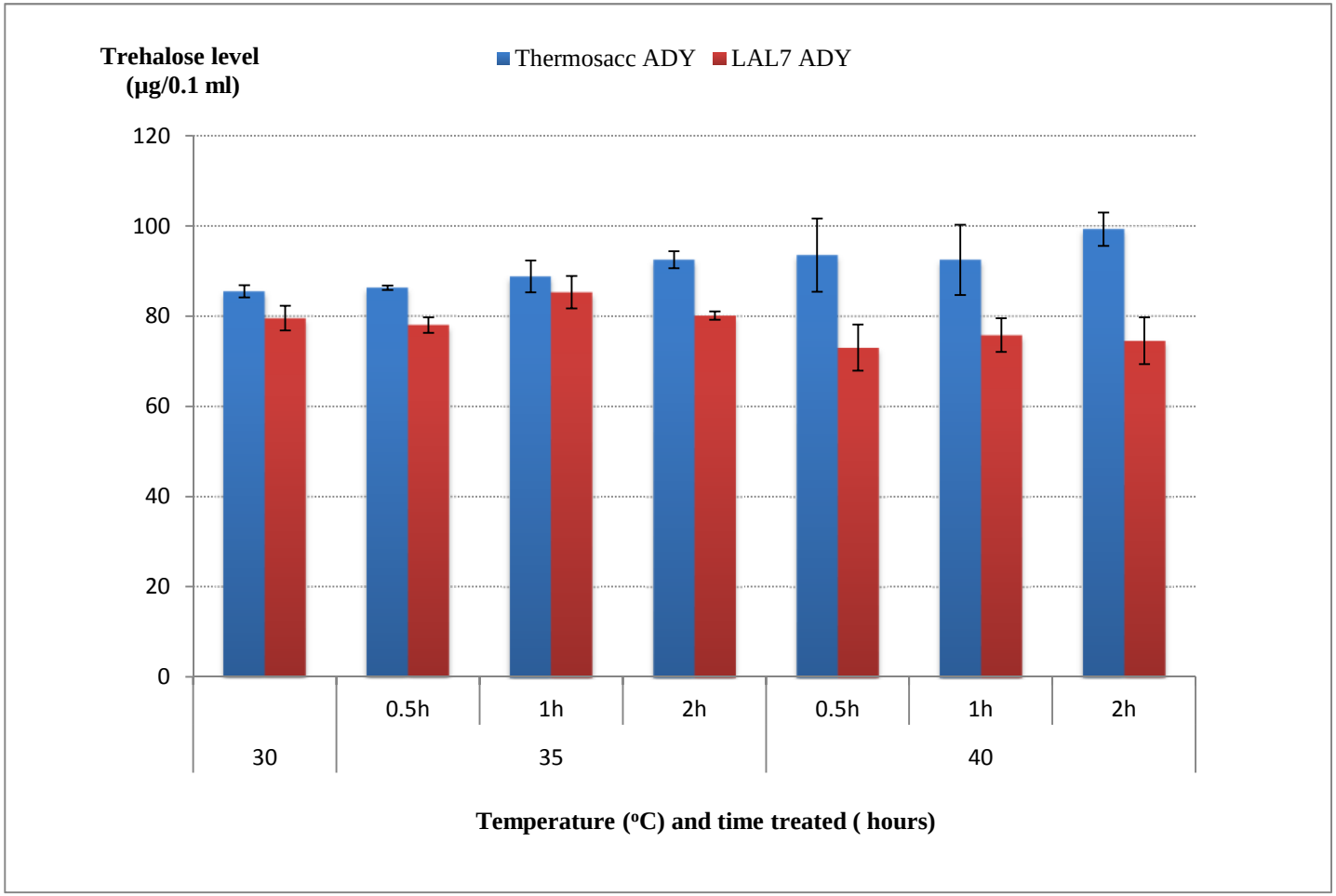


Figure 5.2: Trehalose level of two active dried yeast strains LAL7 and Thermosacc before (30^{o}C) and after heat treatment (35 and 40^{o}C) for 0.5, 1 and 2 hours. ADY LAL7 and ADY Thermosacc were rehydrated as described in section 2.2.2 and incubated at 35 and 40^{o}C for 0.5, 1 and 2 hours. Error bars indicate the standard deviation of triplicate samples.

5.2.2 Effect of yeast pre-treatment at 35°C on fermentation progression

Although the heat treatment of cells only led to a small increase in trehalose for one yeast strain, there are known to be other molecules which are involved in the heat shock response, such as Hsps which perform molecular chaperoning functions to prevent protein aggregation (Parsell and Linquist, 1994). Consequently, in order to examine the effect of heat treatment on fermentation performance, each cell population which had been subjected to temperature shock was pitched into corn mash fermenters using the flask-based shaken fermentation system developed in Chapter 4.

Analysis of sugar utilisation curves (as determined by weight loss over time (Section 2.8.5)) indicated that fermentations were finished within 72 hours, corresponding to previous data (Chapter 4). From Figure 5.3, it can be seen that for Thermosacc, cultures which had been pre-treated at 35°C for various times all produced lower final attenuation levels than the control samples. Specifically, yeast treated with heat for 2 hours gave the best weight and, in most instances, the longer the heat treatment the lower the final attenuation (Figure 5.3 and 5.4). The effects of heat treatment were less pronounced for LAL7, which showed quite similar fermentation profiles at each of the three heat treatment time points, however 2h treatment continued to produce the lowest final gravity.

The viability of all cultures was also assessed to determine the effect of pre-heat treatment affects on yeast biomass production and viability. The results of this analysis indicated that, for Thermosacc, viability before heat treatment was 75% (Table 5.1). After heating at 35°C the viability was between 71-74%

irrespective of the length of time for treatment, however at the end of fermentation the viability had reached approximately 86% indicating that heating had no long term effects on culture health, and furthermore that the culture had been able to recover during the fermentation itself. Interestingly, the control culture was observed to recover to an even greater extent, with viability rising from 78% before fermentation to 94% afterwards (Table 5.1).

