The Role of Inositol in Yeast Adaptation

A Thesis Submitted by

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ABSTRACT

Inositol is required for better yeast growth, stress tolerance and fermentation performance. This study focused on investigation of the role of inositol as a stress tolerance inducer, rather than as an essential growth factor. Three *Saccharomyces cerevisiae* strains with different properties were used in the present study, namely A12, A15 and K7. These strains were selected based on their different stress tolerance and ethanol productivity.

The first step of the present study was to determine the best conditions whereby the effects of supplementation of inositol can be differentiated from the control, where no inositol was added to the fermentation media. Since fluidity of the yeast cellular membranes was examined using fluorescence spectroscopy as one of the parameters in the present study, yeast nitrogen base (YNB) was used as basal media. YNB is a chemically-defined fermentation medium that does not interfere with fluorescence spectroscopy measurements in the UV to blue range. It is a useful medium for *in situ* monitoring, during fermentation, of cell physiology by fluorescence methods, however compared to rich media it is considered to have poor nutritional availability, which could affect the yeast ability to convert sugar to ethanol. Glucose concentrations ranging from 5 to 15% (w/v) were applied to investigate the highest concentration of glucose able to be efficiently converted to ethanol by each yeast strain. Growth and fermentation performance of the yeast strains were different. The fermentation performance could be ranked (highest to lowest) as strains A15, A12 and K7, while the growth performance could be ranked K7, A12, and A15. In general, fermentation with 15% initial sugar in the minimal medium led to lower sugar conversion to ethanol. The medium containing 10% glucose was considered the best to optimally differentiate fermentation performance of yeast strains.

The main objective of the present study was to investigate the effect of inositol supplementation on growth, fermentation performance and cellular membrane fluidity. Yeast cells were grown in a chemically-defined fermentation medium based on YNB but with no inositol and with 10% (w/v) glucose, and with varying levels of inositol supplementation (0.05, 0.10, 0.20, 0.40 and 0.80 g/L). Cell density, cell viability, glucose consumption and ethanol production were monitored for 96 hours. Cellular membrane fluidity was monitored at 24 hours fermentation, representing the respiro-fermentative growth phase, by measuring generalized polarization (GP) of laurdan. The effect of ethanol on membrane fluidity was also monitored by measuring GP after exposing cells to 18% (v/v) ethanol. When analysing the effect on fermentation kinetics it was found that inositol supplementation did not have the same effect on all strains, with A15 affected least. Although inositol supplementation did not seem to improve fermentation performance of yeast strain A15, it did improve cell growth leading to higher cell densities. Yeast strains A12 and K7 also evidenced higher cell densities with inositol supplementation, confirming the reported necessity of inositol for yeast growth. Unlike the preliminary experiment of the present study which used standard YNB medium containing 0.002 g/L inositol, the main experiments included analysis of multiple concentrations of inositol to better define the minimal requirement. While inositol-supplemented cells had higher growth rates and cell densities, they had significantly lower viability, thus the viable cell counts were similar with and without supplementation. Fluidity of the yeast cell cellular membranes responded differently to inositol supplementation. It was expected that inositol supplementation can increase membrane fluidity of all yeast strains. However, it was found that for yeast strains A12 and K7, inositol seemed to decrease cellular membranes fluidity, while for yeast strain A15 inositol supplementation led to increased membrane fluidity. When exposed to high

ethanol concentrations, yeast strain A12 with inositol supplementation showed a significantly greater increase in membrane fluidity compared to cells grown without inositol.

Further analysis of the effect of inositol on fermentation performance of the three yeast strains showed that final ethanol concentration produced by yeast strain A15 was not significantly different when the fermentation media were supplemented with inositol, while yeast strains A12 and K7 produced significantly higher ethanol with inositol supplementation. Examination of the effect of inositol on yeast stress tolerance indicated that the three strains tested had better tolerance against ethanol, hyperosmotic and acetic acid stress when the fermentation media were supplemented with inositol. This led us to conclude that inositol acts as a general stress protector. One possible mechanism for the increased stress tolerance against hyperosmotic stress could be stimulation of synthesis of the osmoprotectant glycerol, as indicated by the significantly higher extracellular glycerol produced by inositol-supplemented cells. Inositol was also found to affect the fatty acids composition of the total cell lipid, where each strain showed a different response. No change was seen in the unsaturated fatty acid proportion for yeast strain A12, while yeast strain K7 showed a marked increase in C18:1, and yeast strain A15 had lower C16:1, but significantly higher C18:1.

In summary, the present study found that, compared to the industrial situation in which rich media are used, osmotic stress is evidenced at a lower sugar concentration (15% w/v, compared to 27%) when minimal media are used for fermentation. Therefore, to be able to distinguish the effect of inositol supplementation, we used a lower sugar concentration (10% w/v). At this concentration the sugar may not be exhausted, but the osmotic stress is not too severe. Inositol addition experiments indicated that even though inositol supplementation did not affect fermentation performance, it did increase cell

growth and affect cellular membrane fluidity. Specifically, yeast strains A12 and K7 showed increased fluidity while A15 showed decreased membrane fluidity when grown in inositol-supplemented media. Further investigation on inositol supplementation indicated that inositol acts as general stress protector. Inositol supplementation also affects cellular fatty acid composition, where each strain showed a different response. Therefore, we conclude that inositol effects are strain-specific.

CERTIFICATION OF THESIS

This Thesis is entirely the work of Safri Ishmayana except where otherwise acknowledged. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: Dr. Mark Lynch

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Student and supervisors signatures of endorsement are held at the University.

ASSOCIATED PUBLICATIONS

Part of the work in this thesis has been published as follows:

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ABBREVIATIONS

ADH	alcohol dehydrogenase
ANOVA	analysis of variance
ATCC	American type culture collection
ATP	adenosine tri phosphate
C6:0	caproic acid, hexanoic acid
C8:0	caprylic acid, octanoic acid
C10:0	capric acid, decanoic acid
C12:0	lauric acid, dodecanoic acid
C14:0	myristic acid, tetradecanoic acid
C14:1	myristoleic acid, teradecenoic acid
C16:0	palmitic acid, hexadecanoic acid
C16:1	palmitoleic acid, hecadecenoic acid
C18:0	stearic acid, octadecanoic acid
C18:1	oleic acid, octadecenoic acid
C18:2	linoleic acid, octadecadienoic acid
C18:3	linolenic acid, octadecatrienoic acid
C20:0	arachidic acid, eicosanoic acid
CDP-DAG	cytidine diphospho-diacylglycerol
CL	cardiolipin
DLPC	dilauroyl-phosphatidylcholine
DNA	deoxyribonucleic Acid
DPH	1,6-diphenyl-1,3,5-hexatriene
EPR	electron paramagnetic resonance
ER	endoplasmic reticulum
FAD2	Δ12-fatty acid desaturase encoding gene
FAME	fatty acid methyl ester
FTIR	fourier transform infrared
G	instrument grating factor
g	gram, unit of mass
G	gravity, relative centrifugal force
GC-MS	gas chromatography-mass spectrometry
GK	glycerol kinase
GP	generalized polarization
GPI	glycosylphosphatidylinositol

GPO	glycerol-3-phosphate oxidase
GRAS	generally recognized as safe
HOG	high osmolarity glycerol
HPLC	high performance liquid chromatography
HSD	honestly significant difference
HSP	heat shock proteins
INO1	*myo*-inositol-1-phosphate synthase encoding gene
IPC	inositol-phosphoceramide
I_{VH}	fluorescence emission intensity measured in the plane perpendicular to the plane of vertically polarized excitation
I_{VV}	fluorescence emission intensity measured in the plane parallel to the plane of vertically polarized excitation
L	litre, unit of volume
Laurdan	6-lauroyl-2-dimethylamino naphthalene
LCB	long chain base
MIPC	mannose-inositol-phosphoceramide
$M(IP)_2C$	mannose-(inositol-P)$_2$-ceramide
Mg	milligram, 10^{-3} gram
mL	millilitre, 10^{-3} litre
MW	molecular weight
NAD^+	micotinamide adenine dinucleotide (oxidized form)
NMR	nuclear magnetic resonance
OD_{600nm}	optical density at 600 nm
OLE1	Δ9-fatty acid desaturase encoding gene
OPI1	negative regulatory factor of the *INO1* structural gene encoding gene
opm	orbital per minute
P	polarization
p	p-value, probability value
PA	phospatidic acid
PC	phosphatidylcholine
PDA	photo diode array
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PMMA	polymethyl methacrylate
PS	phosphatidylserine

Qs	glucose consumption rate
Qp	ethanol production rate
R	Pearson correlation coefficient
r	anisotropy
RID	refractive index detector
SD	standard deviation
TPC	total plate count
UI	unsaturation index
VHG	very high gravity
% w/v	percent of weight of solute in the total volume of solution
YEP	yeast extract peptone
YNB	yeast nitrogen base
YNBNG	yeast nitrogen base without glucose
YPD	yeast extract peptone dextrose
YPDE	yeast extract peptone dextrose with ethanol
Yp/s	ethanol productivity

CHAPTER ONE: LITERATURE REVIEW AND BACKGROUND

1.1 Introduction

There are emerging needs to find alternative renewable energy sources due to declining fossil fuel reserves (Shafiee & Topal 2009). Among the alternative fuels that have been developed, bioethanol is widely known and used around the world. Bioethanol as a fossil fuel substitute has become more important not only because of the decreasing availability of fossil fuels, but also because of increasing fossil fuel price and environmental issues (Bai, Anderson & Moo-Young 2008; Thomsen, Medina & Ahring 2003). In fuel applications, bioethanol is mostly mixed with gasoline, with common proportions ranging from 5 - 95% v/v ethanol (Hammel-Smith *et al.* 2002; Kumar, Singh & Prasad 2010; Solomon, Barnes & Halvorsen 2007), even though application of high level ethanol blends requires a suitable specialized design of vehicles (Bailey 1996).

Bioethanol is ethanol derived from sugary, starchy or cellulosic material feedstocks. These feedstocks are converted to bioethanol through fermentation processes by the activity of microorganisms, especially baker's yeast, *Saccharomyces cerevisiae* (Smith 2006; Thomsen, Medina & Ahring 2003). Some studies also applied genetically-modified mutants of the bacterium *Zymomonas mobilis* capable of xylose fermentation for ethanol production, especially in cellulosic-derived feedstocks, due to its ability to convert 5 carbon sugars to ethanol (Dien, Cotta & Jeffries 2003; Fu & Peiris 2008; Wirawan *et al.* 2012). However, *S. cerevisiae* is still preferred over non-genetically modified *Z. mobilis* for industrial fermentation processes because of the following reasons. Firstly, *Z. mobilis* has a narrow substrate preference, utilizing only D-glucose, D-fructose and sucrose as substrates (Bai, Anderson & Moo-Young 2008). When sucrose is used

as the substrate, formation of by-products decreases ethanol productivity. *Z. mobilis* is not an ideal microorganism for industrial process since industrial feedstocks are generally complex sugar mixtures (Bai, Anderson & Moo-Young 2008). Secondly, biomass waste generated from the fermentation process using *S. cerevisiae* can be used as animal feed, as the yeast belongs to the generally recognized as safe (GRAS) microorganisms and is accepted as animal feed. However, even though *Z. mobilis* is also defined as a GRAS microorganism, it is not generally accepted as animal feed, and therefore will create problems with waste management. Lastly, *Z. mobilis* is reported to be oscillatory (cycles of increased and decreased metabolites during fermentation) when applied in continuous fermentation. This property can negatively impact on fermentation performance, leading to incomplete utilization of sugar. Therefore *S. cerevisiae* is still preferred over *Z. mobilis* at the industrial bioethanol fermentation scale (Bai, Anderson & Moo-Young 2008).

Compared to the other microorganisms, *S. cerevisiae* is known for its high tolerance against environmental stress, and is therefore more suitable for the conditions prevailing in industrial ethanol fermentations (Bhadana & Chauhan 2016). Ethanol, as the final product of the fermentation process, is also toxic for the yeast itself by affecting membrane fluidity as well as key cytosolic and membrane-bound proteins that have important roles in cellular homeostasis (Learmonth & Gratton 2002; Nagodawithana & Steinkraus 1976). Many attempts have been conducted to increase yeast tolerance to the stresses endured during fermentation as well as improve ethanol productivity, including supplementation of the fermentation media and genetic engineering of the yeasts, or a combination of both approaches. Some supplements added to the fermentation media to improve stress tolerance include metal ions (Xue *et al.* 2008), trehalose (Hottiger *et al.* 1994), proline (Takagi *et al.* 2005; Taylor *et al.* 2008) and inositol (Chi, Kohlwein &

Paltauf 1999; Ji *et al.* 2008). Genetic engineering approaches include modifying enzymes involved in biosynthesis of stress protectant molecules (Krause *et al.* 2007; Takagi *et al.* 2000; Takagi *et al.* 2005) or incorporating and/or stimulating synthesis of proteins involved in increasing tolerance (Alper *et al.* 2006; Çakar *et al.* 2005). To enhance stress tolerance, a combination of genetic engineering and supplement addition has also been performed (Krause *et al.* 2007; You, Rosenfield & Knipple 2003).

Myo-inositol (referred to simply as inositol in this thesis) has been used as supplement in fermentation processes and was found to increase ethanol tolerance and ethanol productivity (Caridi 2002; Nikolić *et al.* 2009a). Inositol supplementation reportedly led to altered phospholipid composition of the yeast plasma membrane, leading to increased ability to tolerate ethanol (Chi, Kohlwein & Paltauf 1999; Gaspar *et al.* 2006). Yeasts grown in inositol-supplemented media had a higher proportion of phosphatidylinositol (PI) (Chi, Kohlwein & Paltauf 1999). This observation indicates that PI has an important role in adaptation of the yeast cell against ethanol. This was supported by the work of Krause *et al.* (2007) who developed a yeast mutant strain with capability to synthesize PI constitutively and found that the mutant strain had higher ethanol tolerance than its parent strain.

The yeast plasma membrane is an important barrier when yeast cells are exposed to stress. Therefore, maintaining its integrity is crucial for cell survival when the cells are exposed to various environmental stresses. Changes in phospholipid proportions in the yeast plasma membrane may lead to changes in its physical properties, including membrane fluidity. Jurešić, Blagović & Rupčić (2009) found that yeast cells with different proportions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) had different unsaturation indices (UI) which has been inferred to reflect different membrane fluidity. Therefore, it is also

interesting to investigate whether the changes in lipid composition due to inositol supplementation also lead to changes in membrane fluidity.

Many studies in the literature have inferred membrane fluidity indirectly by determining only the fatty acid unsaturation index, while only few have included direct measurement of membrane fluidity. According to Alexandre, Rousseaux & Charpentier (1994) and Learmonth (2012) utilization of the unsaturation index value as an indication of membrane fluidity is not recommended, since while the phospholipid and fatty acid composition are dominant factors in determining membrane fluidity numerous other factors may also make major contributions to the fluidity, for example sterol and protein composition as well as membrane environmental factors. It has been recommended that direct measurement, using techniques such as fluorescence spectroscopy, is required to enable accurate representation of the membrane fluidity. In a recent study we investigated effects of inositol supplementation on yeast ethanol tolerance and membrane fluidity, although lipid compositional analysis was beyond the scope of the study and findings were inconclusive at the range of inositol concentrations studied (Ishmayana 2011; Ishmayana, Kennedy & Learmonth 2011). In the present study, we aimed to assess membrane lipid composition as well as membrane fluidity measured using a spectrofluorometry technique, in order to further investigate the effects of inositol supplementation on the yeasts.

1.2 Yeast Fermentation

Sugars, as the main energy source for most microorganisms, can be converted to various end products depending on the microorganism's specific metabolic enzyme pathways as well as the prevailing cellular conditions. The end product can be, for example, acetic acid, lactic acid, ethanol, succinic acid, fumaric acid or carbon dioxide. Generally, in sugar dissimilation there are three stages of

reactions: (i) hydrolysis, (ii) cleavage and oxidation and (iii) reduction or oxidation. In the first stage, sugar from the environment is transported into the cell. Generally, the monosaccharide form of the sugar can be directly transported into the cell. Disaccharides or other complex sugars could be cleaved to monosaccharides by secreted or cell-wall bound enzymes, or by transport into the cell followed by intracellular hydrolysis. Within the cell, monosaccharides are phosphorylated to form sugar phosphates in the second stage. The sugar phosphate is generally converted into pyruvate through cleavage and oxidation. Finally, in the last stage, depending on the metabolism of the microorganism, the pyruvate is converted to various end products as noted previously (Nelson & Cox 2013; Pronk, Steensma & van Dijken 1996). Even though there are three different metabolic pathways for catabolism of phosphorylated sugars, all sugar metabolizing microorganisms share a common pathway: the lower part of Embden-Meyerhof pathway (glycolysis), which is the conversion of triose phosphate to pyruvate. The main differences between microorganisms are in the further metabolism of pyruvate (Pronk, Steensma & van Dijken 1996). The possible metabolism of sugar in microorganisms is presented in Figure 1.1.

Yeast is the most widely known microorganism used in industrial ethanol fermentation processes. According to their ability to produce ethanol as related to the presence of oxygen, yeasts are divided into two groups, Crabtree-positive and Crabtree-negative. Crabtree-positive yeasts are yeast species capable of accumulating ethanol by fermentation under aerobic conditions. Crabtree-negative yeasts only degrade sugar to CO_2 via respiration in aerobic conditions (Piškur *et al.* 2006; Pronk, Steensma & van Dijken 1996). *S. cerevisiae* belongs to the Crabtree-positive yeast group, since it has the capability to convert sugar with 6 carbon atoms (6C) to ethanol (2C) molecules, in the presence of oxygen without completely oxidising it to CO_2. Note that the ethanol is produced by what

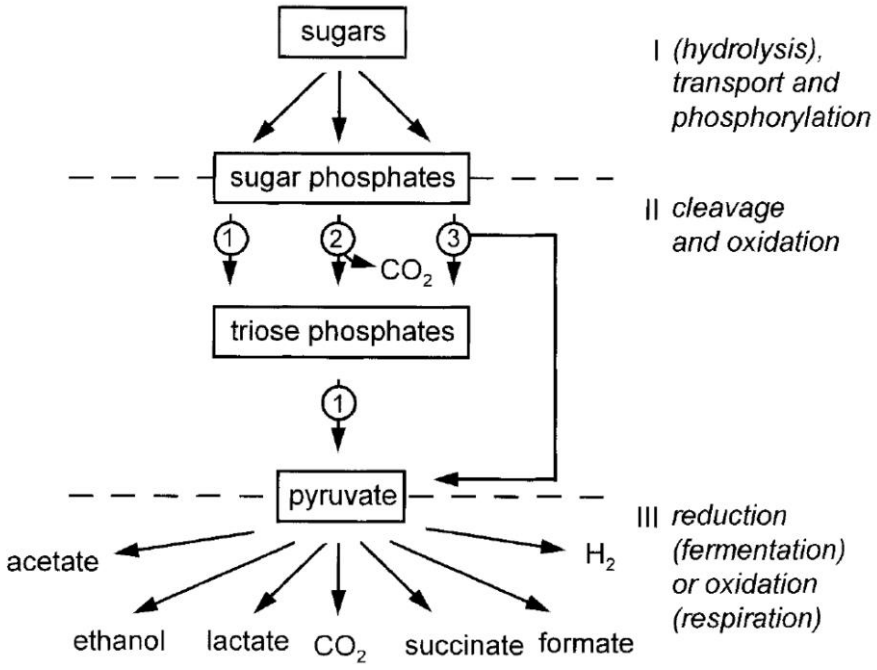


Figure 1.1 Scheme representing the diversity of sugar dissimilation pathways in microorganisms, in which each pathway consists of three levels of reactions. Numbers in circles represent (1) the Embden-Meyerhof pathway, otherwise known as glycolysis, (2) the hexose monophosphate pathway and (3) the Entner-Doudoroff pathway (figure reproduced from Pronk, Steensma & van Dijken 1996).

is termed respiro-fermentative metabolism whereby respiration is repressed but some minimal activity continues (Lewis *et al.* 1993). *Schizosaccharomyces pombe* also belongs to this class of yeast. Some yeasts that belong to the Crabtree-negative group include *Kluyveromyces lactis* and *Candida albicans* (Piškur *et al.* 2006). There are some differences in the metabolic pathways between Crabtree-positive and Crabtree-negative yeasts. In Crabtree-positive yeasts, dihydroxyacetone phosphate from the glycolysis pathway can be converted to glycerol and excluded from the cell, and ethanol is produced through reduction of acetaldehyde. Furthermore, in Crabtree-positive yeasts the metabolites produced from pyruvate (ethanol, acetaldehyde and acetate) can also be excluded from the cell. However, these processes cannot occur in Crabtree-negative yeasts (Piškur *et al.* 2006). The comparison of different metabolic pathways in Crabtree-positive and Crabtree-negative yeasts is presented in Figure 1.2.

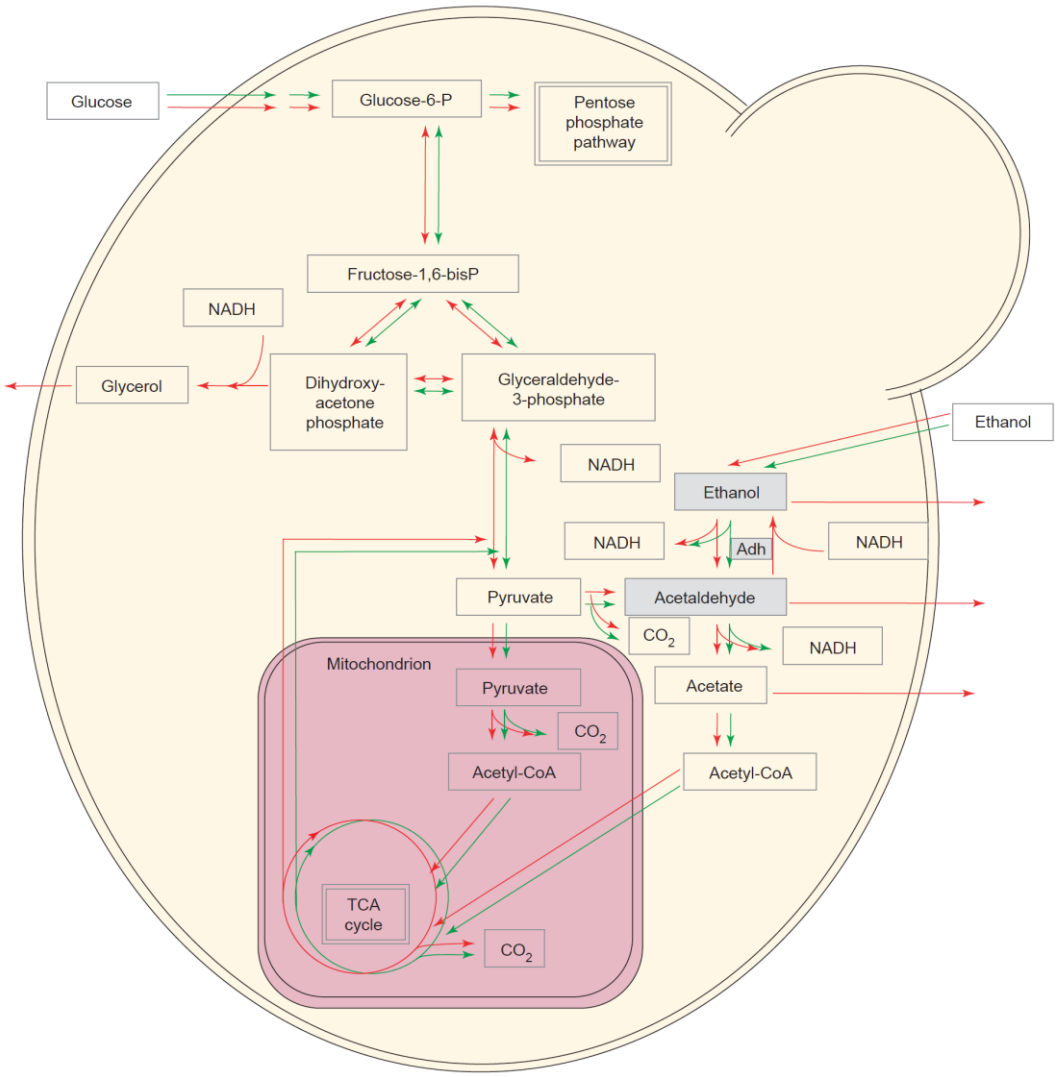


Figure 1.2 Scheme representing the different pathways involved in dissimilating glucose under aerobic conditions in Crabtree-positive yeasts (represented by red arrows) and Crabtree-negative yeasts (represented by green arrows) (figure reproduced from Piškur *et al.* 2006).

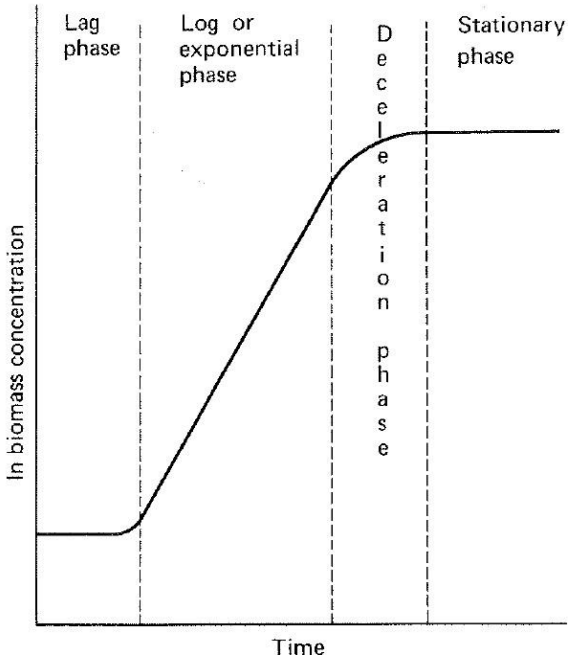
Energy obtained by *S. cerevisiae* in the ethanol fermentation process is mainly from two processes, the glycolysis pathway (i.e. conversion of glucose to pyruvate) and the fermentation pathway (conversion of pyruvate to ethanol) rather than the oxidative respiration pathway (Piškur *et al.* 2006). Glucose is considered as a very important factor that regulates whether the yeast undertakes the oxidation or the fermentation pathway. When glucose is present as substrate, it can repress gene expression for proteins involved in respiration pathways and enzymes catalysing

utilization of other sugars (e.g. maltose or galactose). On the other hand, glucose will activate the enzymes involved in the glycolysis pathway and glucose transport which therefore will enhance glucose utilization (Johnston 1999). The activation of fermentation and the repression of the respiratory pathway is considered as an advantage because it inhibits the growth of competing microorganisms (Verstrepen *et al.* 2004).

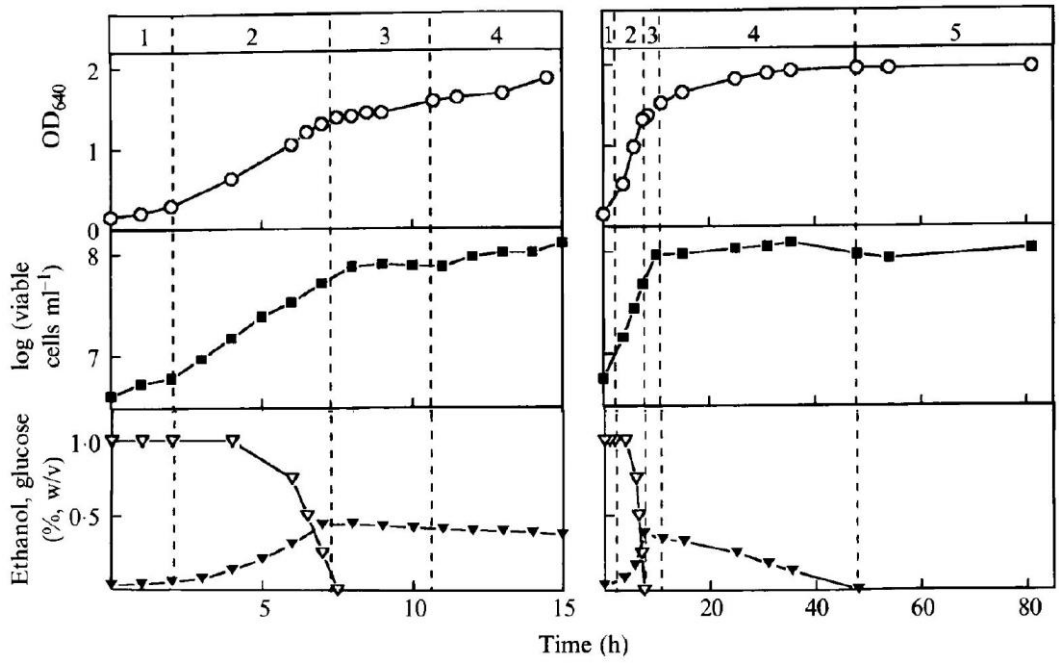
When glucose is depleted, Crabtree-positive yeasts start to utilize ethanol as their substrate and degrade it to obtain energy. It should be noted that ethanol utilisation may only occur in the presence of oxygen, as it is a respiratory activity involving oxidation of ethanol to acetaldehyde, oxidation of the acetaldehyde to acetate which is then converted to Acetyl CoA and oxidised to CO_2 via the citric acid cycle. This generates numerous reduced cofactors which feed into oxidative phosphorylation with needs oxygen as the terminal electron acceptor (see Figure 1.2). Therefore, in growth phase of Crabtree-positive yeasts (especially *S. cerevisiae*) cultured under aerobic conditions, while it was traditionally considered that there are only four growth phases, in fact six growth phases can be observed. The first phase is the initial lag phase, which was referred to as lag phase in the "traditional" nomenclature. In this phase the yeast adjusts to the new conditions and substrate(s) in the fermentation media. This is followed by the respiro-fermentative phase which is characterized by exponential cell growth and high fermentative activity with substantially repressed respiration. This phase was known as "exponential" or "logarithmic" phase in the "traditional" nomenclature. The third phase is diauxic lag phase, which is characterized by the depletion of sugar as the primary substrate and change of carbon source to ethanol as the primary substrate. At this stage the cell growth is retarded, as in the initial lag phase, since the yeast must adjust to the new substrate. This phase does not appear in the "traditional" assessment of growth phases. The diauxic lag phase is

then followed by a phase where the cell grows on ethanol as substrate, termed respiratory growth phase. This fourth phase is usually incorrectly termed “stationary phase” in the traditional nomenclature, since it started when the initial fermentable carbon source is exhausted from the media and it was assumed that no further growth occurred. The fifth and sixth phases, stationary and death phase, respectively, actually have the same terms as in the “traditional” terminology. The stationary phase refers to termination of growth due to exhausted nutrients but maintenance of high viability, while the death phase describes the condition in which the viability decreases after prolonged starvation (Lewis *et al.* 1993). It must be stressed, as noted above, that the diauxic lag and respiratory growth phases can only occur for Crabtree-positive yeasts under aerobic conditions. Under anaerobic conditions, in the respiro-fermentative phase no respiration can actually occur, furthermore after the exhaustion of the initial carbon sources oxidation of ethanol is not possible and the cells would in fact proceed directly to stationary phase in essentially the “traditional” model. Comparison of the different models for microorganism growth phases is presented in Figure 1.3.

In the ethanolic fermentation process, pyruvate from the glycolysis pathway is converted to acetaldehyde through decarboxylation by pyruvate decarboxylase and this is followed by reduction of the acetaldehyde to ethanol by hydrogenation catalysed by alcohol dehydrogenase. In overall ethanol fermentation from glucose, each glucose molecule will produce two molecules of ethanol and two molecules of CO2. Theoretically, each 180 g (1 mole) of glucose used in the fermentation process will produce 92 g (2 moles) of ethanol and 88 g (2 moles) of CO_2. However, this theoretical amount cannot be achieved since there may be some possible contamination, production of other metabolites as byproducts, cell growth and also ethanol evaporation during fermentation. In an ideal fermentation condition, 95% of the sugar available in the fermentation media can be converted



(A)



(B)

Figure 1.3 (A) A typical traditional growth curve of microbial culture (figure reproduced from Stanbury, Hall & Whitaker 1995) and (B) growth curve nomenclature proposed by Lewis *et al.* (1993) (reproduced from the Lewis publication, see text for details). The left figure represents growth from 0 to 15 hours, while the right figure represents growth from 0 to 75 hours. Legend: ○, OD 640 nm; ■, log viable cells/mL; ▽, glucose concentration (%w/v); ▼, ethanol concentration (%w/v). Note that the death phase was not included in either of these examples.

to ethanol and CO_2, 1% to cellular components, and the rest to other metabolites such as glycerol (Beltran 2005). The fermentation efficiency may further decrease when the fermentation media has a high sugar concentration and/or is poor in nutrients (Batistote, da Cruz & Ernandes 2006; da Cruz, Batistote & Ernandes 2003; Ishmayana, Learmonth & Kennedy 2011).

1.3 Bioethanol

Bioethanol is considered as an environmentally sustainable alternative fuel, complementing biodiesel and biogas (Antoni, Zverlov & Schwarz 2007). It is produced through biomass utilization by microbial fermentation; the yeast *S. cerevisiae* is the most widely used and preferred microorganism (Bai, Anderson & Moo-Young 2008; van Maris *et al.* 2006).

Various feedstocks, namely sugary, starchy and lignocellulosic materials can be used for bioethanol production. Sugary material, such as sugarcane, can be used directly as feedstock without any other treatments before conversion to bioethanol. Simple 6-carbon (6C) sugars in these types of material are easily converted, via the metabolic pathways of microorganism, to ethanol. On the other hand, starchy and lignocellulosic materials need pre-treatment before they can be converted to ethanol by microbial activity (Bonin & Lal 2012; Thomsen, Medina & Ahring 2003).

Starchy material requires a hydrolysis process to degrade starch to produce glucose. This step is very important, since effective hydrolysis produces glucose that is readily available for ethanol fermentation by yeasts, which cannot digest and assimilate starch. Prior to the hydrolysis process, gelatinization is usually required, requiring high amount of energy to disrupt starch granules which will release amylose and amylopectin molecules. Once the amylose and amylopectin molecules are released from the granules, they can then be hydrolysed either by

enzymatic or acid hydrolysis, which also usually conducted at high temperature to maintain amylose and amylopectin solubility (Tester, Karkalas & Qi 2004; van der Maarel *et al.* 2002). However, gelatinization and hydrolysis at high temperature is not desirable due to high energy inputs and costs. Therefore, hydrolysis at lower temperatures, and even at room temperature, is being developed using amylases active towards starch granules at low temperature (van der Maarel 2006). The latter technology is also known as non-cooking bioethanol production. A comparison of conventional and non-cooking bioethanol production processes is presented in Figure 1.4. Bioethanol produced from starch is known as “first generation” bioethanol.

As starchy materials are important food sources for humans, there are many concerns that in the end the requirement for biofuel will compete with food production thereby leading to food shortages. Therefore, the “second generation” of bioethanol is being developed, using lignocellulosic materials as feedstocks, largely from agricultural wastes (Bonin & Lal 2012). However, lignocellulosic materials are more resistant to hydrolysis than starch, due to their lignin content. Pre-treatments based on physical, physicochemical, chemical, biological or combinations of these methods are required to remove the lignin part of lignocellulosic materials, so that the cellulose and hemicellulose are more accessible for hydrolysis process (Sarkar *et al.* 2012). Similar to starch hydrolysis, hydrolysis of cellulose and hemicellulose can be achieved using acid or enzyme. However, acid hydrolysis is less preferred, firstly because it is not environmentally friendly and secondly because it produces toxic substances such as furfural derivatives which inhibit fermentation processes (van Maris *et al.* 2006). As hemicellulose not only contains 6C sugars, but also 5C sugars, *Z. mobilis* has better performance for converting sugars from this type of feedstock. However,

many efforts have also been devoted to develop yeast strains with 5C sugar fermentation ability (Sarkar *et al.* 2012).