For LAL7, a similar pattern was observed. Analysis of viability before and after treatment indicated a live cell percentage of 71.6% and 67% respectively (Table 5.2). However, after fermentation all cultures were around 80% viable, similar to the control sample (Table 5.2). It is unknown if this reflects the good recovery of heat treated cells, or poor performance of the control sample and further experimentation would be required to verify the results.

When comparing yeast strains it was interesting to note that for both yeasts a longer heat treatment gave a greater yeast viability post fermentation. For Thermosacc 85.83% viability was observed after 1h treatment whilst for LAL7 84.45% viability was observed after 2h treatment (Table 5.2). In each instance cultures treated for 0.5h treatment gave the least viability (Tables 5.1 and 5.2). Although further experimentation would be required to ensure this was a reproducible event, this may indicate that heat treatment had a beneficial effect on yeast health.

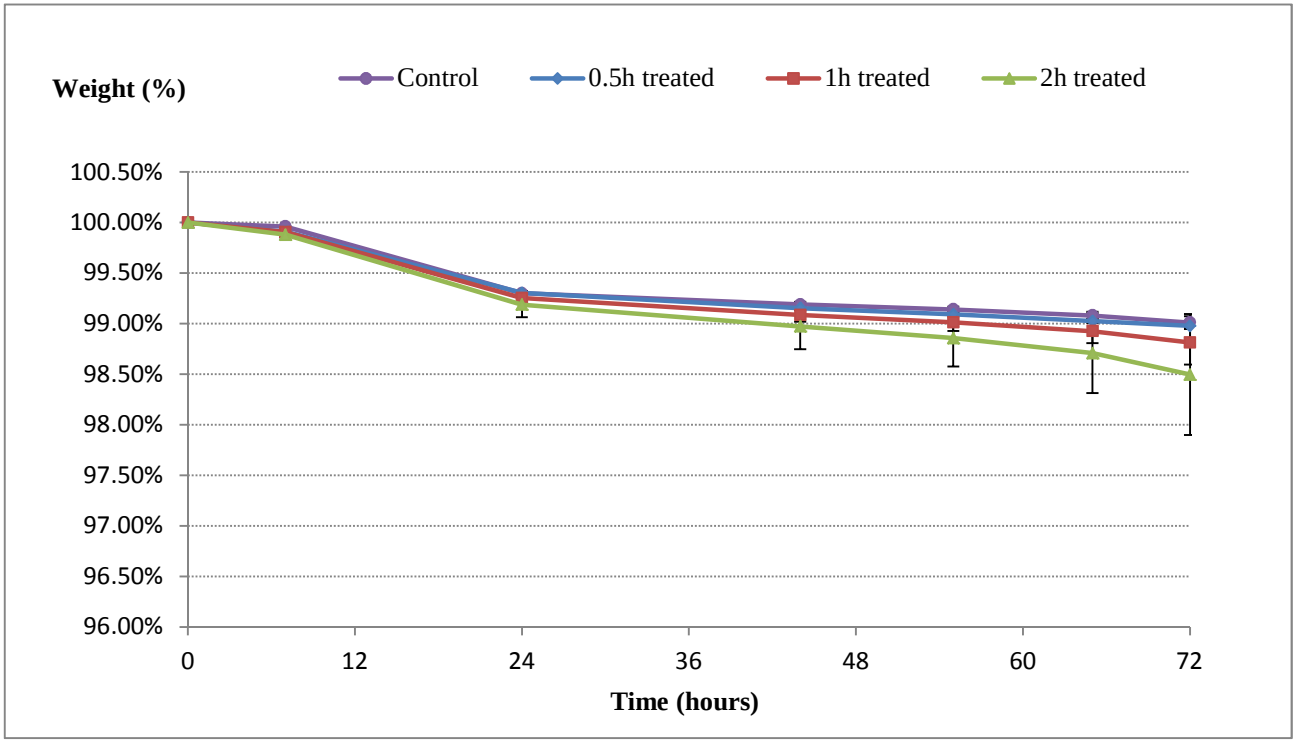


Figure 5.3: Comparison fermentation profiles of active dried yeast strain Thermosacc at 30^{o}C with heat treatment at 35^{o}C for 0.5, 1 and 2 hours. Shake flask fermentation method was carried out with inoculation rate at 1x10^{7} cells per millilitre of mash. Error bars indicate standard deviation of triplicate samples.