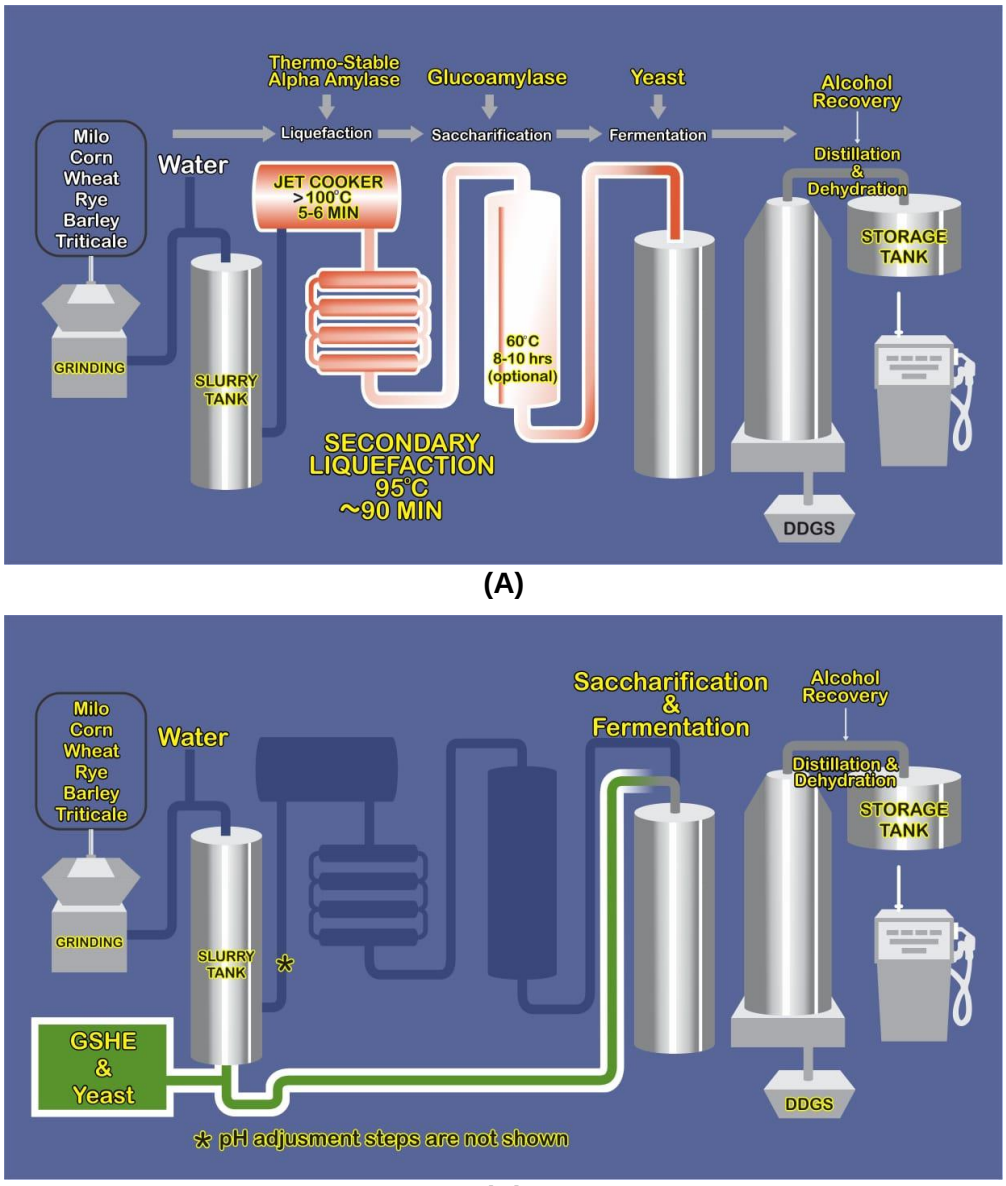


Figure 1.4 (A) Conventional bioethanol production using separated hydrolysis and fermentation process. (B) non-cooking bioethanol production (reproduced from van der Maarel 2006). Abbreviation: GSHE = granular starch hydrolysing enzyme, DDGS = dried grains with solubles.

Once the ethanol is produced by the fermentation process, separation of ethanol from the fermentation media is required since only high purity ethanol can

be used as biofuel. This step involves distillation followed by separation using selective membranes. Again, high amounts of energy are required when distillation is used for purification of the ethanol (Franceschin *et al.* 2008). Lower ethanol yields from fermentation thus require more energy at this step. Therefore, fermentation processes which lead to higher ethanol yields are desirable to reduce energy requirements. The proposed study seeks to improve the ethanol concentration provided by yeast fermentation, which would lead to decreased distillation costs in terms of both economics and environment (reduced fuel used for heating). Some manipulations of fermentation conditions have already been developed, including fermentation under very high gravity (VHG) condition or supplementation of media with compounds (such as yeast extract, glycine, or metal ions) that tend to increase ethanol yield (Chan-u-tit *et al.* 2013; Deesuth *et al.* 2012; Thomas *et al.* 1993). While there are indications that inositol supplementation may improve fermentation yield, the data have been inconclusive (Caridi 2002; Chi, Kohlwein & Paltauf 1999; Ishmayana 2011; Ji *et al.* 2008), leading to our objective to better elucidate whether inositol has the ability to improve fermentation yield.

1.4 The Yeast Plasma Membrane

The plasma membrane is one of the most important organelles of eukaryotic cells, including yeast cells. Plasma membranes comprise thin, flexible and relatively stable structures that encapsulate all living cells. The thickness of plasma membranes varies between organisms. For yeast cells the thickness is about 7.5 nm (McKee & McKee 2003; Nipper 2007; van der Rest *et al.* 1995). The plasma membrane contains a mixture of lipids and proteins and interactions between these determine the structure of the plasma membrane (van der Rest *et al.* 1995). It separates the cell from its surrounding environment and represents the first semipermeable barrier between the cell and the external environment.

Consequently, this component becomes the first to be damaged when the cells are exposed to environmental stress (Learmonth 2012; Learmonth & Gratton 2002; Rodríguez-Vargas *et al.* 2007). The plasma membrane has important roles in transport of molecules into and out of the cell, signal transduction, maintaining cell shape, interaction between cells and overall metabolism of the cell (Elliot & Elliot 2009; Nipper 2007).

Basically, the yeast plasma membrane is a lipid bilayer composed of mainly three classes of lipids which are glycerophospholipid, sphingolipids and sterols (Dickson, Sumanasekara & Lester 2006; van der Rest *et al.* 1995). Glycerophospholipid structure is composed of two distinctive parts, which make them suitable for their structural role to form the bilayer; a hydrophilic group and a hydrophobic group composed of two fatty acyl chains (Daum *et al.* 1998). Membrane proteins, which also contribute to the structure of the plasma membrane, can be inserted into the bilayer structure (intrinsic) or partially embedded in the plasma membrane and extended on the extracellular side of the plasma membrane (extrinsic). Therefore, the location of the protein on the plasma membrane is considered asymmetric (van der Rest *et al.* 1995). Membrane proteins have important functions in transporting solute molecules, signal transduction, cytoskeleton anchoring, and synthesis and repair of the outer part of the plasma membrane (Daum *et al.* 1998; van der Rest *et al.* 1995).

1.4.1 Glycerophospholipid

Glycerophospholipid is the main component of the lipid bilayer of the yeast plasma membrane comprising about 70% of the total phospholipid in the plasma membrane (Patton & Lester 1991). Glycerophospholipid is composed of a glycerol with sn1 and sn2 positions esterified to fatty acids, and sn3 position is esterified to a phosphate group which in turn may be attached to a hydrophilic molecule forming

a polar head group (Beltran 2005; Daum *et al.* 1998). Fatty acids attached to the sn1 and sn2 are commonly different in length and saturation. A saturated fatty acid is commonly found attached to the sn1 position while an unsaturated fatty acid is usually present in the sn2 position. The fatty acid component of the yeast phospholipid is mainly composed of palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1), and oleic (C18:1) acids (Beltran 2005). Only minor amounts of other fatty acid are found in the *S. cerevisiae* membrane, including myristic acid (C14:0) and C-26 fatty acid (Daum *et al.* 1998).

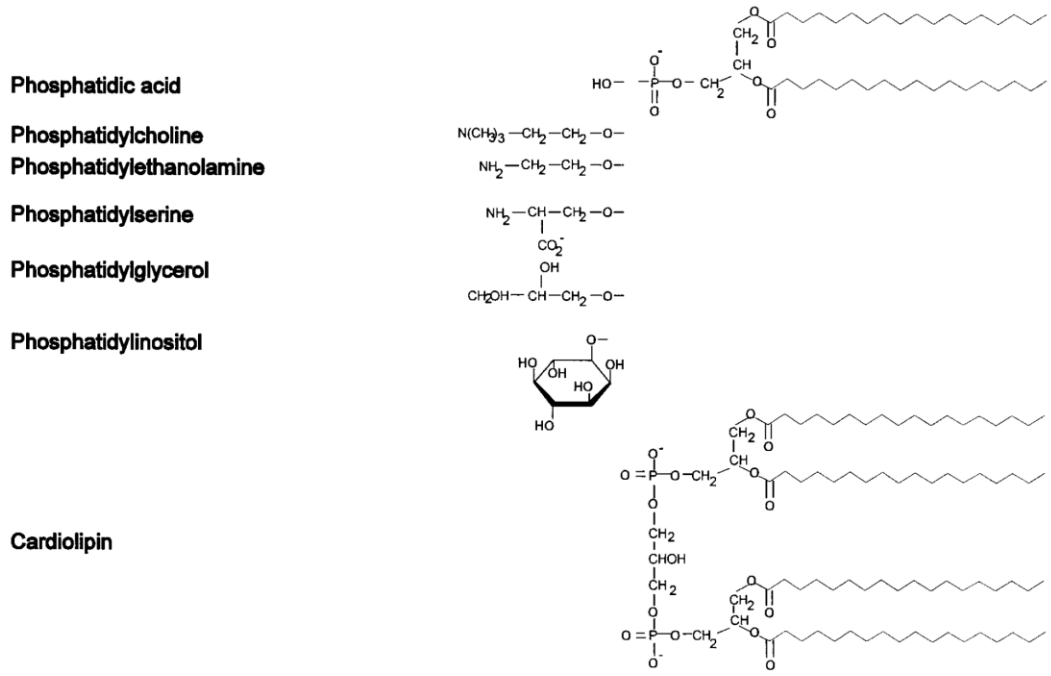


Figure 1.5 Glycerophospholipid found in the *S. cerevisiae* plasma membrane (reproduced from van der Rest *et al.* 1995).

One of the oxygens of the phosphate group attached to sn3 of the glycerol can be linked to a polar molecule via a phosphodiester linkage and contribute to the hydrophilic part of the glycerophospholipid and thereby helping to determine the physical properties of the phospholipid. This polar group can be used as a basis for glycerophospholipid classification (Daum *et al.* 1998). There are four main polar molecules that can be attached to the phosphate group of yeast plasma membrane

phosphoacyl glycerol which are ethanolamine, choline, serine and inositol, forming phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI), respectively (Beltran 2005). Figure 1.5 shows the main classes of glycerophospholipid that can be found in the *S. cerevisiae* plasma membrane. The part of the bilayer that faces into the interior part of the cell is rich in PE, PI and PS, while the exterior part of the bilayer is rich in PC and sphingolipid (van der Rest *et al.* 1995).

Glycerophospholipids of the yeast plasma membrane are synthesized through the CDP-DAG pathway (Carman 2005; Gaspar *et al.* 2006). PC, PE, PI and PS are primarily synthesized in the endoplasmic reticulum (ER), while cardiolipin (CL), phosphatidylglycerol (PG) and small amount of PE are synthesized in mitochondria. The precursor for glycerophospholipid biosynthesis is glycerol-3-phosphate, which is transformed to phospatidic acid (PA) catalyzed by glycerol-3-phosphate acyltranferase. From this point, PA can be converted to CDP-DAG or DAG by CDP-DAG synthase or phosphatidic acid phosphatase, respectively. With inositol, CDP-DAG forms PI, catalyzed by PI synthase. Alternatively, CDP-DAG can also form PS assisted by PS synthase. PE is then formed by decarboxylation of PS by the activity of PS decarboxylase. Furthermore, PE also can be converted to PC through three steps including a methylation reaction catalyzed by PE *N*-methyltransferase, a second methylation catalysed by phospholipid *N*-methyltransferase and a dephosporilation catalysed by PGP phosphatase. There are alternative pathways for PE and PC synthesis beside the pathway described above. PE can be synthesized from DAG and ethanolamine, catalyzed by ethanolaminephosphotransferase, while PC can be synthesized from DAG and choline, catalyzed by cholinephosphotransferase (Daum *et al.* 1998; van der Rest *et al.* 1995). The biosynthesis pathways for the various glycerophospholipid classes are presented in Figure 1.6.

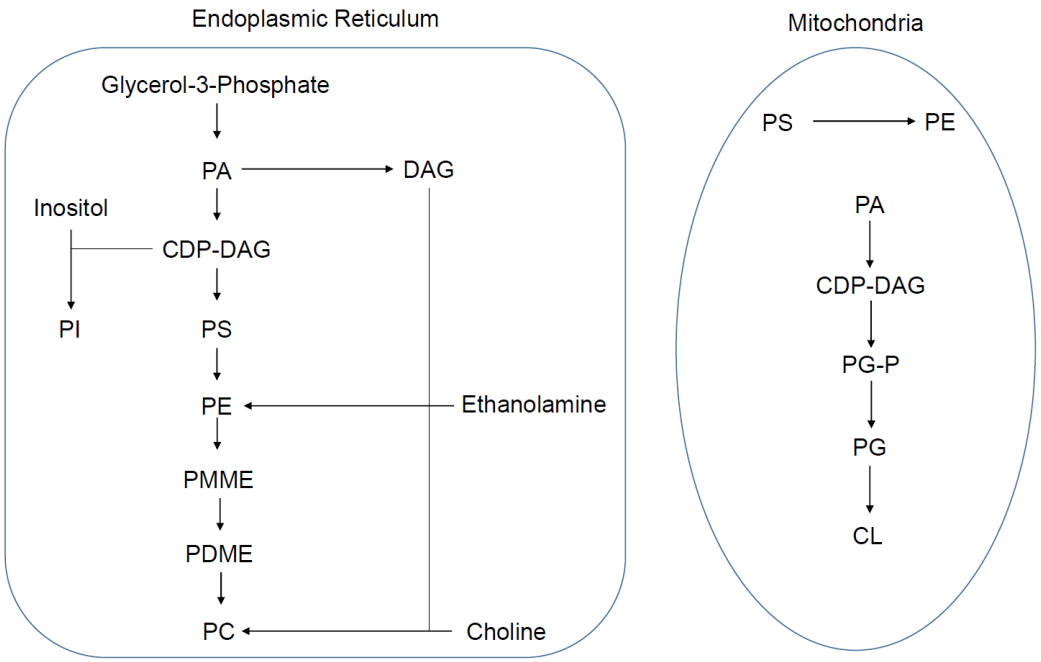


Figure 1.6 The biosynthesis pathways for the various glycerophospholipid classes. Most biosynthesis reactions occur in the ER while only a few occurs in mitochondria (modified from van der Rest *et al.* 1995 and Daum *et al.* 1998).

Several authors have reported different glycerophospholipids compositions of the *S. cerevisiae* plasma membrane. The composition of the glycerophospholipids in the plasma membrane can be different due to differences in yeast strain, growth conditions, lipid extraction procedures etc. (van der Rest *et al.* 1995). The phospholipids composition of the plasma membrane reported by several studies is presented in Table 1.1.

The present study focused on possible effects of inositol supplementation on yeast, including potential modification of cellular membrane phospholipid composition and cell physiology. In yeast metabolism, inositol can be synthesized from glucose-6-phosphate by the activity of inositol-3-phosphate synthase (encoded by *INO1* gene), followed by dephosphorylation of inositol-3-phosphate by inositol monophosphatase (Michell 2008). Inositol then can be used to synthesize PI and converted to various inositol-containing compounds through

Table 1.1 Phospholipid composition of the *S. cerevisiae* plasma membrane

Phospholipid	% Composition according to				
	Patton & Lester (1991)	Zinser *et al.* (1991)	Tuller *et al.* (1999)	Blagović *et al.* (2005)	Butcher (2008)
Phosphatidylcholine	17.0	16.8	11.3	18.7	27.2
Phosphatidylethanolamine	14.0	20.3	24.6	16.6	19.8
Phosphatidylinositol	27.7	17.7	27.2	36.6	24.7
Phosphatidylserine	3.8	33.6	32.2	5.0	28.4
Cardiolipin	4.2	0.2	ND*	6.2	NA[#]
Phosphatidic acid	2.5	3.9	3.3	13.4	NA[#]

*ND = not detected
[#]NA = not assayed

different pathways. Despite inositol biosynthesis by *S. cerevisiae*, supplementation with inositol has been found to alter the proportions of the various glycerophospholipids in the yeast plasma membrane, which found to influence stress tolerance and also the activity of membrane bound proteins (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004).

According to Gaspar *et al.* (2006), inositol addition increases PC turnover. When the medium was supplemented with inositol, the PI proportion increased and the PC proportion decreased, while no significant changes were observed for the other phospholipid classes. Inositol can act as a non-competitive inhibitor at the major pathway branch point by inhibiting PS synthase (See Figure 1.6). This inhibition occurs by lowering the amount of CDP-DAG available for PC formation, since the same substrate is used by *PIS1*-encoded phosphatidylinositol synthase to form PI. Other than that, a more rapid degradation of PC as a response to inositol supplementation was observed in relation to increased activity of phospholipase B (Gaspar *et al.* 2006). Thus, the presence of inositol in growth media will lead to a higher rate of PI synthesis and lower rate of PS synthesis. This may affect the formation of other phospholipids, i.e. PE and PC, since these two phospholipids

are the products of the next step of PS processing in the CDP-DAG pathways (Gaspar *et al.* 2006). Eventually, the presence of inositol may alter the phospholipid composition of the yeast membrane. Other changes in phospholipid composition have been reported following inositol supplementation. In the presence of inositol, the PI content increased while PC and PE levels decreased (Chi, Kohlwein & Paltauf 1999). Alteration of plasma membrane glycerophospholipid composition due to inositol supplementation is believed to be responsible for better stress tolerance, plasma membrane integrity and also increasing activity of plasma membrane H^+-ATPase which has a very important role in cell homeostasis (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Krause *et al.* 2007).

1.4.2 Sphingolipid

Sphingolipids are highly localized in the plasma membrane and not found in other membrane organelles. They comprise about 30% of the total phospholipid in the plasma membrane (Patton & Lester 1991). Similar to glycerophospholipid, sphingolipid also has a polar head group and a non-polar tail, but unlike glycerophospholipid they do not contain glycerol as their backbone structure but instead have sphingosine (Nelson & Cox 2013).