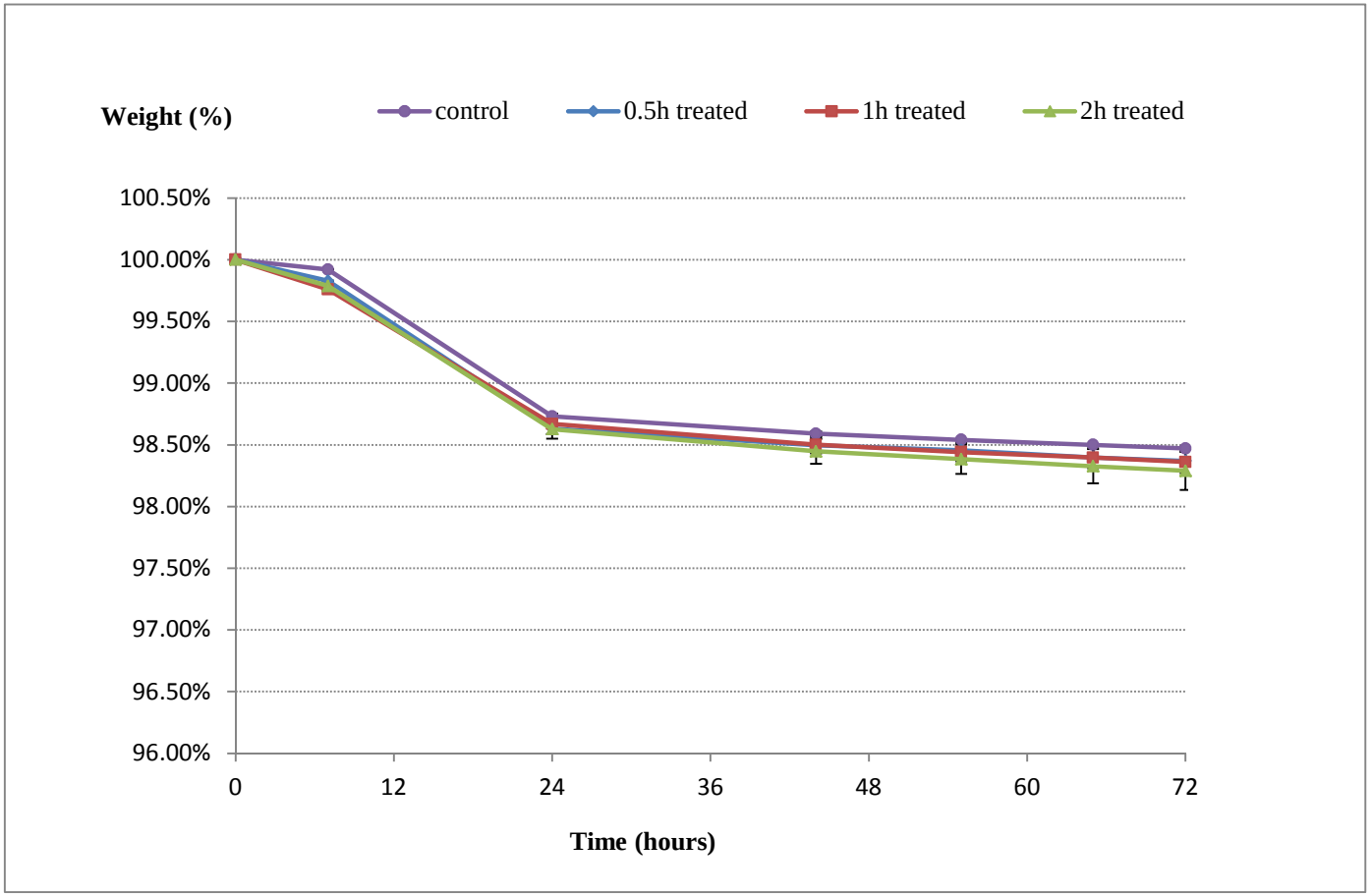


Figure 5.4: Comparison fermentation profiles of active dried yeast strain LAL7 at 30^{o}C with heat treatment at 35^{o}C for 0.5, 1 and 2 hours. Shake flask fermentation method was carried out with inoculation rate at 1x10^{7} cells per millilitre of mash. Error bars indicate standard deviation of triplicate samples.

Strain and conditions	Time	% Viability before heat treatment	% Viability after heat treatments	% Viability after fermentation	Total Cell number after fermentation
Thermosacc Control	NA	77%	NA	94%	2.93×10^8
Thermosacc 35°C	0.5h	75%	74%	80%	1.37×10^8
	1h	75%	72%	79%	1.92×10^8
	2h	75%	71%	85%	1.57×10^8
Thermosacc 40°C	0.5h	76%	73%	90%	2.43×10^8
	1h	76%	74%	90%	3.06×10^8
	2h	76%	72%	90%	3.56×10^8

Table 5.1. Viability of Thermosacc before and after heat treatment for 0.5, 1 and 2 hour at 35^{o}C and 40^{o}C. The viability and total cell number after fermentation are also expressed. In each instance viability was determined using methylene blue stating and enumeration of >400 cells.

Strain and conditions	Time	% Viability before heat treatment	% Viability after heat treatments	% Viability after fermentation	Total Cell number after fermentation
LAL7 Control	NA	74.34%	NA	81.52%	1.1×10^8
LAL7 35°C	0.5h	72%	68%	80%	5.28×10^7
	1h	72%	68%	79%	6.08×10^7
	2h	72%	66%	84%	8.80×10^7
LAL7 40°C	0.5h	71%	70%	84%	9.52×10^7
	1h	71%	71%	85%	7.56×10^7
	2h	71%	71%	84%	7.12×10^7

Table 5.2. Viability of LAL7 before and after heat treatment for 0.5, 1 and 2 hour at 35^{o}C and 40^{o}C. The viability and total cell number after fermentation are also expressed. In each instance viability was determined using methylene blue stating and enumeration of >400 cells.

5.2.3 Ethanol production of yeast subjected to pre-treatment at 35°C

At the end of fermentation, corn mash media was filtered in order to obtain clear liquid samples. These samples were then analysed for ethanol concentration using HPLC (Chapter 2) in order to examine the effects of 35°C pre-treatment on the ability of cultures to convert sugars to alcohol. The results in Figure 5.5 show that the amount of ethanol yielded by LAL7 at 0.5, 1 and 2 hours was within a similar range, between 4.2 - 4.3 ± 0.2%. However cultures which had been pre-treated showed an increase in ethanol over the control sample (4.3 ± 0.2% compared to 3.0 ± 0.1% for the control). For the strain Thermosacc, it is clear to see that the ethanol concentration increased as the time for heat treatment was increased (Figure 5.5), however in this instance in comparison to the control, there was a decrease in ethanol concentration.