There are two main functions of sphingolipids. The first function is as a structural component of the membrane which contributes to the physical properties of the lipid bilayer and regulates the activity of transmembrane proteins. Due to this function, sphingolipid has a role in many membrane-associated cellular processes including endocytosis, intracellular trafficking and signal transduction by membrane receptors. A second function of sphingolipids is that they have important roles as signalling molecules in many cellular processes (Breslow & Weissman 2010). Long chain base (LCB, in yeast it is found as dihydrosphingosine and its 4-hydroxy derivative, phytosphingosine), a turnover product of sphingolipid,

is found to have a role in heat stress tolerance in yeast cells by acting as a signalling molecule (Dickson, Sumanasekara & Lester 2006).

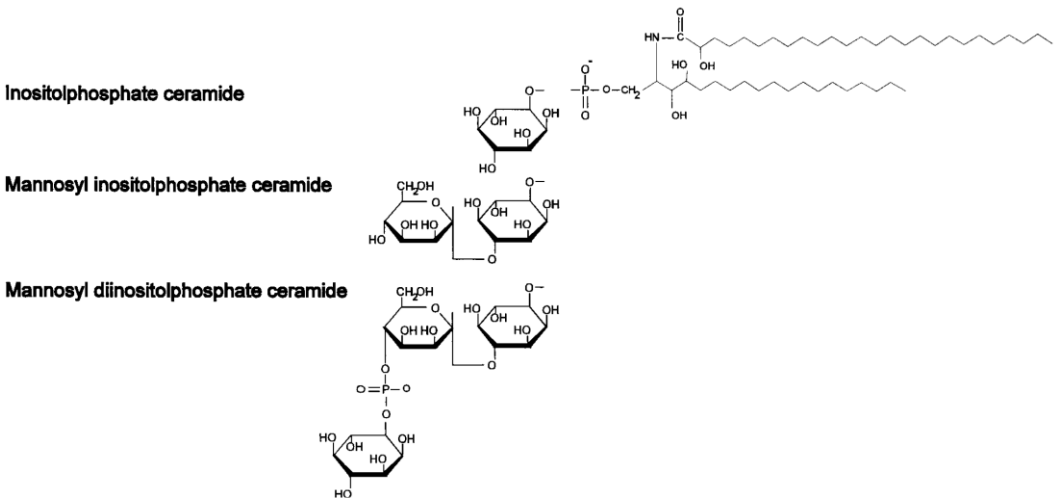


Figure 1.7 Types of sphingolipid found in the plasma membrane of *S. cerevisiae* (reproduced from van der Rest *et al.* 1995).

There are three main types of sphingolipid in *S. cerevisiae* and all of them contain the inositol molecule. They are inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC) and mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C) (van der Rest *et al.* 1995). The structure of the types of sphingolipid found in the yeast plasma membrane is presented in Figure 1.7. The tail component of a sphingolipid is known as ceramide.

1.4.3 Sterol

Sterol is one of the important classes of lipids present in the yeast plasma membrane. The main sterol found in yeast plasma membrane is ergosterol, comprising about 70% of the total sterol (Aguilera *et al.* 2006). It is found to be responsible for various physical properties of the plasma membrane. Sterol is considered as one of factors that determine the rigidity of the plasma membrane and therefore it is believed to be one of the important regulators of membrane

fluidity and permeability (Daum *et al.* 1998; Sharma 2006; van der Rest *et al.* 1995). Changes in the sterol content of plasma membranes were found to affect protein mobility and also their function (i.e. activity in term of membrane-bound enzymes) (Alexandre, Mathieu & Charpentier 1996). Polypeptides embedded in the plasma membrane are believed to be inserted in a region rich in sterols (van der Rest *et al.* 1995). Some studies also found that modification of sterols in the plasma membrane can affect the ability of the yeast to utilize different energy sources (Lees *et al.* 1980) and the activity of the plasma membrane-bound ATPase (Alexandre, Mathieu & Charpentier 1996).

1.4.4 Plasma Membrane Protein and Plasma Membrane H^+-ATPase

Using highly-purified yeast plasma membranes, Rank & Robertson (1983) found approximately 150 unique polypeptides, of which transport proteins are most likely the major component (van der Rest *et al.* 1995). The protein component of the plasma membrane accounts for only 1-2% of the protein in the whole yeast cell (Zinser & Daum 1995). Plasma membrane proteins are either integral membrane proteins, which span the lipid bilayer, or peripheral proteins connected to the membrane surface by noncovalent interactions or covalently-linked to membrane lipids or to integral membrane proteins (Premsler *et al.* 2009). The functions of membrane proteins include sensing of the external environment, signalling, nutrient transport processes, endo-/exocytosis, maintenance of membrane electrochemical potential, cell wall synthesis and maintenance, as well as providing anchors for the cytoskeleton (Delom *et al.* 2006; Premsler *et al.* 2009).

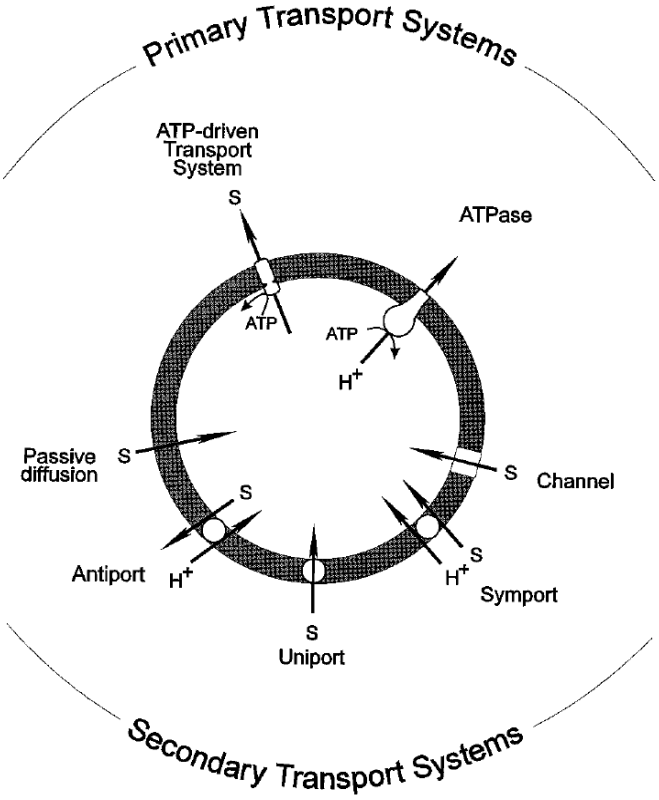


Figure 1.8 Primary and secondary transport systems in *S. cerevisiae* (reproduced from van der Rest *et al.* 1995).

As noted above, a large proportion of plasma membrane proteins are transport proteins. There are two major types of transport system in *S. cerevisiae*, which are the primary and secondary transport systems (Figure 1.8). In primary transport chemical energy is used to create solute or ion concentration gradients (van der Rest *et al.* 1995). The primary transport systems are facilitated by two groups of proteins, plasma membrane ATPase and ATP-binding cassette (ABC) proteins. In secondary transport, the solutes traverse the membrane driven by the electrochemical energy of the membrane and facilitated by protein, either an ion channel or secondary transport protein (van der Rest *et al.* 1995).

Plasma membrane ATPases are the primary active transporters involved in maintaining steep concentration gradients of cations. The membrane potential and ion gradients form the basis for a range of essential cellular processes. The ion

gradients may be applied to maintain pH homeostasis and cell volume (Morth *et al.* 2011). The plasma membrane (P-type) ATPases form a large family of more than 50 membrane proteins which are responsible for active transport of cations across the cell plasma membrane. These enzymes transport cations against their electrochemical gradient at the expense of ATP hydrolysis (Catty, d'Exaerde & Goffeau 1997; Lutsenko & Kaplan 1995).

In the yeast *S. cerevisiae*, 16 open reading frames encoding P-type ATPases have already been identified. Phylogenetic analysis followed by topology prediction, amino acid sequence analysis and phenotype analysis revealed 6 distinct families which are the ATPases transporting either H^+ (2 members), Ca^{2+} (2 members), Na^+ (3 members), heavy metals (2 members), possibly amino phospholipid (5 members) and unknown substrate(s) (2 members). The latter family was proposed as a new group called P4-ATPases (Catty, d'Exaerde & Goffeau 1997).

There are two H^+-ATPases found in the yeast *S. cerevisiae*. The first one is encoded by *PMA1* (Pma1p) which is the first sequenced gene encoding a yeast P-type ATPase and was characterized by Serrano, Kielland-Brandt & Fink (1986). This is essential for cell growth since it generates the proton motive force for nutrient transport across the plasma membrane (Morth *et al.* 2011). Because of its important role in cell growth, knockout of the *PMA1* gene is lethal (Ambesi *et al.* 2000). This enzyme is the major protein in the plasma membrane, accounting in for exponentially growing cells almost 50% of the plasma membrane protein content (van der Rest *et al.* 1995). It is estimated that 10-15% of the ATP produced during growth is consumed by Pma1p and the reaction stoichiometry is one proton extruded per one molecule ATP hydrolysed (van der Rest *et al.* 1995).

Schlesser *et al.* (1988) identified the second gene encoding H^+-ATPase, *PMA2.* Pma2p is 90% identical in amino acid sequence to PMA1P, but has distinct

enzymatic properties (Schlesser *et al.* 1988). It consists of 947 amino acid residues (Catty, d'Exaerde & Goffeau 1997), and unlike Pma1p, this enzyme is expressed in small amounts and has only a minor role in cell growth. The physiological functions and conditions that allow the expression of this enzyme are still unknown (van der Rest *et al.* 1995). However, the high affinity of this enzyme for Mg-ATP indicates that this enzyme is regulated by glucose availability and it may play a role in survival under starvation conditions, when the cellular concentration of ATP is low (Supply, Wach & Goffeau 1993).

1.5 Yeast Stress Response and Tolerance

In the fermentation process, yeast cells are exposed to various stress conditions such as changes in temperature, unfavourable osmolarity (either hypoosmotic or hyperosmotic), organic acids in feedstocks or produced during fermentation, free radicals, low nutrient availability (Siderius & Mager 2003) and also the ethanol produced can increase to concentrations stressful to the cell (Dinh *et al.* 2008; Learmonth 2012; Learmonth & Gratton 2002; Taylor *et al.* 2008). These stress conditions will be sensed by the yeast cell, and induce signal transduction that leads to changed gene expression and ultimately to changed cell metabolism. These events can lead to repair of the damage caused by the stress and/or future protection of the cell components through the induction of stress tolerance. Once these responses become effective, cell growth and the other functions of the cell can be resumed (Siderius & Mager 2003). The generalized scheme of yeast response to stress is presented in Figure 1.9.

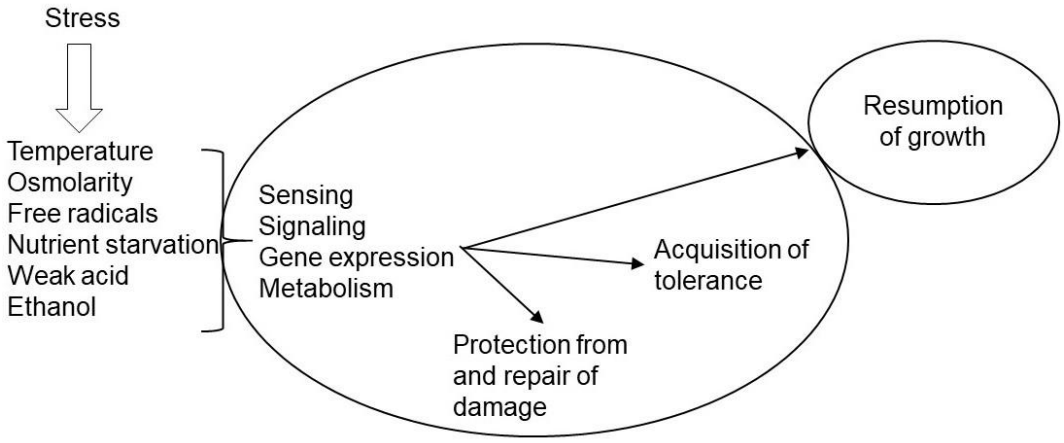


Figure 1.9 General scheme illustrating the main principles of yeast response to stress (reproduced from Dinh *et al.* 2008; Siderius & Mager 2003).

To counteract the effect of stress conditions, yeast has developed adaptation mechanisms. Many researchers have attempted to improve the resistance of yeast to various stresses via genetic manipulation (Zhao & Bai 2009). Expression of heat shock proteins (HSP) when yeast cells are exposed to heat stress is known to protect the cell (Kim *et al.* 2006). These proteins are also expressed when yeast cells are exposed to other stress factors, including high hydrostatic pressure and high ethanol concentration (Fernandes 2005; Piper *et al.* 1994), and as in other organisms, the HSPs provide cross-protection against other stresses.

Even though ethanol is the main product of yeast fermentation, it is known to be one of the stress conditions that affect yeast growth and fermentation performance. Ethanol can be toxic by several means, including damaging mitochondrial DNA and inactivating enzymes such as hexokinase (You, Rosenfield & Knipple 2003) and alcohol dehydrogenase (Nagodawithana & Steinkraus 1976). It was also found that ethanol can inhibit amino acid and glucose transport systems which in turn can inhibit cell growth and reduce cell viability (Lei *et al.* 2007). The plasma membrane of the yeast cell also become more fluid when ethanol is present

(Learmonth & Gratton 2002), leading to leakage of cellular components out of the cell (Furukawa *et al.* 2004).

An increase in unsaturation index in response to ethanol stress is one of the adaptations to ethanol that has been extensively studied (Dinh *et al.* 2008; Rodríguez-Vargas *et al.* 2007; You, Rosenfield & Knipple 2003). It was observed that when yeast cells are exposed to ethanol, the proportion of unsaturated fatty acids in the plasma membrane increases. Since the fatty acid composition of the yeast plasma membrane is rather simple, mainly palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1) and oleic (C18:1) acids, changes in the proportion of these fatty acids can be easily monitored.

In the presence of a high ethanol concentration, the level of C18:1 increased dramatically, while C16:1 level remained relatively constant. The change was found to correlate with decreases in C16:0 and C18:0 level, with the former showing more dramatic changes. It was shown that the unsaturation index value increased by about 30%. Interestingly, despite the increasing unsaturation index value, the average fatty acyl chain length increased, due to an increase in C18:1 level and a reduction in C16:0 levels (Alexandre, Rousseaux & Charpentier 1994).

Ergosterol is considered to be one of the agents that protects against stressful conditions. It has been reported that when ethanol was present in the culture media, the proportion of ergosterol in the yeast cell increased, at the expense of other sterols (Alexandre, Rousseaux & Charpentier 1994). This observation suggests that a higher proportion of ergosterol may correlate with better ethanol tolerance. When ratios of sterol:protein and sterol:phospholipid were calculated, it was revealed that both values decreased relative to the control when yeast cells were grown in the presence of ethanol, i.e. there was a lower proportion of sterols compared to phospholipids and proteins. These changes were considered in combination to lead to the higher fluidity of the plasma membrane that was

measured (Alexandre, Rousseaux & Charpentier 1994). Another study investigating the relationship between floc size and ethanol tolerance found that populations of cells with the highest ethanol tolerance had the highest ergosterol content. Cells with smaller or bigger size than the optimum floc size had lower ergosterol content and were also more susceptible to ethanol stress (Lei *et al.* 2007).

Some authors have also recorded morphological differences of yeast cells in relation to different degree of ethanol tolerance (Canetta, Adya & Walker 2006; Dinh *et al.* 2008; Lei *et al.* 2007). When yeast cells are exposed to stepwise increasing ethanol concentrations, they seem to develop tolerance by means of increasing their size. It was observed that yeast cells already adapted to high ethanol concentrations were larger compared to non-adapted cells. Even though the shortest diameter of adapted and non-adapted cells were similar, their longest diameters were clearly different (Dinh *et al.* 2008). In addition, Canetta, Adya & Walker (2006) found that when the yeast were directly exposed to a very high ethanol concentration, i.e. 30%, they shrunk and the shape became irregular as indicated by increasing cell roughness. Thus, it can be seen that the tendency is for ethanol to shrink cells, so an active response must be activated to enlarge the cells.

Lei *et al.* (2007) used different mechanical stirring rates to manipulate the flocculation process in a bioreactor such that it resulted in different floc populations each of which contained yeast with different sizes and degrees of ethanol tolerance. Four different yeast floc sizes (100, 200, 300 and 400 µm) were compared for their ethanol tolerance. It was observed that the floc population with the smallest cell size had the lowest ethanol tolerance. Up to 300 µm, the ethanol tolerance of the cell was increased, but further increases in size from this point

were associated with a decrease in ethanol tolerance. The relationship between yeast floc cell size and ethanol tolerance is presented in Figure 1.10.

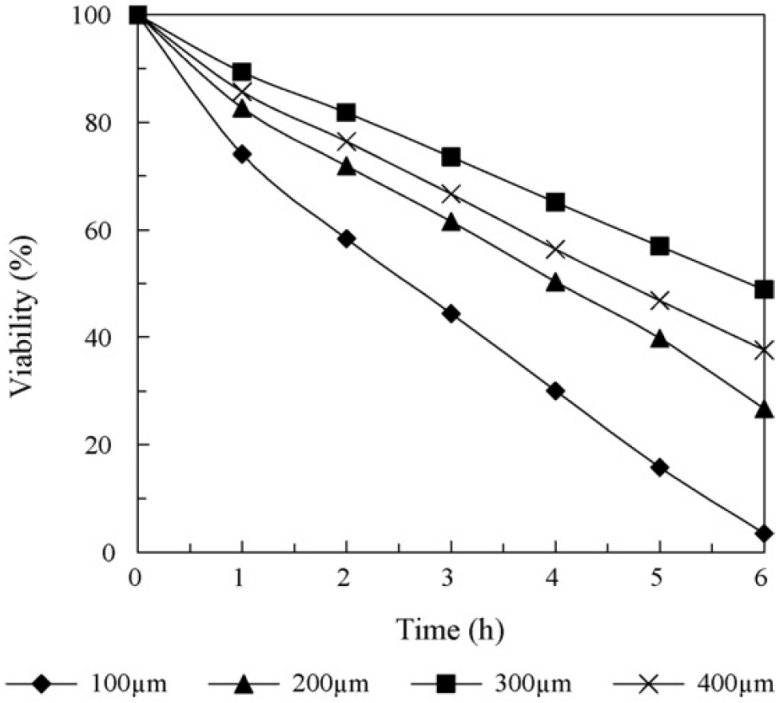


Figure 1.10 The viability of the various yeast floc populations after exposure to 20% (v/v) ethanol shock at 30°C. Different floc cell sizes are indicated in the graph by different symbols (reproduced from Lei *et al.* 2007).