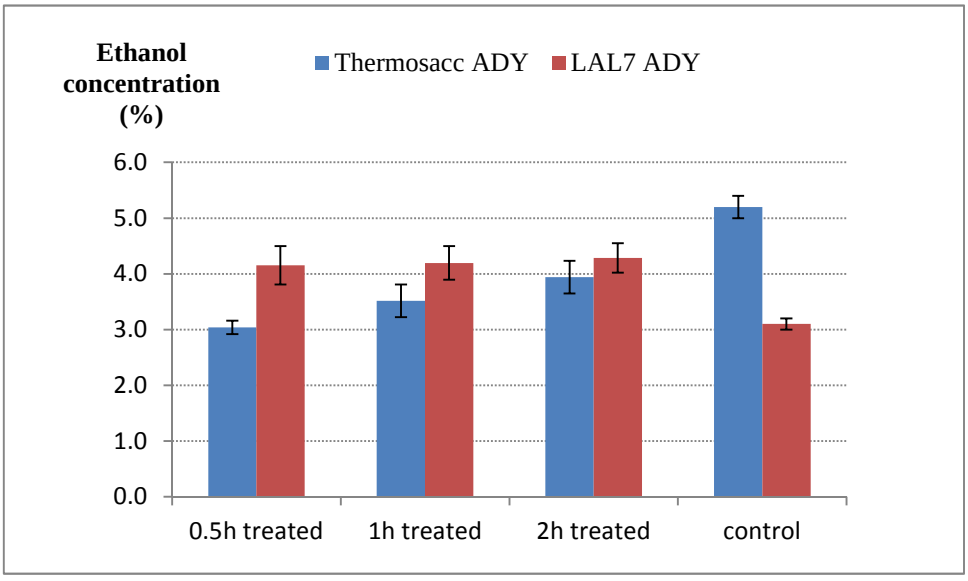


Figure 5.5: Ethanol concentration of 2 yeast strains pre-heated for 0.5, 1 and 2 hours at 35°C at the end of fermentation. Error bars indicate standard deviation of triplicate samples.

5.2.4 Effect of yeast pre-treatment at 40^{o}C on fermentation progression

In order to investigate the effect of heat treatment at 40^{o}C on yeast prior to and during fermentation capacity, yeasts were subjected to this temperature for 0.5, 1 and 2 hours in a similar fashion to that performed previously. Fermentation progression was again measured by determining weight loss due to carbon dioxide released during fermentation.

Analysis of sugar utilisation curves indicated that fermentations were finished within 72 hours, corresponding to previous data from this and previous Chapters (Chapter 4). From Figure 5.6, it can be seen that for Thermosacc, cultures which had been pre-treated at 40°C for various times each culture produced a lower final attenuation levels than the control samples. However, in contrast to the effect of 35°C, fermentations were best when treatment was conducted for 1h (Figure 5.6). Specifically, yeast treated with heat for 0.5h hours gave a final weight of (98.4%), while after 1h it was (97.5%). After 2h there was a seemingly negative effect on the yeast with weight loss similar to 0.5h treatment (98.3%). The effects of heat treatment followed the same pattern for LAL7, with 1h treatment yielding best results (97.1%), with the control sample again yielding the lowest weight loss (98.4%).

The viability of all cultures was also assessed to determine the effect of pre-heat treatment affects on yeast biomass production and yeast health. The results of this analysis indicated that, for Thermosacc, the viability before heat treatment was 75.56% (Table 5.1) while after heating at 40°C it was between 71-74% irrespective of the length of time for treatment, similar to that observed at 35°C. However at the end of fermentation the viability had

reached approximately 90% indicating again that heating had no long term effects on culture health, and furthermore that the culture had been able to recover during the fermentation itself. Interestingly, this was nearer to the viability observed in the control culture which was approximately 94% as reported earlier (Table 5.1).

For LAL7, the same pattern of results was seen as for treatment at 35°C. Before and after treatment there was a viability of 71% and 70% respectively (Table 5.2). However, after fermentation all cultures were again higher than the control sample of 81.5%, all greater than 84% viable (Table 5.2). As mentioned previously, it is unknown if this reflects the good recovery of heat treated population, or poor performance of the control sample and further experimentation would be required to verify the results.

When comparing yeast strains it was interesting to note that for both yeasts a longer heat treatment did not appear to influence viability either post treatment or post fermentation. Consequently, it is suggested that viability cannot be the cause of the difference in sugar utilisation observed, but that some other factor associated with heat treatment may play an important role.