As discussed in Section 1.4, the plasma membrane is the first barrier of the yeast cell exposed to various stress conditions. Therefore, the composition of the plasma membrane is a very important factor in determining the stress tolerance as discussed above. Changes in phospholipid composition during ethanolic fermentation were observed by Chi, Kohlwein & Paltauf (1999). They monitored the phospholipid composition and found that the level of PI increased while the level of the other phospholipids (PA, PS, PC and PE) decreased, especially those of PE and PC which decreased rapidly. The reason for this will be explained in the next section. This observation is consistent with the expectation based on the biosynthetic pathways of these phospholipid classes as detailed in Section 1.4.1.

As noted above, a high ethanol yield at the end of fermentation is desirable to reduce distillation energy costs. However, this is not easy to achieve. For example, when VHG is used in a fermentation process, yeast cells will be exposed to high osmotic stress, which will lead to lower viability of the yeast population and expenditure of significant energy by viable cells in countering the osmotic pressure, which is generally achieved by synthesis of glycerol (Nevoigt & Stahl 1997). This consequently lowers the ethanol yield. Originally, the lower ethanol yield was thought to be due to diversion of glycolytic output to glycerol rather than ethanol, however recent evidence indicates that ethanol production may not be directly affected as glycerol production occurs at the expense of biomass accumulation rather than ethanolic fermentation (Petelenz-Kurdziel *et al.* 2013). This would lead indirectly to longer fermentations with lower ethanol yields, as lower biomass effectively means less “fermenting units”.

1.6 Inositol in the Development of Stress Tolerance

The inositols (Figure 1.11) are a group of cyclohexanehexol isomers where six carbon atoms form a cyclic structure and one hydroxy group is attached to each carbon atom, with the empirical formula of $C_6H_{12}O_6$. There are nine isomeric structures of this compound. Six of them have biological roles, which are *myo*-inositol, *scyllo*-inositol, *epi*-inositol, *neo*-inositol, D-*chiro*-inositol and *muco*--inositol. Among the six inositols, *myo*-inositol (herein referred to as inositol) is the most biologically important (Michell 2008).

In yeast cells, inositol can be found in various forms including one of the phospholipid classes of the yeast plasma membrane, PI, and further phosphorylated phosphatidyl inositol phosphate compounds which have roles in signalling processes. These compounds play crucial functions in yeast, and

therefore inositol availability is considered crucial for cell development (Michell 2008; van der Rest *et al.* 1995).

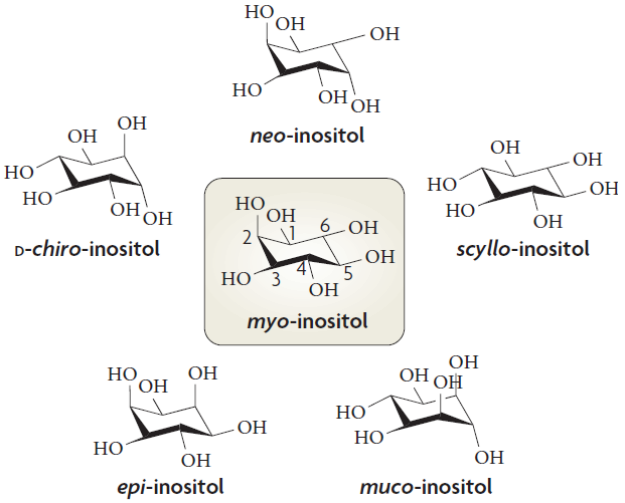


Figure 1.11 Structures of inositol (reproduced from Michell 2008)

In yeast metabolism, inositol can be synthesized from glucose-6-phosphate by the activity of inositol-3-phosphate synthase (encoded by the *INO1* gene), followed by dephosphorylation of inositol-3-phosphate by inositol monophosphatase. Inositol then can be used to synthesize PI and converted to various inositol-containing compounds through different pathways (Michell 2008). In the phospholipid biosynthesis pathway (as detailed in Section 1.4.1), PI and PS share the same precursor, cytidine diphosphate-diacylglycerol (CDP-DAG), and therefore the biosynthetic pathway of these two phospholipid classes are influenced by the availability of either inositol or serine in the growth media. Inositol supplementation was found to have a marked effect on increasing the proportion of PI while serine supplementation only slightly increases PS synthesis. As PS is a precursor for other phospholipid classes, i.e. PE and PC, reduction of PS synthesis can also changes the proportions of PE and PC in the plasma membrane (Kelly *et al.* 1988).

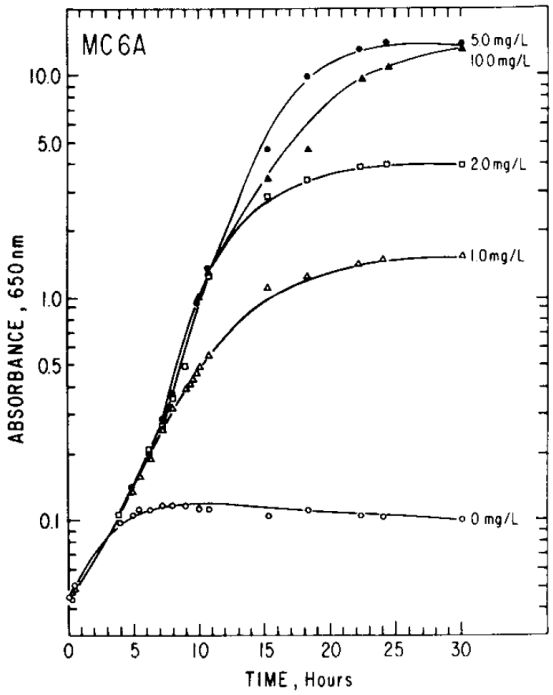


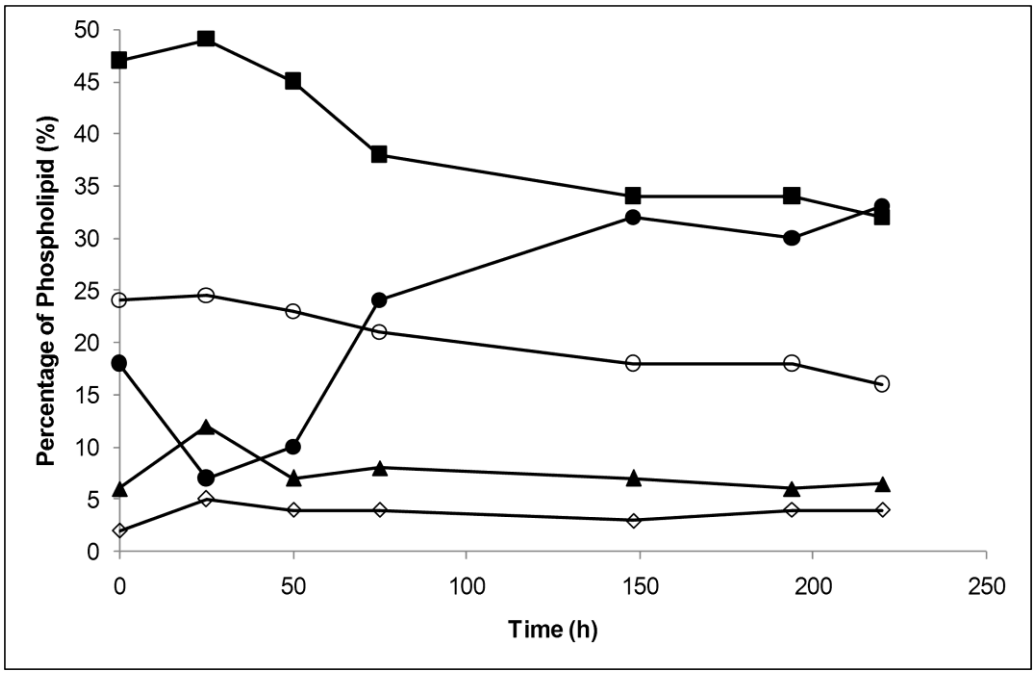
Figure 1.12 Growth of an inositol-requiring strain (MC6A) on media supplemented with different concentrations of inositol (reproduced from Becker & Lester 1977).

Early work by Becker & Lester (1977) found that excess inositol may have negative effects on yeast cell growth. When an inositol-requiring *S. cerevisiae* yeast strain was grown in the presence of increasing inositol concentrations, viz 0, 1, 2, 5 and 10 mg/L, it was observed that increased growth correlated with increasing inositol concentration only up to 5 mg/L. The growth becomes lower when 10 mg/mL inositol was supplemented into the fermentation media (Figure 1.12). A similar phenomenon was also observed for another yeast species, *Pachysolen tannophilus*, where the inositol supplementation observed to be optimal for growth (150 mg/L) was higher than the inositol supplementation that was observed to be optimal for ethanol production (100 mg/L). When inositol supplementation was increased further, both growth and ethanol production decreased (Ji *et al.* 2008).

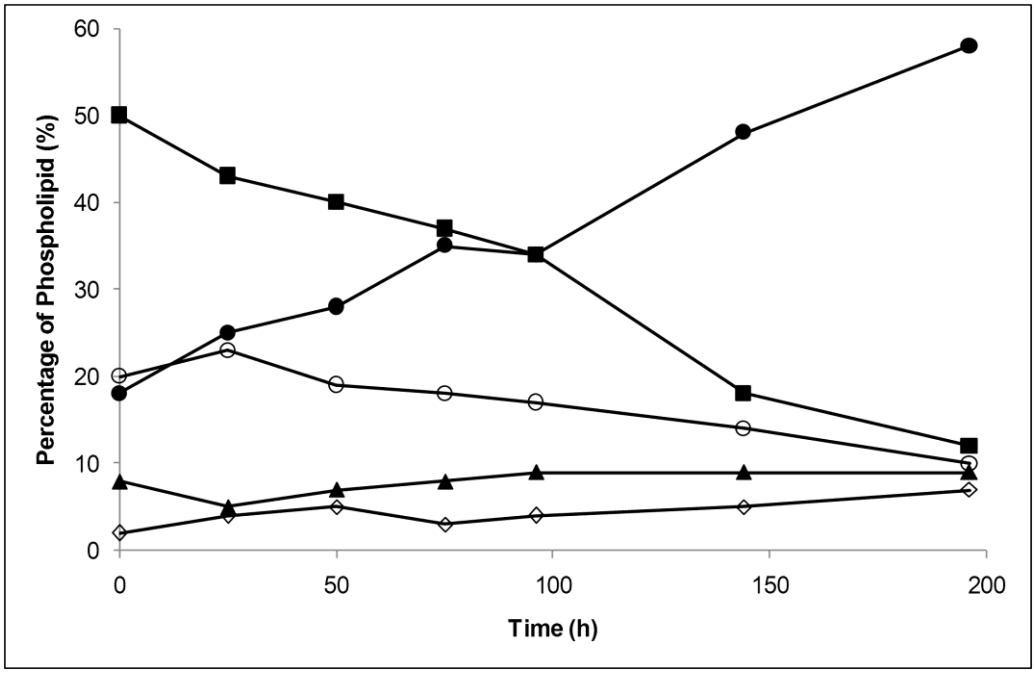
As noted above, supplementation of fermentation media with inositol has been reported to increase yeast ethanol tolerance as well as to modify the phospholipid

balance in the yeast plasma membrane (Chi, Kohlwein & Paltauf 1999; Krause *et al.* 2007). Inositol supplementation led to markedly higher levels of PI, markedly lower levels of PC and slightly lower levels of PS, while PE levels either decreased or increased slightly depending on the study (Chi, Kohlwein & Paltauf 1999; Gaspar *et al.* 2006; Kelly *et al.* 1988).

Chi, Kohlwein & Paltauf (1999) supplemented fermentation media with 100 mg/mL inositol and compared yeast grown on it with yeast grown on the unsupplemented control media. They monitored phospholipid composition and viability of the cells when exposed to 18% ethanol (Figure 1.13). These researchers found that as the culture progressed the plasma membranes of cells grown in control media exhibit an increase in the proportion of PI, which peaked at about 30%, and this increase correlated with a decrease in the proportions of PC and PE. For cells grown media supplemented with 100 mg/mL inositol, the proportion of PI in the plasma membrane increased more rapidly and peaked at more than 50%. For the cells grown in inositol-supplemented media, a more rapid decrease in levels of PC and PE were observed compared to cells grown in the unsupplemented control media. For both cell grown on inositol supplemented and unsupplemented control media, the proportions of PA and PS were relatively unchanged (Chi, Kohlwein & Paltauf 1999). It was also found that the cells grown in inositol-supplemented media had higher ethanol tolerance (Figure 1.14). From their experiment, Chi, Kohlwein & Paltauf (1999) suggested that PI plays a very important role in yeast tolerance to ethanol stress.



(A)



(B)

Figure 1.13 The changes in the proportions of various phospholipids in the absence (A) and presence (B) of inositol supplementation during fermentation. PS (–◊–), PA (–▲–), PE (–○–), PI (–●–), PC (–■–) (reproduced from Chi, Kohlwein & Paltauf 1999).

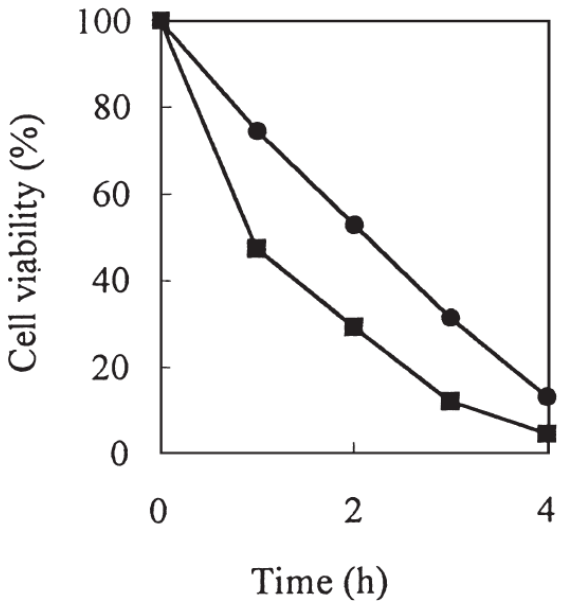


Figure 1.14 Cell viability during high ethanol shock treatment (18.0% v/v ethanol). (–●–) Cells from 24 h fermentation cultures with inositol supplementation, (–■–) cells from 24 h fermentation cultures without inositol supplementation (reproduced from Chi, Kohlwein & Paltauf 1999).

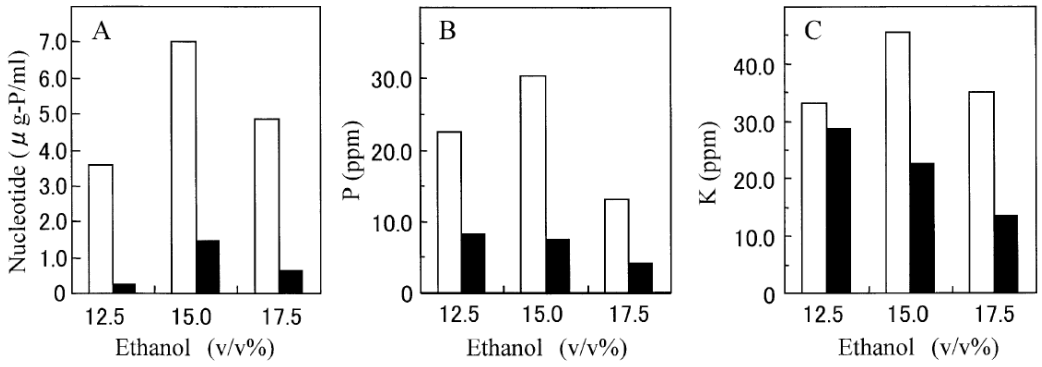


Figure 1.15 Effect of level of supplementation of media with inositol on leakage of intracellular components in the presence of ethanol. Cells were cultured in the synthetic medium supplemented with 1.8 mg/mL (white bars) or 16.2 mg/mL (black bars). Cells were incubated in 12.5% (v/v) ethanol for 3 days, 15.0% (v/v) ethanol for 2 days and 17.5% (v/v) ethanol for 1 day, at 15°C. Nucleotide (A), phosphate (B) and potassium (C) concentrations in the supernatant were determined (reproduced from Furukawa *et al.* 2004).

Another inositol supplementation study, in this case by Furukawa *et al.* (2004), investigated the effect of inositol on plasma membrane permeability by measuring leakage of cell components including nucleotide, phosphate and potassium, when yeast cells grown on media with different levels of inositol supplementation were

exposed to high ethanol concentrations. In their experiment, Furukawa *et al.* (2004) used two inositol concentrations, i.e. 1.8 mg/mL and 16.2 mg/mL. The result of their experiment is presented in Figure 1.15. They found that lower amounts of nucleotide, phosphate and potassium leaked from the cell when yeast cells were grown in media supplemented with 16.2 mg/mL inositol. Therefore, they suggested that cell leakage due to ethanol exposure can be prevented when cells are grown in the presence of sufficient inositol. The authors observed lower level of cell component leakage when the yeast cell exposed to increasing ethanol concentration, i.e. 15 to 17.5% (v/v). This is most likely because the time of exposure for each concentration of inositol supplementation was different. Cells exposed to 15% (v/v) ethanol were incubated for 2 days, while the cells exposed to 17.5% (v/v) were incubated only for 1 day. Therefore, exposure time might have lowered the component leakage into to the media. As for the incubation temperature, the authors also did not discuss why they used an unusual temperature (15°C). It is most likely that they used the temperature since the main purpose of the experiment was to see the effect of cell leakage, not growth of the cells, and therefore they tried to reduce the growth of the cell by incubating it in low temperature

Another aspect that they studied was the correlation between level of inositol supplementation and the activity of the plasma membrane H^+-ATPase. They found that increasing inositol supplementation from 1.8 mg/mL to 16.2 mg/mL in the fermentation media increased inositol content and also increased the activity of the plasma membrane H^+-ATPase twofold (Table 1.2) (Furukawa *et al.* 2004). With respect to better ethanol tolerance (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Krause *et al.* 2007), it is clear that the H^+-ATPase plays an important role. When yeast cells are exposed to high ethanol concentrations, protons (H^+) can enter the cell by passive diffusion and therefore decrease the intracellular pH. The

plasma membrane H^+-ATPase acts to maintain intracellular and extracellular proton balance by pumping excess protons out of the cell. Therefore, increasing the activity of H^+-ATPase can lead to more ethanol tolerant yeast.

Table 1.2 Effect of cellular inositol level on plasma membrane H^+-ATPase (Furukawa *et al.* 2004)

Inositol supplementation (mg/mL)	Inositol Content (μg/mg-protein)	H^+-ATPase activity (Unit/mg-protein)
1.8	6.84	8.76
16.2	16.60	16.70

In the present study, inositol will be used as a supplement in the fermentation media, since previously published studies reported that inositol supplementation may lead to increasing ethanol stress tolerance and increasing ethanol productivity. However, the optimal concentrations of inositol which resulted in positive effects varied between studies (Caridi 2002; Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Krause *et al.* 2007) and, furthermore there were also indications that excess inositol may lead to negative effects (Becker & Lester 1977; Ji *et al.* 2008). Therefore, one aim of the present study is to identify the concentration of supplemented inositol that provides optimal positive effects while also precisely identifying the concentration above which there may be negative effects.

A full understanding of the role of inositol in yeast adaptation is yet to be elucidated, especially in relation to changes in plasma membrane fluidity as a consequence of changes in plasma membrane lipid composition. Unlike previous studies, the present study will attempt to reveal the relationship between fatty acid composition and membrane fluidity by direct measurement of all of these parameters.

1.7 Membrane Fluidity Measurement

Membrane fluidity is very important for cell health, and therefore changes in membrane fluidity when yeast cells are exposed to stress conditions needs to be monitored, to better understand the effect of stress on membrane physiology. There are several spectroscopic methods that can be used to monitor membrane fluidity, including electron paramagnetic resonance (EPR) (Kandušer, Šentjurc & Miklavčič 2006; Turk *et al.* 2004), nuclear magnetic resonance (NMR) (Baer, Bryant & Blaschek 1989; Lee *et al.* 2006), Fourier transform infrared (FTIR) (Inaba *et al.* 2003; Leheny & Theg 1994) and fluorescence (Alexandre, Berlot & Charpentier 1994; Butcher 2008; Learmonth & Gratton 2002). In the present study, membrane fluidity was monitored using fluorescence spectroscopy, specifically using the generalized polarization of the laurdan probe in the yeast cellular membranes.

When exposed to electromagnetic radiation of appropriate frequency, a molecule can absorb a photon which will cause an excitation of an electron from the electronic ground state (S_0) to a higher energy electronic state (S_1, S_2, etc.), known as an excited state. The Perrin-Jablonski diagram, as presented in Figure 1.16, demonstrates the excitation process. The absorption of electromagnetic radiation and excitation of an electron take place in about 10^{-15} s (Croney, Jameson & Learmonth 2001; Learmonth, Kable & Ghiggino 2009). Due to changes in electron cloud distribution in the excited state, the structure of the molecule will change. This will lead to vibrational levels, altered dipole moment and a change in molecular shape (Learmonth, Kable & Ghiggino 2009).

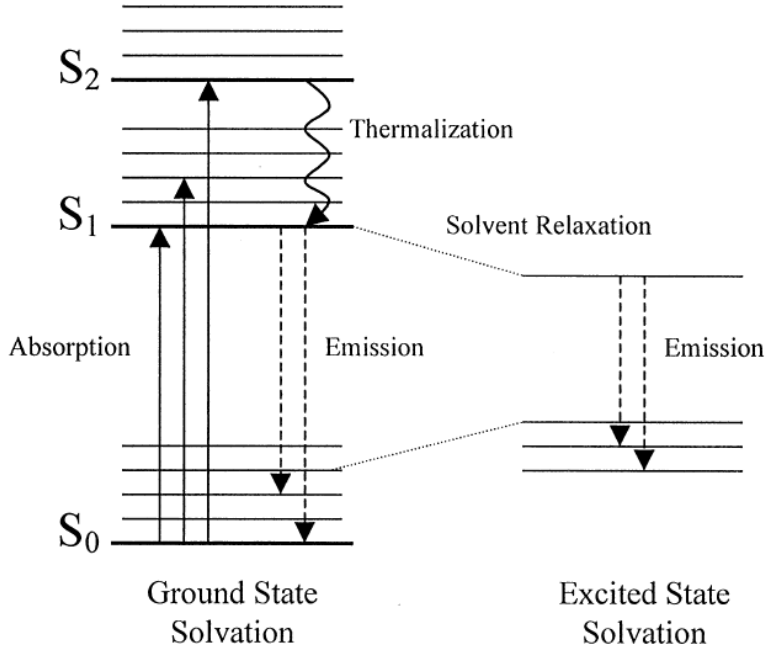


Figure 1.16 Simplified Perrin-Jablonski energy level diagram showing absorption (—) and emission (---) processes as well as thermalization and solvent relaxation (reproduced from Croney, Jameson & Learmonth 2001).

The excited molecule will rapidly lose energy to the environment through non-radiative modes involving nuclear movement, and will revert to the lowest vibrational level of the lowest excited electronic state (S_1). This event is known as thermalization. The electron can settle in the lowest vibrational level for a period of time known as fluorescence lifetime, which can last for picoseconds to hundreds of nanoseconds (Croney, Jameson & Learmonth 2001).

Since the excited state is a non-stable condition, it may relax back to the ground state by emitting a photon (emission process) as can be seen in Figure 1.16. The emitted photon will have energy corresponding to the difference between the final and initial energy states of the molecule. This photon emission can be observed as a fluorescence or phosphoresence event (Learmonth, Kable & Ghiggino 2009; Nipper 2007). The emission process is not dependent on the wavelength of the light absorbed by the molecule or the energy level reached in the excited state. Instead, the emission process always takes place from the lowest

vibrational level of the first excited state (S_1) to the ground state (S_0) (Croney, Jameson & Learmonth 2001).

Therefore, emitted fluorescent light always has lower energy (longer wavelength) than the absorbed light. The differences between absorption and emission maxima are known as the Stokes shift (Learmonth, Kable & Ghiggino 2009). An example of the Stokes shift of a fluorophore is presented in Figure 1.17. A larger fluorophore will cause a shift in absorption to a longer wavelength (Butcher 2008), for example the maximum absorption for 1,6-diphenyl-1,3,5-hexatriene (DPH, MW = 232.32 g/mol) is at 350 nm whereas 6-lauroyl-2-dimethylamino naphthalene (laurdan, MW = 353.54 g/mol) absorbs maximally at 364 nm.

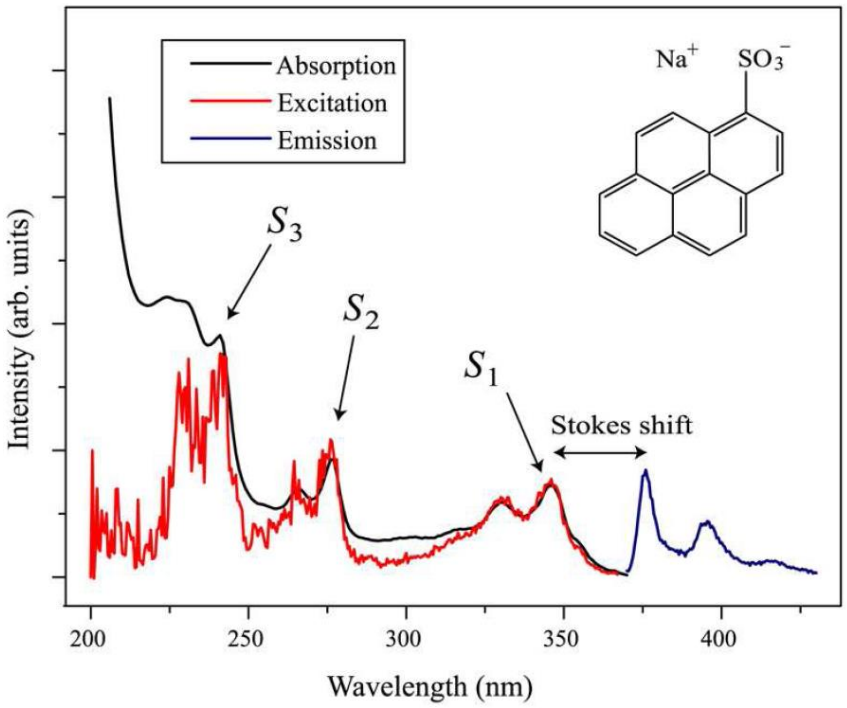


Figure 1.17 Absorption, excitation and emission spectrum of pyrene sulfonic acid (pictured top right). Three excitation states are observed for the molecule. Fluorescence occurs when the molecule shifts from the lowest excitation state (S_1) to the ground state, resulting in a Stokes shift (reproduced from Learmonth, Kable & Ghiggino 2009).

The main fluorophores that have been widely used for investigation of cellular membrane fluidity are DPH (Carratu *et al.* 1996; Najjar, Chikindas & Montville 2007) and laurdan (Learmonth & Gratton 2002; Parasassi *et al.* 1998). Laurdan is considered a better alternative to DPH since it exhibits less cell-density dependent scattering of polarized light during measurement of membrane fluidity (Learmonth & Gratton 2002). For this reason, laurdan was chosen as fluorophore in the present study.

The excitation and emission spectra of laurdan in membranes are very sensitive to the extracellular environment and this has led to the use of laurdan for measurement of fluidity in various membrane systems (Parasassi, Conti & Gratton 1986). Laurdan has a markedly higher quantum yield when dissolved in membranes than in aqueous solution. Laurdan also has low solubility in water and this leads to efficient partitioning of the probe in membranes and decreases the background fluorescence in cellular imaging of membrane structure (Yu *et al.* 1996).

A membrane can be in a liquid crystalline (fluid) or gel state. Laurdan fluorescence can be used to distinguish whether the membrane is in either of these states (Parasassi, Conti & Gratton 1986). When laurdan is inserted into a membrane and the emission spectrum is recorded, it is noted that when the lipid bilayer changes state from gel state to liquid-crystalline state, the laurdan emission spectrum shifts as much as 50 nm, i.e. from ~440 nm to ~490 nm (Parasassi *et al.* 1990; Parasassi *et al.* 1998). The shift in emission spectrum and this associated change in colour of the emission of the membrane-embedded laurdan due to changes in temperature and membrane fluidity are presented in Figure 1.18.

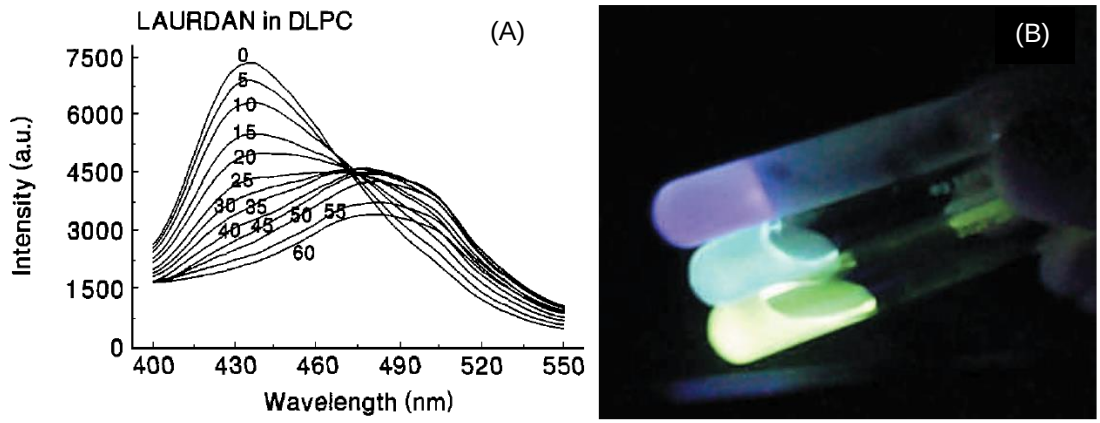


Figure 1.18 (A) Emission spectrum of laurdan in dilauroyl-phosphatidylcholine (DLPC) vesicles as a function of temperature from 0 to 60°C (Parasassi *et al.* 1998). (B) Colour changes of laurdan dissolved in glycerol. The mixture of laurdan and glycerol are frozen to -70°C (top), kept at room temperature (middle) and heated to 80°C (bottom) (reproduced from Croney, Jameson & Learmonth 2001).

Since an important approach for the determination of membrane fluidity utilizes polarized light, it will be useful here to introduce the theoretical basis of this approach to facilitate comparison of previously published data to our findings in this study. The fluorescence polarization approach determines the degree of polarization/depolarization of incident polarized light emitted by fluorophores such as DPH and laurdan. Light itself is a form of electromagnetic radiation that consists of oscillating electric waves perpendicular to oscillating magnetic waves (Learmonth, Kable & Ghiggino 2009). Normal light is unpolarised and can be illustrated as a wave with half of its vibration on the horizontal plane and the other half on the vertical plane (Butcher 2008; Learmonth, Kable & Ghiggino 2009), as presented in Figure 1.19. To polarize the light , polarizing optics such as prisms and filters can be used. After passing through the polarizing optics, the light become linearly polarized (Learmonth, Kable & Ghiggino 2009).

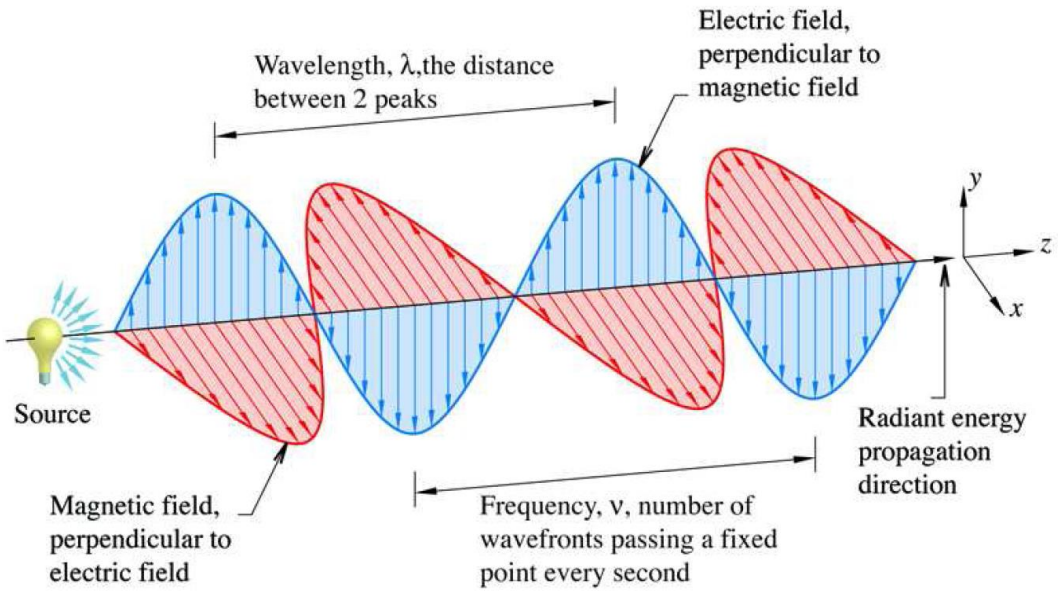


Figure 1.19 An electromagnetic ray showing wavelength, velocity and frequency (reproduced from Learmonth, Kable & Ghiggino 2009).

Membrane fluidity can be determined using excitation of the fluor by polarized light and calculation of the degree of polarization (or alternatively, anisotropy) of the light emitted by the fluor. When a molecule is illuminated using polarized light and it can rotate during the lifetime of the excited state, the emitted light will be depolarized relative to the absorbed light. Thus, the degree of polarization is dependent on the mobility of the emitting species; higher mobility will lead to lower polarization (Learmonth, Kable & Ghiggino 2009). The polarisation value can be calculated using Equation 1.1.

Equation 1.1:

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

where P : Polarization value

I_{VV} : fluorescence emission intensity measured in the plane parallel to the plane of polarization of vertically-polarised excitation light

I_{VH} : fluorescence emission intensity measured in the plane perpendicular to the plane of polarization of vertically-polarised excitation light

Or, alternatively, anisotropy can be calculated using Equation 1.2.

Equation 1.2: $$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$

where r : anisotropy value

When using polarisation or anisotropy values, the results of the calculations need to be corrected using an instrument grating factor (G), which can be calculated using Equation 1.3.

Equation 1.3: $$G = \frac{I_{VH}}{I_{HH}}$$

Anisotropy and polarisation are two ways to express the same property of light and can be easily interconverted as presented in Equation 1.4.

Equation 1.4: $$r = \frac{2P}{3 - P}$$

The anisotropy parameter is preferred over the polarisation parameter as the anisotropy parameter permits direct addition of individual components. Therefore, the mathematical equations describing multi-component systems are simpler when expressed using the anisotropy parameter (Learmonth, Kable & Ghiggino 2009).

As previously mentioned in this section, when laurdan is embedded in a membrane, it exhibits 50 nm shifts to the red end of the spectrum when the environment surrounding the laurdan molecules shifts from a gel to a liquid-

crystalline phase. Based on this property, the membrane fluidity can be calculated by measuring fluorescence intensity at the blue and red ends of the emission spectrum. This approach is known as generalized polarization (GP). The GP value is calculated by measuring the emission intensity at 440 nm and 490 nm, which represent the gel and the liquid-crystalline states, respectively, and the value is calculated using Equation 1.5.

Equation 1.5:

$$GP = \frac{I_{gel} - I_{lc}}{I_{gel} + I_{lc}}$$

Where I_{gel} is the fluorescence intensity at the blue end (440 nm) and I_{lc} is the fluorescence intensity at the red end (490 nm). Theoretically, the GP value can vary from -1 to +1, and inversely correlate with membrane fluidity, i.e. a high GP value means low membrane fluidity (Butcher 2008; Learmonth & Gratton 2002; Yu *et al.* 1996). This method is considered simpler, more rapid and more sensitive, due to the sensitivity of laurdan to its environment. Therefore, this method was chosen to monitor the membrane fluidity of the cells in the present study.

1.8 Membrane Fluidity and Yeast Adaptation to Environmental Stress

According to Los & Murata (2004) membrane fluidity is the degree of molecular disorder and molecular motion within a lipid bilayer. Qualitative characteristics of a lipid bilayer can be investigated to assess its fluidity. The rate of movement of particles in a membrane bilayer is influenced by the fluidity of the membrane. Movement of these particles, such as membrane proteins, plays an intrinsic role in their function (Nipper 2007).