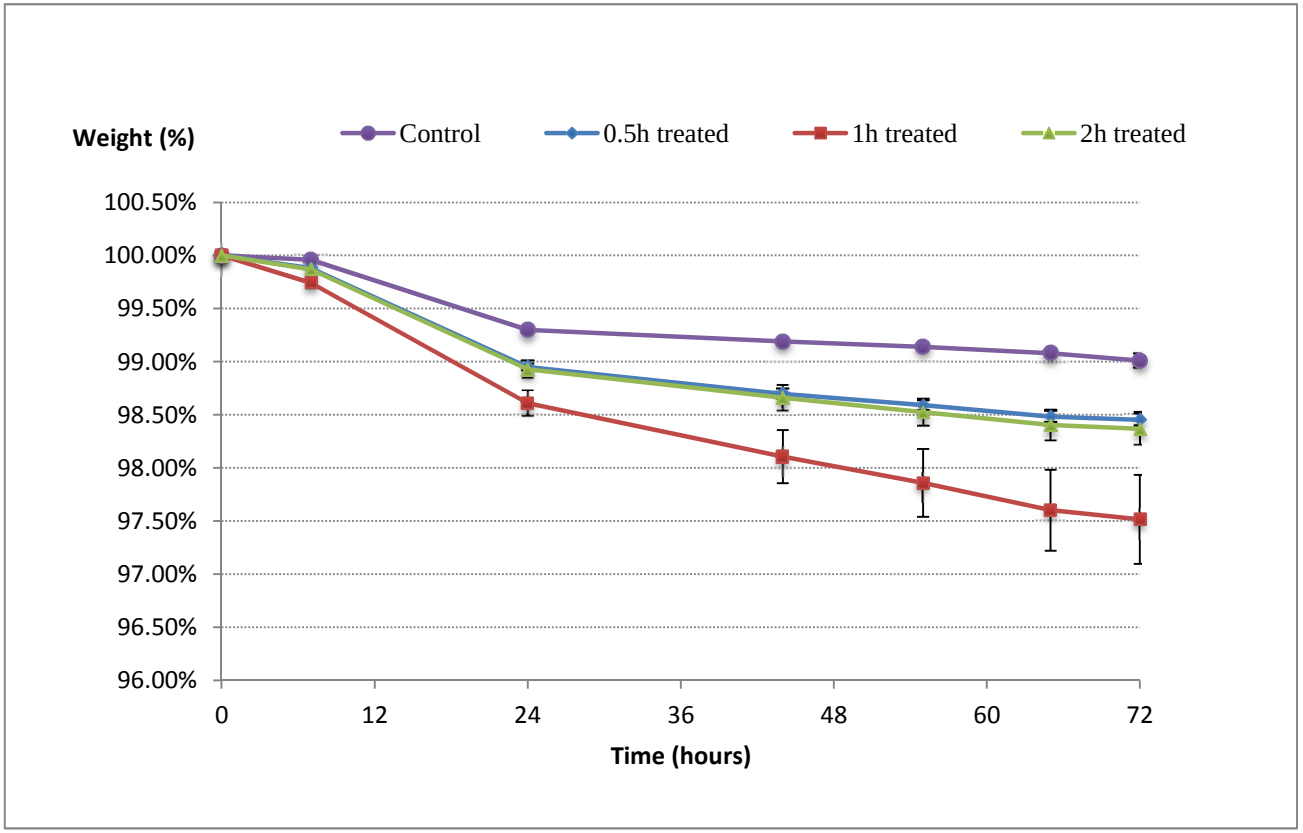


Figure 5.6: Fermentation progression of active dried yeast strains Thermosacc with 0.5, 1 and 2 hours treated with heat at 40^{o}C. An inoculation rate at 1 x 10^{7} cells per ml of mash was pitched into 100ml corn mash. Fermentations were then carried out using the flask fermentation method. Error bars indicate the standard deviation of triplicate samples.

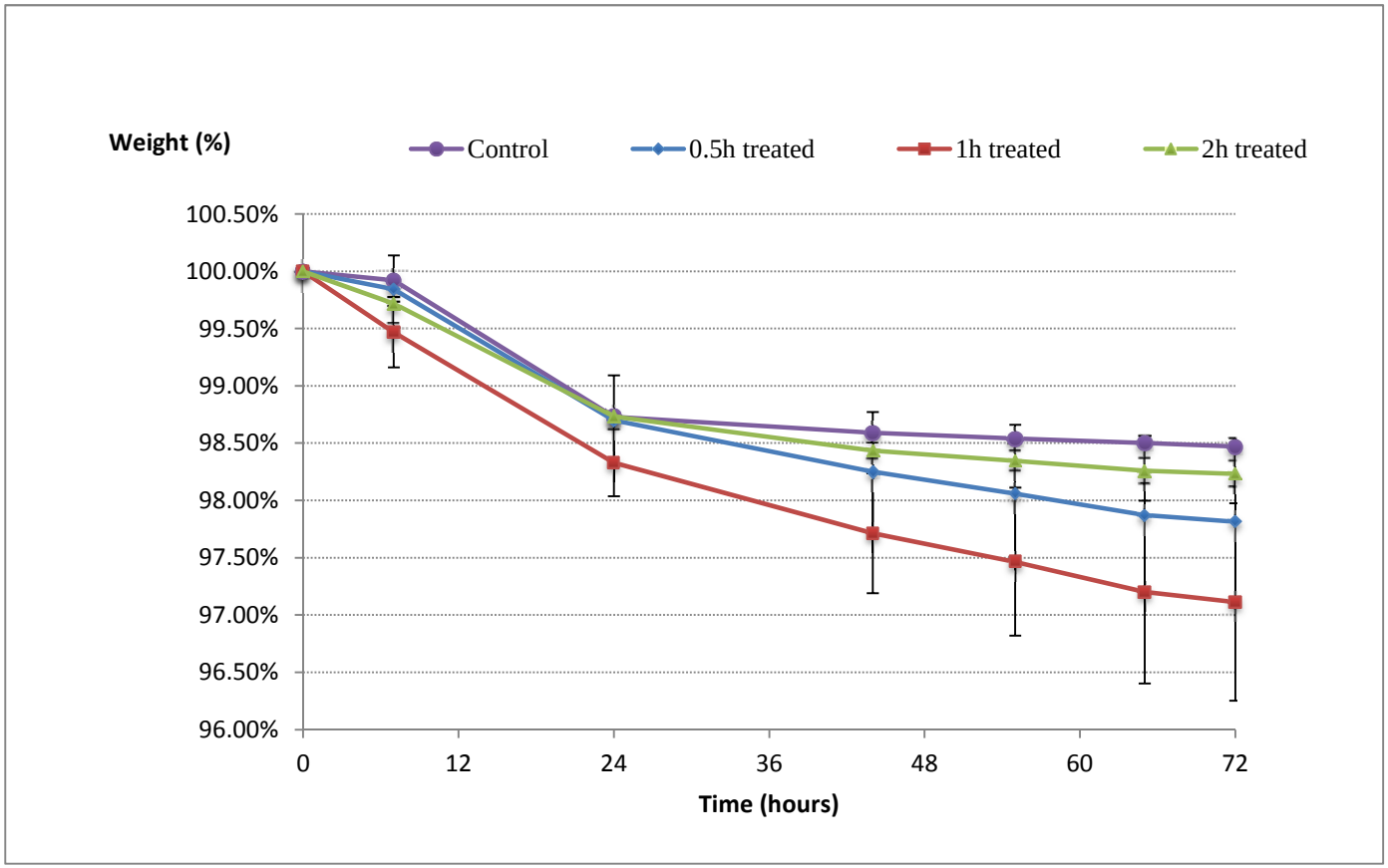


Figure 5.7: Fermentation progression of active dried yeast strains LAL7 with 0.5, 1 and 2 hours treated with heat at 40^{o}C. An inoculation rate at 1 x 10^{7} cells per ml of mash was pitched into 100ml corn mash. Fermentations were then carried out using the flask fermentation method. Error bars indicate the standard deviation of triplicate samples.