Even though lipid composition (phospholipid class distribution, phospholipid fatty acyl saturation, sterols, etc.) is very important and is a major factor that

determines membrane fluidity and phase (Turk *et al.* 2004), several other factors including temperature, ionic strength, pH, the presence of protectant molecules, membrane proteins, and cellular metabolic status also contribute to maintenance of membrane fluidity (Learmonth 2012). In order to survive, a cell must maintain its cellular membrane fluidity in its optimal state even under severe conditions.

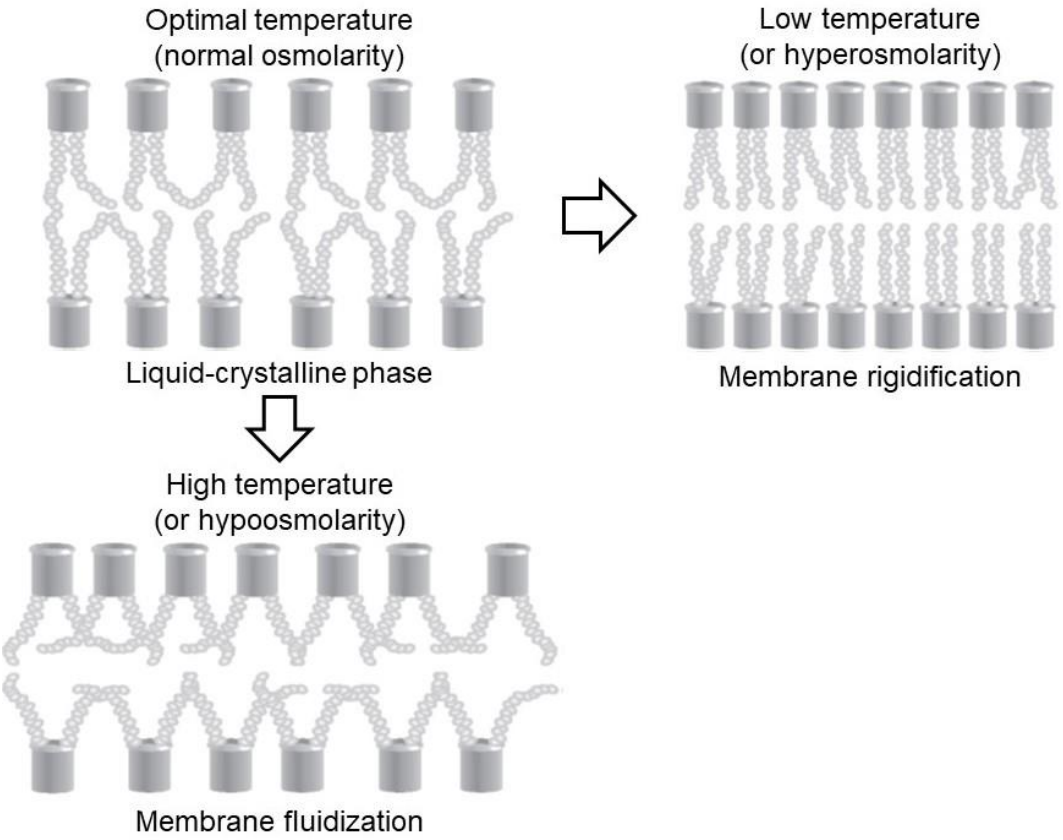


Figure 1.20 Schematic diagram showing changes in membrane order when cells are subjected to various stress conditions (reproduced from Los & Murata 2004).

Some studies reported changes in membrane fluidity when yeast cells are subjected to stress conditions, e.g. ethanol (Alexandre, Rousseaux & Charpentier 1994), freezing, salt, hyperosmotic conditions and heat (Learmonth 2012). Membrane fluidity may increase or decrease when yeast are exposed to stress conditions, depending on the stress. For example, when a membrane is subjected

to low temperature or hyperosmotic conditions the membrane will become less fluid (more rigid), whereas when the membrane is subjected to high temperature or hypoosmotic conditions, the membrane will become more fluid (Los & Murata 2004). A schematic representation of these events is presented in Figure 1.20.

The yeast cell plasma membrane is considered to be the first barrier against various external stress factors and therefore the integrity of this membrane is vital for yeast adaptation and maintenance of high viability (Learmonth 2012; Rodríguez-Vargas *et al.* 2007). The composition of the yeast plasma membrane is considered to have a high impact on the ability of the yeast cell to survive when the yeast cell is exposed to various stress factors (Redón *et al.* 2009). Some investigators found that increasing plasma membrane unsaturated fatty acid levels can increase yeast tolerance to ethanol (Kajiwara *et al.* 2000; You, Rosenfield & Knipple 2003), freezing or osmotic stress (Rodríguez-Vargas *et al.* 2007). However, not all unsaturated fatty acids provide a similar positive influence on yeast stress tolerance. For example, some researchers found that oleic acid (C18:1) protected yeast cells against ethanol stress, while palmitoleic acid (C16:1) did not (You, Rosenfield & Knipple 2003), while other researchers (Aguilera *et al.* 2006) found that ATPase activity and ethanol tolerance correlated with levels of ergosterol, C18:1 and C16:1.

Using the anisotropy value of DPH, Alexandre, Rousseaux & Charpentier (1994) studied the effect of increasing ethanol concentration on membrane fluidity. It was found that when subjected to an increasing ethanol concentration the anisotropy value decreased, which indicates that plasma membrane fluidity increased. The results are presented in Table 1.3. Besides determining the anisotropy value, they also investigated the unsaturation index of the plasma membrane. They found that exposure to 10% (v/v) ethanol increased the unsaturation index, which has a negative correlation with anisotropy value, so led

to increased membrane fluidity (Alexandre, Rousseaux & Charpentier 1994). Other studies of yeast exposed to heat and high ethanol concentration using laurdan generalized polarisation and/or DPH anisotropy also concluded that these conditions fluidize the cellular membranes (Learmonth 2012).

Table 1.3 Anisotropy values for the plasma membrane of yeast cells subjected to ethanol shock with or without prior culture in the presence of 10% (v/v) ethanol as measured using DPH as a membrane probe (reproduced from Alexandre, Rousseaux & Charpentier 1994).

Medium	Ethanol Concentration (% v/v)						
	0	2	4	6	8	10	12
YPD*	0.173	0.161	0.153	0.144	0.141	0.138	0.131
(n=3)	±0.004	±0.004	±0.003	±0.001	±0.002	±0.002	±0.008
YPDE**	0.142	0.141	0.141	0.142	0.141	0.137	0.138
(n=7)	±0.002	±0.001	±0.003	±0.003	±0.003	±0.002	±0.003

* YPD growth medium without added ethanol, ** YPDE growth medium containing 10% (v/v) ethanol.

In order to survive when exposed to stress conditions, microorganisms, including yeast, must develop mechanisms to adapt. One of these is to adjust their membrane fluidity to restore an optimal state. Some studies found that when the membrane fluidity of yeast cells changes due to a change in fatty acid composition, it can alter the ability of the cells to maintain viability when subjected to various stress conditions, such as freezing (Rodríguez-Vargas *et al.* 2007), high ethanol concentrations (Kajiwara *et al.* 2000), heat and oxidative stress (Steels, Learmonth & Watson 1994).

Kajiwara *et al.* (2000) developed genetically modified yeast strains capable of synthesizing large amounts of monounsaturated (monoenoic) fatty acids (by overexpression of the yeast *OLE1* gene) or diunsaturated (dienoic) fatty acids (by overexpression of the *Arabidopsis thaliana FAD2* gene) and also a combination of

both. These authors discovered that a genetically modified yeast strain overexpressing both of these genes had a very high proportion of unsaturated fatty acids (82%) with 54% of the unsaturated fatty acids being dienoic fatty acids. This genetically modified strain also had a higher tolerance to 15% (v/v) ethanol (Kajiwara *et al.* 2000). The results of this study indicate that increasing membrane fluidity can increase ethanol tolerance of yeast.

Rodríguez-Vargas *et al.* (2007) studied the effect of membrane fluidity on tolerance of yeast to freezing stress. They developed a genetically modified yeast strain capable of synthesizing dienoic fatty acids by introducing two desaturase genes from *Helliantus annuus*, *FAD2-1* and *FAD2-3,* into a wild type *S. cerevisiae* strain, W303. They found that introduction of the genes decreased the DPH polarisation value which indicates an increase in plasma membrane fluidity, in this case in agreement with the increasing unsaturation index. The polarisation values for wild type, W303 *FAD2-1* and W303 *FAD2-3* were 0.160 ± 0.016, 0.116 ± 0.033 and 0.125 ± 0.020, respectively. In terms of stress tolerance, they found that the genetically modified strains had higher tolerance to freezing stress compared to the parent strain. Therefore, they suggested that higher membrane fluidity leads to better tolerance to freezing stress.

You, Rosenfield & Knipple (2003) investigated which fatty acids provide the best protection against ethanol stress. They developed several genetically modified yeast strains with the ability to synthesize different unsaturated fatty acids due to the expression of different *S. cerevisiae* and insect fatty acid desaturases. The genetically modified strains were engineered to express Δ^9Z desaturase from *S. cerevisiae* (*OLE1*), acyl-CoA Δ^9 desaturase from *Helicoverpa zea* (*Hzea*KPSE), acyl-CoA Δ^9 desaturase from *Trichoplusia ni* (*Tni*NPVE), acyl-CoA Δ^{11} desaturase from *H. zea* (*Hzea*LPAQ) and acyl-CoA Δ^{11} desaturase from *Trichoplusia ni* (*Tni*LPAQ). The Δ^9 and Δ^{11} desaturases catalyze the formation of double bond at

carbon number 9 and 11 of fatty acid, respectively. Analysis of fatty acid composition indicated that each genetically engineered strain had a different membrane fatty acid composition, especially with respect to unsaturated fatty acids, as presented in Table 1.4. The result showed that strains with Δ^9 desaturase activity have an expected result, except for *Tni*NPVE strain. *Hzea*KPSE strain has similar fatty acid composition as the reconstituted OLE1 strain and surprisingly *Tni*NPVE strain has opposite composition with OLE1 and *Hzea*KPSE strains in which the ratio of C16:1 to C18:1 was 1:2. Further investigation including exposure of the genetically modified strains to 5% (v/v) ethanol revealed that the strain expressing *Tni*NPVE, mutant which had the highest C18:1 content, also had the best tolerance to ethanol, followed by strain expressing the *OLE1,* which had the second highest C18:1 fatty acid content, while the strains expressing other fatty acid desaturase did not show any change in their tolerance to ethanol. Growth

Table 1.4 Relative amounts of the major fatty acids in the plasma membrane of fatty acid desaturase-deficient yeasts transformed with plasmids that express various integral membrane fatty acid desaturases. The Δ^9Z and Δ^{11}Z transformants has their double bond at carbon number 9 and 11 of the fatty acids with *cis*-conformation, respectively (Redrawn from (You, Rosenfield & Knipple 2003).

Transformant	Fatty acid content (%)			
	Saturated		Unsaturated	
	C16:0	C18:0	C16:1	C18:1
Δ^9Z expressing				
OLE1	45.5 ± 2.2	4.7 ± 2.4	34.9 ± 0.8	14.9 ± 1.0
*Hzea*KPSE	49.5 ± 5.5	7.9 ± 2.2	31.7 ± 5.6	11.0 ± 2.0
*Tni*NPVE	46.9 ± 4.0	8.6 ± 3.9	12.8 ± 1.9	31.7 ± 5.8
Δ^{11}Z expressing				
*Hzea*LPAQ	45.6 ± 3.6	11.9 ± 2.8	42.6 ± 2.3	ND
*Tni*LPAQ	49.7 ± 4.8	12.5 ± 0.1	41.8 ± 11.8	11.2 ± 1.5

Note: ND = not detected

curves of the genetically modified strains during ethanol stress are presented in Figure 1.21. These findings indicate that tolerance to ethanol may correlate with an increase in the content of C18:1 fatty acid in the plasma membrane and this may somehow compensate for the increase in membrane fluidity caused by ethanol. Perhaps the longer-chain of C18:1 act to enhance membrane stability.

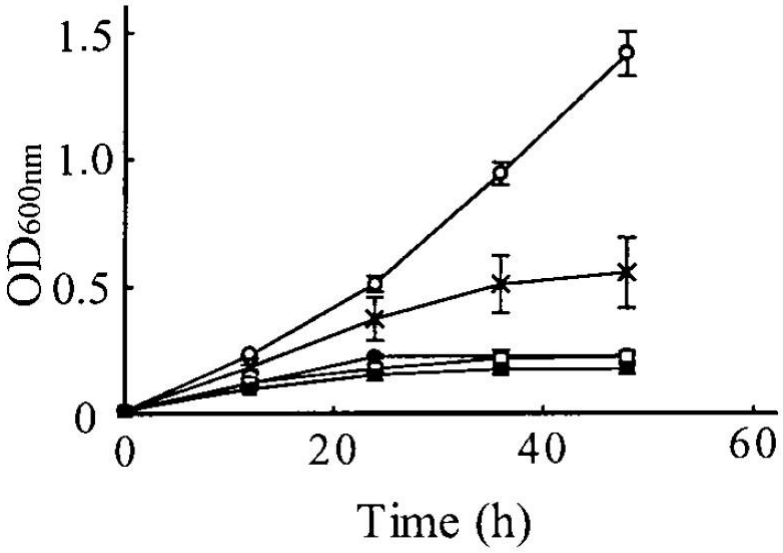


Figure 1.21 Growth curves of fatty acid desaturase-deficient yeast strains transformed with plasmids expressing *OLE1* (×), *Hzea*KPSE (●), *Tni*NPVE (○), *Hzea*LPAQ (■) and *Tni*LPAQ (□) grown in YPD medium containing 5% (v/v) ethanol with 300 rpm shaking speed at 30°C (reproduced from You, Rosenfield & Knipple 2003).

1.9 Outline of Investigation in this Project

Even though several studies reported positive effects of inositol supplementation, the concentrations of inositol reported to provide optimal positive effects varied between the studies (Caridi 2002; Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Ji *et al.* 2008; Krause *et al.* 2007). Furthermore, there were also indications that excess inositol may lead to negative effects (Becker & Lester 1977; Ji *et al.* 2008). Therefore, one aim of the present study was to determine

precisely and conclusively the concentration of supplemented inositol that provides optimal positive effects while also to precisely determine the concentration of inositol above which negative effects ensue. As far as the writer is aware, in the previous studies the effect of supplementation with inositol was not tested for tolerance to any stress factors other than ethanol stress. The present study will also investigate whether inositol supplementation may have positive effects on tolerance to other stresses, including tolerance to osmotic and acetic acid stresses.

Based on previous studies (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004), we hypothesize that inositol supplemented into the fermentation media will be consumed by yeasts and utilized to synthesise PI and other inositol-containing compounds, leading to increased membrane stability through increasing the proportion of PI in the plasma and other cellular membranes. Furthermore, we predict that this will correlate with increasing membrane fluidity, in part through increasing the proportion of unsaturated fatty acids in the cellular membranes, as Christie (2010) suggested that PI has the highest unsaturated fatty acid composition compared to the other phospholipid. Through these proposed mechanisms, the yeast will better tolerate and adapt to environmental stress, leading to improved ethanol productivity.

Given that PI levels rise in response to inositol supplementation, it could be asked whether the mechanism itself responsible for the observed higher ethanol tolerance relates to the direct biophysical impact of the presence PI anchored on the membrane, or its role as an anchor for glycosylphosphatidylinositol (GPI) proteins, or via signalling mechanisms based on phosphatidylinositol 4,5-bisphosphate and inositol polyphosphates (mainly inositol 1,4,5-trisphosphate). Furthermore, inositol may also be assimilated into inositol pyrophosphates, which also play roles in intracellular signalling (Wilson, Livermore & Saiardi 2013). Regardless of the mechanism, we expect to find changes in membrane fluidity,

lipid composition and function, as even the intracellular signalling mechanisms will involve membrane changes. We believe that a better understanding of the precise nature of the future membrane-associated changes observed will aid in the elucidation of the underlying molecular mechanisms.

1.9.1 Aims and Objectives

To test the hypotheses described above, the present study is focused on achieving the following specific aims:

1. To determine the inositol concentration in fermentation media that is optimal for enhancement of fermentation performance under laboratory conditions. Fermentation performance will be assessed in terms of ethanol production rate, final ethanol concentration achieved and efficiency (ethanol output compared to feedstock input)
2. To determine whether inositol supplementation improves fermentation performance by enhancing yeast tolerance to ethanol and osmotic stresses
3. To determine the molecular mechanisms that underlie the observed improved stress tolerance and whether they involve an increase in the level of cellular membrane PI and/or unsaturated fatty acids and resultant changes in membrane fluidity

CHAPTER TWO: MATERIALS AND METHODS

2.1 Yeast Strains and Maintenance

Three yeast (*S. cerevisiae*) strains were used in the present study. These were selected on the basis of known characteristics which make them promising candidates for use in ethanol production and also on the basis of a range of tolerances to key stresses such as ethanol, heat and osmotic stress. The strains are A12, a baker's yeast (Lewis *et al.* 1997), A15 (also known as SG195 and Y273, originally isolated from canned cherries; ATCC 38554, American Type Culture Collection, Rockville, USA) and K7, a Sake strain (ATCC26422). Each of these strains was included in preliminary trials of the effect of supplementation on bioethanol fermentation (Ishmayana 2011) with A12 and K7 being studied extensively.

Table 2.1 Yeast strains used in the present study and their properties

Yeast strain	Type	Ethanol tolerance	Heat tolerance	Osmotic tolerance	Notes
A12	Baker's	65 ± 2%[a,b]	4 ± 2%[a,c]	60 ± 11%[a,d,e]	Noted for good growth at high temperatures. Generally tolerant, most tolerant to ethanol, H_2O_2, slow freeze, acid[a].
A15 (ATCC)	Fruit spoilage	40%[f]	thermotolerant strain (Close to 100%)[f,g]	N.A.	ATCC 38554 – originally isolated from canned cherries, strain designation: SG195, Up to 80% freeze-thaw tolerance[f]
K7	Sake	~ 17.5%[b]	~ 65%[c]	N.A.	ATCC 26422

[a]data from Lewis *et al.* (1997)
[b]Ethanol tolerance - % survivors of 20% ethanol, 1 h, room temp (~25°C)
[c]Heat tolerance - % survivors of 52°C, 5 minutes with growth temp 25°C
[d]Osmotic tolerance – growth in 1.5 M NaCl
[e]from Lewis (1993)
[f]data from Lewis, Learmonth & Watson (1993) and Lewis *et al*. (1993)
[g]survival after heat treatment at 62.5°C for 20 minutes (Put *et al.* 1976)
N.A. = not available

Yeast were maintained on slopes of a complete medium, yeast extract peptone (YEP). YEP comprised of (w/v) 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 1% glucose and 1.5% agar. Slopes were stored at 4°C and sub-cultured every 6 months. Master cultures were stored in a Sanyo -80°C freezer.