5.2.5 Ethanol production of yeast subjected to pre-treatment at 40^{o}C

In order to examine the effects of 40°C pre-treatment on the ability of cultures to convert sugars to alcohol, each sample at the end of fermentation was analysed for ethanol concentration as described previously (Chapter 2). It can be seen from Figure 5.8 that the amount of ethanol yielded by LAL7 at 0.5, 1 and 2 hours was within a similar range, between 4.8 ± 0.2% and 5.3 ± 0.3. Furthermore, similarly to pre-treatment at 35°C, these cultures showed an increase in ethanol over the control sample (3.0 ± 0.1%). Furthermore, the level of alcohol observed at 40°C was greater than at 35°C, indicating a possible advantage of high temperature pre-treatment with this strain. For the strain Thermosacc, similarly to that observed at 35°C, the ethanol concentration was lower than the control sample (Figure 5.5), even though the sugar utilisation profiles above indicated that a greater proportion of fermentable sugars had been utilised. Interestingly, for Thermosacc, ethanol production was also greater at 40°C than at 35°C, even though both sets of fermentations yielded less ethanol than the control sample without pre-treatment.

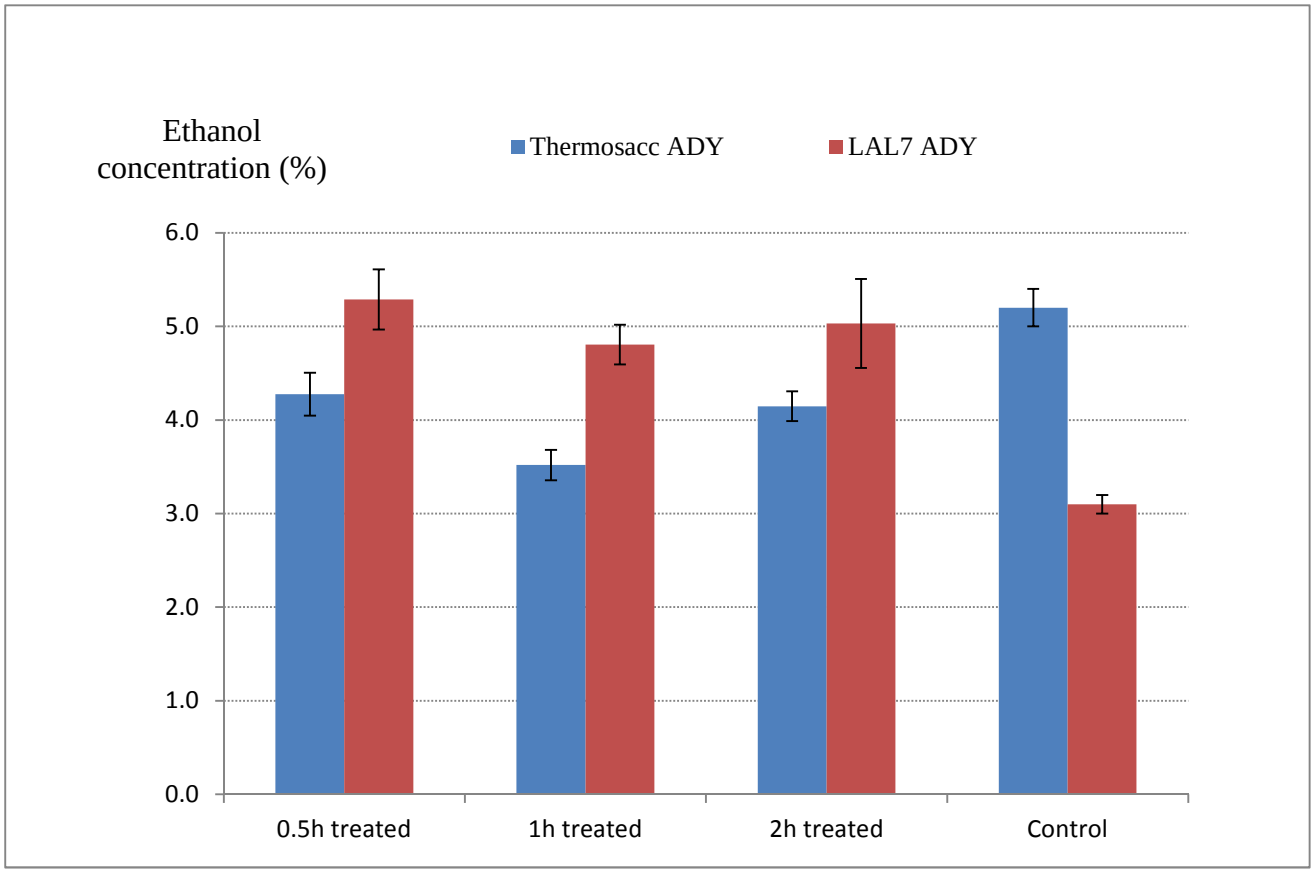


Figure 5.8: Ethanol concentration at the end of fermentation of two active dried yeast strains Thermosacc and LAL7 at 40^{o}C with 0.5, 1 and 2 hours treated with heat. An inoculation rate at 1 x 10^{7} cells per ml of mash was pitched into 100ml corn mash. Fermentations were then carried out using the flask fermentation method. Error bars indicate the standard deviation of triplicate samples.

5.3 Discussion

It is known that the efficiency of corn mash to ethanol fermentation is affected by a number of parameters such as yeast quality and temperature (Chapter 1). Indeed, for commercial bioethanol fermentation, the temperature is usually set between 25-35^{o}C and higher temperatures can maximize ethanol production (Rao and Pasha, 2005). However, if the fermentation temperature is higher than 35^{o}C the denaturation of yeast proteins and enzymes can occur along with a reduced resistance to ethanol stress, reduced ethanol production (Hughes *et al*., 1984; Rao and Pasha, 2005; Araque *et al*., 2008; Yah *et al*., 2010) and the possibility of stuck fermentations (Laluce *et al*., 1991; Thomas *et al*., 1993; Jones and Ingledew, 1994). Despite the negative effects of heat on fermentation, short sub-lethal stress is known to procure a benefit to cells in certain situations (Chatterjee *et al.*, 2000; Birch and Walker, 2000). However, the effect of yeast pre-treatment using temperature on corn mash fermentation and ethanol yield has not previously been investigated fully. In order to understand more about the effects of sub-lethal heat treatment on yeast health and performance during corn mash fermentation, yeast strains Thermosacc and LAL7 were subjected to a variety of heat treatments and analysed for fermentation characteristics.

Analysis of glycogen and trehalose before and after heat treatment indicated that, generally, the results agreed with previous reports that heat induction can trigger trehalose accumulation in yeast (Hottiger *et al*, 1987a, b; D'Amore 1991). However, intracellular glycogen content remained similar, which is not surprising since this compound is believed to function as a storage carbohydrate rather than have a role in protecting cells (Boulton and Quain

2000, Francois and Parrou, 2001). The trehalose levels were observed to increase as the pre-treatment temperature was increased from 35 and 40^{o}C, although this increase was smaller than expected. In addition, the longer the treatment time, the more trehalose was produced, for example the amount of trehalose present after conditioning at 35^{o}C for 2 hours was higher than both 0.5 and 1 hour. However, this increase was dependent on the yeast strain and, as already mentioned, was only small when compared to the control sample. Indeed, although strain LAL7 showed an increase in trehalose when heat induction was performed at 35^{o}C, its level was reduced at 40^{o}C. This may be explained by the heat tolerance of this strain which was previously shown to be lower than Thermosacc (Chapter 3). This may have caused LAL7 to be unable to function as efficiently at 40^{o}C, perhaps caused by cell components being damaged by heat stress, leading to genes and enzymes responsible for the production of trehalose being not fully functionable. Furthermore, LAL7 did not show any increase in trehalose at all, typically exhibiting between 79.59 ± 2.74 µg/1x10^{8} cells before treatment to a maximum of 85.33 ± 3.59 µg/1x10^{8} cells afterwards (Figure 5.2). The data were unexpected given that under similar circumstances, specifically after switching temperature from a similar range (27^{o}C to 40^{o}C) to introduce the heat shock, Hottiger *et al*. (1987b) observed a three-fold increase in trehalose. However, the same authors noted that trehalose was rapidly degraded once temperature was shifted back from 40^{o}C to 27^{o}C and it is possible that a similar event happened in these samples prior to analysis, although care was taken to process samples immediately.

Although trehalose levels were not as elevated as expected, fermentation performance analysis indicated some differences between each of the pre-treated cultures. In each instance, heating cells appeared to result in greater sugar uptake with identical patterns for both yeast strains. At 35°C this was observed after 2h treatment, and at 40°C it was found after 1h treatment. This difference may have been due to the level of stress applied. It is possible that 2h treatment was too long at the higher temperature leading to less positive effects. Irrespective, it was interesting to note that despite greater sugar uptake, for Thermosacc less ethanol was produced in each instance. This data was not expected but it may be explained by the original analysis indicating that this strain is simply more resistant to temperature (Chapter 3). Lower conversion of carbon to ethanol could indicate a shift in carbon flux to respond to stress and to trigger cell repair mechanisms rather than producing ethanol or cell mass (Walker, 2009). However it is also possible that combinations of stress (ethanol and temperature) may have led to the poor performance in the current study. In contrast to Thermosacc, for LAL7 more ethanol was produced in each instance after pre-treatment, with greater temperature leading to higher alcohol, although the time of treatment did not appear to influence results. It is possible that for this strain, short heat treatment at 40^{o}C can improve stress tolerance and therefore LAL7 is able to convert glucose to ethanol more efficiently. This indicates that, for this strain, pre-treatment may be an effective means of increasing yield.

It was also interesting to find that pre-heating of yeast could influence the viability of the culture, although this improvement was again dependent on the yeast strain used. When pre-treating at 35^{o}C, both Thermosacc and LAL7

yeast cultures treated for 1 and 2 hours yielded 5% higher viability than cultures treated for 0.5h. Although pretreatment had a positive effect on LAL7, it was perhaps greater for Thermosacc, again suggesting that this strain perhaps devoted more energy to repair than ethanol production.

Chapter 6. Conclusion and Future works

Over recent years the demand for alternative fuels has increased due to concerns regarding the security of petroleum and oil supplies. In order to supplement existing fuels, ethanol is now widely produced and used as an environmentally friendly energy source (Zhao and Bai, 2009). However the bioethanol industry is still relatively new and fermentation process innovations are required to maximize ethanol yield and allow ethanol to be produced economically (Yu *et al*., 2003; Cardona and Sanchez, 2007). One such innovation is the pre-treatment of yeast slurries to optimise fermentation performance. In this thesis, the main aim was to determine if sub-lethal heat treatment could be applied to induce the production of anti-stress compounds which would provide a benefit to yeast during corn to ethanol fermentations. Consequently, four industrial commercial yeast strains; LAL7, Ethanol Red, Thermosacc and 2592 were initially examined for their response to heat stress and subsequently for the impact of temperature preconditioning on yeast physiology and ethanol yield during corn mash fermentations.

In Chapter 3, a number of different assays were used to assess the effect of temperature on the growth characteristics of each strain. Spot plate and growth curve analyses indicated that each yeast was able to grow and survive under a wide range of temperatures, although their growth rate was dependent on the temperature set. Yeast growth was observed to be optimal between 25 and 35^{o}C and was poor at 40^{o}C or higher in all strains, although the strain Thermosacc was able to tolerate up to 45^{o}C to some extent. Further analysis indicated that the volume of yeast cells and accumulation of protective compounds in response to heat was also variable. In particular, the content of

the known stress protectant molecule trehalose (Wiemken, 1990) in Thermosacc and 2592 was observed to be significantly higher than in Ethanol Red and LAL7. This was particularly true when cultivated at 40^{o}C, where Thermosacc contained significantly higher levels of this molecule than any of the other strains. This initial work suggested that Thermosacc may be able to respond well to high temperatures, potentially giving these cells greater heat resistance. In contrast, strain LAL7 accumulated the least trehalose, and also demonstrated some unusual characteristics when compared to the other strains. LAL7 accumulated more glycogen under high temperature conditions and produced cells which were larger in size than any of the other yeasts examined. This was exacerbated at higher temperatures where the cell size of all strains except LAL7 began to be reduced. The precise reasons for this are unknown, however the fact that the other three strains were able to tolerate heat better than LAL7 and produced smaller cells indicated that size may actually play a role in heat tolerance or stress resistance in general. This supported the previous observation that strains which naturally form smaller cells may be more successful at fermenting particularly high gravity substrates (Ingledew 2009).

Due to the interesting characteristics of LAL7 and Thermosacc, these strains were selected and examined further in Chapter 4, specifically for their fermentation characteristics using a simultaneous saccharification and fermentation (SSF) system. However, analysis of wet and ADY yeasts using the initial stirred fermentation vessels yielded unusual results. Although fermentations were finished early, yeast health was observed to be extremely poor and a low ethanol yield was achieved. The poor results obtained using

stirred fermentations were unexpected, however it was evident that the viscosity of the corn mash had an effect, with poor agitation leading to poor yeast dispersal and poor final attenuation. The use of shaken fermenters as a means of agitation produced more realiable data. The reason may be explained improved oxygen transfer due to the method of agitation employed. As the original density of yeast was also lower than in the stirred fermenters it is also possible that increased cell division may have enabled cells to adapt to the environment, allowing them to cope with stresses more gradually. Irrespective of the reasons behind the differences in fermentation, the shaken fermenters were deemed to be more robust and were used for subsequent experiments.

In order to assess the effect of pre-treatment on the efficiency of corn mash fermentations and yeast physiology, yeasts were subjected to heat at 35 and 40^{o}C for 0.5, 1 and 2 hours prior fermentation (Chapter 5). The results suggested that pre-heat treatment on yeast had both negative and positive effects on corn mash fermentation, yeast viability and ethanol yield. In general, heating yeast prior to fermentation caused attenuation to be lower and improved cell viability post fermentation. To determine potential reasons for this, glycogen and trehalose concentrations were analysed. Analysis of glycogen before and after heat treatment indicated that levels of this compound were not affected, which was not unexpected as this molecule is typically not linked to the heat stress response. However analysis of trehalose only yielded a minor increase compared to the control sample. This was particularly unexpected for Thermosacc given that in Chapter 3 it was demonstrated that this strain was capable of accumulating this molecule to a high level. However it is important to note that the preliminary data

considered trehalose accumulation over 3 days (Chapter 3), rather than <2h as performed in the later experiments (Chapter 5). Consequently the possibility remains that the temperature and conditions applied were not severe enough to induce the stress response in Thermosacc or LAL7, even though similar conditions cause a three-fold increase in industrial strains studied previously (Hottiger *et al*., 1987b). Despite this concern, heat treating both strains at 40°C for 2h yielded fermentations which were not as efficient as those treated at 40°C for 1h, indicating that these conditions may actually have been stressful, although perhaps not strong enough to warrant production of trehalose to significant levels. Due to this discrepancy it would be important to analyse both strains further using different time and temperature regimes in an attempt to determine the exact conditions appropriate to produce trehalose in these strains and then to analyse the effect of this on fermentations.

It should be noted that strain LAL7, which was initially noted as having a poor response to heat actually performed better than Thermosacc, producing elevated levels of ethanol. Again, this data was not expected but it may be explained by the higher concentration of glycogen in this strain, perhaps leading to a higher initial carbon pool, resulting in greater ethanol yield. Alternatively, the improved stress resistance of Thermosacc to temperature may have caused this strain to dedicate more energy to cell repair mechanisms rather than producing ethanol, a hypothesis partially supported by the fact that a higher viability was observed for this strain post-fermentation. Despite this theory, it should also be noted that all fermentations yielded a relatively low ethanol concentration when compared to industrial scale fermentations, which are typically in the range of 8-12%. The reasons for this are unknown, but

could be due to the high concentration of unfermentable sugars initially present in the corn mash. Although enzyme additions were made in excess in the manner of an SSF system, it is possible that some long-chain sugars remained in an non-fermentable form. Consequently future work could be performed to more carefully optimise the concentration of enzyme applied. This may yield more simple sugars for fermentation, resulting in lower attenuation and higher concentrations of ethanol which are more representative of industrial corn to ethanol fermentations

As alluded to above, it is interesting to note that the strain Thermosacc, which was initially noted as being more heat tolerant, accumulating higher trehalose and having smaller cells, did not appear to produce as much ethanol after heat-treatment as LAL7. LAL7 was initially characterised as having high glycogen, low trehalose and large cells, all of which have been implicated in poorer ethanol production. It is possible that for this strain, short heat treatment at 40^{o}C may lead to improved stress tolerance and therefore a greater capacity to convert glucose to ethanol. However, it does also indicate that trehalose alone may not be sufficient and that other molecules such as heat shock proteins and glycerol (Piper, 1995; Li *et al.,* 2009) may play an important role. Further investigation could incorporate a range of analysis to include the study of these molecules. In addition, it would also be interesting to perform yeast vitality analyses as a means of assessing heat-treatment and subsequent fermentations. For example, in this study a high yeast viability at the end of fermentation was shown with different temperature treatment, however it is possible that cell organelles and function could be subjected to various degrees of damage. Vitality analysis by determining enzyme activity or by assessing

cell activity could provide useful information on the precise effects of heat treatment on cells.

While this study has provided some preliminary data on the potential of heat-treatment on fermentation characteristics, there is much that is still to be learnt. The effects of heat on yeast cultures is inevitably strain dependent and the potential benefits are dictated by the strains ability to tolerate combinations of stress factors. One issue which has not been considered thus far is the feasibility of performing controlled heat-treatment in industrial scale vessels. While this is easy to perform accurately at laboratory scale, heat transfer in larger vessels becomes increasingly difficult. Consequently, it may be that adjusting the temperature of the corn mash at pitching may be more easily controlled than trying to adjust the temperature of a yeast slurry. Indeed, even strains with relatively poor heat tolerance may still be able to undergo heat-treatment, perhaps by incorporating a ramped-down temperature profile immediately after pitching. However, it is likely that such strains would require conditions to be applied at a reduced level to prevent any adverse effects from occurring. Despite these concerns, there is some evidence here to suggest that there may be a benefit to yeast cultures by adopting pre-treatment, and, in particular for LAL7, heating cells at a sub-lethal level for a short period of time may be an effective means of increasing ethanol yield.

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