2.2 Growth media and culture conditions

Cells were grown in the defined medium YNB (yeast nitrogen base) broth containing 0.69% YNB without amino acids and inositol (Formedium), and 0.005% amino acid mixture (Sunrise Science, containing L-histidine, DL-methionine, and DL-tryptophan at a ratio of 10:20:20) was added.

YNB media were prepared by weighing out the required amount and dissolving it in MilliQ grade water, filter sterilizing using 0.22 μm pore size sterile syringe filters (Sarstedt) and storing at 4°C. Sterilization via autoclaving could not be performed, as this resulted in an increased autofluorescence which interfered with the interpretation of steady-state fluorescence results. Media was prepared on a monthly basis or as required. Inositol supplement was freshly prepared and sterilized by filtering through 0.22 μm pore size sterile syringe filters.

Starter cultures were inoculated from slopes and grown overnight (~18 h) at 30°C and 180 opm in an orbital shaker (Paton). For inositol addition experiments, inositol was added to the experimental culture at final concentrations of 0, 0.05, 0.1, 0.2, 0.4 or 0.8 g/L at a time designated as 0 h.

2.3 Experimental batch culture conditions and sampling

Aerobic cultures were prepared by aseptically adding YNB media to sterile Erlenmeyer flasks, each sealed with an oxygen-permeable cotton wool bung, and

then inoculating to give an initial viable cell number of ~10^6 cells/mL. The ratio of flask size to culture volume was maintained at 4:1 to ensure adequate oxygen mixing.

Samples from the cultures were aseptically removed by drawing off with a sterile micropipette every 6 hours from 0 to 30 hours and followed by 12 hour intervals until the time indicated in the results chapters (96 or 168 hours). Examination of the samples included measuring growth rate by measuring optical density, cell numbers and viability, % budding and glucose and ethanol concentrations. Detailed analyses including ethanol tolerance and membrane fluidity were performed at 24 h.

2.4 Yeast Growth

Yeast growth was monitored by measuring optical density of the culture at 600nm (OD_{600nm}) using a Beckman DU 650 spectrophotometer, making dilutions where necessary. Measurements were made using 1 mL (10 mm path length) PMMA cuvettes (Sarstedt). An additional indicator of cell growth, viable cell count, was measured and used for the determination of cell viability (Section 2.5).

2.5 Viable Cell Numbers

Viable cell numbers were assessed using the methylene violet staining method and light microscopy (400× magnification) using a Neubauer-type haemocytometer. Methylene violet staining is proposed as a better method for monitoring yeast cell viability compared to the traditional methylene blue staining method (Smart *et al.* 1999).

An equal volume of the sample was mixed with methylene violet solution (0.01% w/v in 2% sodium citrate solution) (Smart *et al.* 1999). Methylene violet crosses the membrane of all cells, but in dead cells is unable to be metabolized,

and as a consequence dead yeast cells stained violet. Viable cells are able to metabolize methylene violet and as a result are unstained under the microscope.

2.6 Percent Viable Cells

When counting, both live and dead cells were recorded, to give the total cells per mL. The number of viable cells was then divided by the total number of cells and multiplied by 100 to give the percentage of viable cells.

2.7 Determination of membrane fluidity by spectrofluorometric analysis

2.7.1 Labelling of cells

Membrane fluidity was assessed using steady-state fluorescence spectroscopy, measuring generalized polarization of 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan) following incorporation of the probe into yeast cellular membranes, as outlined by Learmonth (2012).

For labelling, an aliquot of cells was standardized by diluting with centrifuged (8800 *g*) supernatant to an OD_{600nm} of 0.4 and a volume of 3 mL was placed in a cuvette. Incorporation of the fluorescent probe into yeast cell membranes was accomplished by incubating the standardized washed cell sample with a final concentration of 5 µM laurdan [by adding 6 µL of 2.5 mM laurdan (in ethanol)] for 60 minutes. Samples were incubated at 30°C in the dark with stirring.

2.7.2 Measurement of Generalized Polarization of laurdan incorporated into yeast cellular membranes

In this study, the fluorescent probe laurdan was used to measure Generalized Polarization, as described by Parasassi *et al*. (1990) and applied to yeast by Learmonth & Gratton (2002) and Butcher (2008). Fluorescence measurements

were taken with a PC1 photon-counting spectrofluorometer (ISS Inc., Illinois USA). After calibrating the PC1 spectrofluorometer, the excitation monochromator was set to 340 nm and measurements were taken with emission monochromator wavelengths of 440 and 490 nm, using 8 nm slits for emission and excitation. Generalized polarization spectrofluorimetric measurements were standardized by diluting cells with centrifuged supernatant fermentation culture to an OD_{600nm} of 0.4 immediately prior to analysis. A cuvette containing unlabeled cell suspension was used to measure background fluorescence. Background fluorescence was subtracted from the fluorescence readings obtained from the standardized cell suspension. The results were expressed as generalized polarization (GP) determined using Equation 2.1.

Equation 2.1

$$GP = \frac{I_{440nm} - I_{490nm}}{I_{440nm} + I_{490nm}}$$

where I_{440nm} : Emission intensity at 440 nm

I_{490nm} : Emission intensity at 490 nm

2.8 Stress Tolerance Test

2.8.1 Ethanol tolerance test by total plate count

During growth in batch culture, the composition of the growth medium changes markedly and may affect the tolerance of cells to stress. In order to minimize these types of effects when comparing stress tolerance of cells from different growth phases, stress tolerance of all cells was tested in a standard medium, namely yeast nitrogen base without glucose (YNBNG) (Lewis *et al.* 1997). Samples (1 mL) of 24 h culture were centrifuged at 1500 *g* for 2 minutes, the supernatant growth medium was decanted and the pellet resuspended in the original volume of YNBNG. Resuspended cells were then tested for stress tolerance.

The concentration of ethanol and time of exposure to ethanol used in the ethanol tolerance test were based on the work of Chi & Arneborg (2000) and Lewis (1993) with slight modification. A sample of cells (410 µL) was added to a tube containing 90 µL of absolute ethanol and the sample was mixed immediately, exposing the cells to 18% v/v ethanol. The tube was incubated at 30°C for 60 minutes. The number of surviving cells was determined using total plate count. The concentration of ethanol was chosen, based on previous studies, to provide a level of stress sufficient, but not excessive, to enable discrimination between different yeast strains or supplementation conditions.

2.8.2 Stress tolerance test by optical density

The ethanol tolerance test using total plate count was found to have very high variability, making it difficult to distinguish differences in ethanol tolerance for different strains and treatments. Therefore, an alternative stress tolerance test was trialled and found to provide more consistent results. The assay was based on a method proposed by García *et al.* (1997) and Zheng *et al*. (2011) with slight modification. An additional advantage of this assay was that a similar regime could be used for conducting tolerance test for for hyperosmotic and acetic acid stresses as well as ethanol stress. The basis of these stress tests was essentially evaluation of relative growth rate under stressful and non-stressful conditions.

For the ethanol stress tolerance assay, a 24 h culture was taken and diluted to give a cell density of OD_{600nm} ~0.1 in YNB containing 2% (w/v) glucose and with or without inositol to maintain inositol levels similar to the parental cultures. Additionally, each test included media without ethanol or with 7% (v/v) ethanol as “control” and “stress” media, respectively. The culture was then grown at 30°C and 180 opm in an orbital shaker (Paton) for 24 h. The OD_{600nm} was recorded and the

$OD_{600\ nm}$ values of the ethanol stress cultures were expressed as a percentage of the OD_{600nm} values of the corresponding control cultures.

For the hyperosmotic and acetic acid stress tolerance assays, the same protocol was utilised, however the stress factor was changed to 1.5 M (27% w/v) sorbitol or 67 mM acetic acid.

2.9 Measurement of glucose, ethanol and inositol using HPLC

2.9.1 Instrumentation

The HPLC system (Shimadzu) consisted of a SIL-20A auto sampler, DGU 20A5 in-line degasser, LC-20AD solvent delivery module, CTO 20A column oven, SPD M20A photo diode array detector, RID 10A refractive index detector, and Class-VP software. The apparatus was connected to a personal computer with a CBM 20A communication bus module. The absorbance spectrum was scanned using a photodiode array (PDA) detector and absorbance readings for 195, 200, 210 and 220 nm were monitored. The refractive index detector (RID) spectrum was also recorded.

2.9.2 Column

A Phenomenex Rezek ROA Organic acid H HPLC column (part no. 00H-0138-K0) with dimensions of 300 × 7.8 mm was used for the separation of analytes. The column was maintained at 65°C. A guard column SecurityGuard^{TM} Carbo-H^+ 4 × 3.0 mm cartridge was used to prevent column damage.

2.9.3 Mobile phase

The mobile phase was 5 mM sulfuric acid in deionized water (MilliQ) (resistivity ~ 18 Mohm) filtered through a 0.45 µm-pore-size filter. The mobile phase was passed through an in-line degasser to ensure that the mobile phase was gas free. The flow rate was maintained at 0.6 mL/min.

2.9.4 Sample measurement

Crude samples from fermentation were filtered using a 0.22 µm pore size filter. Aliquots of 10 µL were injected with the HPLC operating at a flow rate of 0.6 mL/min, and with a column temperature of 65°C for 24 minutes analysis run time. A solution of 50% (v/v) methanol was used for the autosampler needle wash step to avoid any cross-contamination between samples.

2.10 Lipid analysis

Lipid extraction was performed according to a protocol proposed by Kolarovic & Fournier (1986). This was followed by determination of fatty acid composition using GC-MS as suggested by Butcher (2008) and Christie (1993).

2.10.1 Total lipid extraction

Total lipid extraction was performed by the method of Kolarovic & Fournier (1986). A volume of cell culture containing ~0.5 g dry weight of cells was removed from the fermentation media and centrifuged at 8800 *g* for 5 minutes. The cell pellet was collected and resuspended in 250 mL milli-Q water and centrifuged again as per the previous centrifugation step. The washing step was repeated four more times. The pelleted cells were transferred to a 50 mL screw-cap test tube and 2 g of glass beads were added. The cells were then suspended in 10 mL of isopropanol. The mixture was boiled for 5 minutes to inactivate degradative enzymes, such as phospholipase. After boiling, the suspension was allowed to cool. Next, the sample was mixed by vortexing for 5 minutes.

The suspension was then placed in a water bath sonicator (50 W) heated to 50°C. As soon as the suspension dispersed, 15 mL of hexane was added to give a hexane-isopropanol ratio of 3:2 (v/v). Sonication of the sample was continued for another 5 minutes. The suspension was then filtered through a Whatman No. 1 filter paper and washed as described previously with hexane:isopropanol (3:2, v/v)

using a Büchner funnel connected to an inline vacuum pump. The clear filtrate was collected in a pre-weighed round-bottomed flask (RBF) and concentrated using a rotary vacuum evaporator (Laborota 4001, Heidolph). The temperature of the rotary evaporator water bath was set at 70°C. The RBF was then placed into a desiccator containing silica gel at room temperature for at least 4 hours to remove residual moisture. The RBF was then weighed to obtain the weight of crude lipid.

The crude lipid sample was then dissolved in chloroform to give a final concentration of 10 mg/mL and transferred to a Teflon-capped glass vial. The vial was then filled with nitrogen gas, tightly sealed and stored in a -80°C freezer until needed.

2.10.2 Determination of fatty acid composition of total lipid extract

The protocol used in this study is based on the protocols of Butcher (2008) and Christie (1993).

A. Methylation of fatty acids

The crude lipid extract from Section 2.10.1 was used for fatty acid methylation. the fatty acid composition was then determined by GC-MS. The crude lipid extract was removed from the freezer. Methylation was carried out according to the procedure described by Christie (1993). Aliquots of 200 µL of the lipid extract were transferred to individual Teflon-capped vials and evaporated to dryness under a constant flow of high-purity nitrogen gas. To each vial, 200 µL of 5% anhydrous HCl in dry methanol was added and the lids were tightly capped. Samples were then incubated overnight at 50°C using a heating block. Then 300 µL of milliQ water and 200 µL hexane were added and the samples were mixed by vortexing and the phases allowed to separate. The hexane layer was washed with dilute potassium bicarbonate solution to remove excess acid and dried over anhydrous sodium sulfate. The fatty acid methyl esters were recovered after removal of the solvent by evaporation under reduced pressure on a rotary evaporator.

B. Gas chromatography-mass spectrometry analysis of fatty acid methyl esters

Fatty acid methyl ester (FAME) samples were analysed using a Gas Chromatograph – Mass Spectrometer (Shimadzu GCMS-QP2010 GCMS) equipped with a Restek Stabilwax®-DA (30 m × 0.25 mm × 0.25 µm) column using the GCMSsolution software. The oven temperature program, GC conditions and MS conditions were set as outlined in Table 2.2, Table 2.3 and Table 2.4, respectively. The carrier gas used was helium. FAME preparations (1 µL) were injected and run. Chromatograms were compared to standard samples (Larodan FAME standards: ME61) and relative percentages of fatty acids were obtained from the chromatograms using the GCMSsolution software.

Table 2.2 Oven temperature program for determination of FAME using GCMS-QP2010

Rate (°C/min)	Temperature (°C)	Hold time (min)
-	30	0.00
25	150	5
4	190	0
2	220	0

Table 2.3 GC conditions for determination of FAME using GCMS-QP2010

Parameter	Setting
Column oven temperature	30°C
Injection temperature	220°C
Injection Mode	Splitless
Sampling time	1 minute
Flow Control Mode	Linear velocity
Pressure	72.0 kPa
Total Flow	4.4 mL/min
Column flow	1.39 mL/min
Linear Velocity	42.3 cm/sec
Purge flow	3.0 mL/min
Split Ratio	-1.0

Table 2.4 MS Conditions for determination of FAME using GCMS-QP2010

Parameter	Setting
Ion Source Temperature	230°C
Interface Temperature	250°C
Solvent cut time	2.00 min
Start time	2.50 min
End Time	35.00 min
Scan Speed	1000
Start m/z	40
End m/z	500

2.11 Determination of Glucose by the Alkaline Ferricyanide Method

This assay is based on reduction of ferricyanide (yellow) to ferrocyanide (colourless) by the hemiacetal or hemiketal functional groups of sugars (Walker & Harmon 1996). Thus, the amount of reducing groups present in a sample will be proportional to the decrease in absorbance at 420 nm, which is the maximum absorbance of potassium ferricyanide. Unlike other methods, this method relies on decreasing absorbance and therefore a standard curve is prepared from the calculated change in absorbance for each standard. The standard curve is plotted as ΔA against glucose concentration, where ΔA is calculated by subtraction of the A value measured for the blank (without sugar) from the A value measured for each standard glucose solution.

A set of tubes was prepared and a 100 µL volume of each sample (or glucose standard) was pipetted into a tube. This was followed by the addition of 300 µL of alkaline ferricyanide reagent (10.6 mM potassium ferricyanide in 2% w/v sodium carbonate) to each tube. The mixture was then heated at 100°C on a hot plate for 10 minutes. After cooling, 2 mL of water was added to each tube and the samples were mixed thoroughly. Absorbance at 420 nm was read using a Beckman DU650 spectrophotometer that had been first blanked (set to zero absorbance) when

measuring the absorbance of water. If the samples were clear following addition of 2 mL of water, then this indicated that the $K_3Fe(CN)_6$ reagent had been exhausted (too much glucose was present in the sample) and the samples were then diluted and re-assayed as described above. A standard curve was then prepared by plotting ΔA against standard glucose concentration. The ΔA value was calculated by subtracting the A reading of the blank from the A reading of each standard or sample. The standards used were glucose at 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL.

2.12 Determination of Ethanol by the Alcohol Dehydrogenase Assay

Ethanol was determined by enzymatic assay using alcohol dehydrogenase as described by Ough & Amerine (1988) and modified by Ishmayana *et al*. (2015). The assay is based on the oxidation of ethanol to acetaldehyde which is accompanied by reduction of NAD^+ to NADH catalysed by alcohol dehydrogenase (ADH). To force the reaction to completion, semicarbazide is added to the reaction buffer to bind the acetaldehyde as it forms. The amount of NADH formed, which equals the amount of ethanol consumed, can be measured by absorbance at 340 nm.

To tubes containing 1.25 mL of semicarbazide buffer solution (3.34 g tetrasodium pyrophosphate, 0.84 g semicarbazide-HCl and 0.16 g glycine dissolved in 100 mL water, pH 8.7), 25 µL of each sample and 25 µL of 24 mM NAD^+ solution were added and the samples were mixed thoroughly. After thorough mixing, 5 µL of alcohol dehydrogenase solution (4000 units/mL) was added to the sample and the sample was again mixed thoroughly. The reaction mixture was then incubated at 35°C for 40 minutes. The absorbance at 340 nm was then read using a Beckman DU650 spectrophotometer after first blanking the spectrophotometer with a reagent blank. The ethanol concentrations were

calculated by comparison of the absorbance values to ethanol standard curve. The ethanol standards used ranged from 0.01 – 0.06% (v/v).

2.13 Determination of Glycerol by Enzymatic Assay

Determination of glycerol was performed using glycerol assay kits purchased from Anpros (Product code: LPGLYC-100T). In this assay, glycerol is phosphorylated by glycerol kinase (GK) in the presence of ATP to form glycerol-3-phosphate. Glycerol-3-phosphate is then converted to dihydroxyacetone phosphate and hydrogen peroxide by glycerol-3-phosphate oxidase (GPO). Hydrogen peroxide then reacts with 4-aminoantipyrene and the sodium salt of N-ethyl-N-(3-sulfopropyl)-3-methoxyaniline (ADPS) forming a chromophore that absorbs light with an absorption maximum at 546 nm.

The assay was modified to make it compatible with the use of microplates. To each well were added 160 µL of reagent 1 (buffer, magnesium acetate, GK, peroxidase, ATP) and 8 µL of either test sample or glycerol standard (0.00, 0.025, 0.05, 0.1 and 0.2 g/L standards were prepared by dilution of the standard glycerol solution provided with the assay kit) and the samples were mixed thoroughly. The samples were then incubated for 10 minutes at 37°C. To each of the wells, 40 µL of reagent 2 (buffer, GPO) was then added and the samples were mixed thoroughly. The samples were then incubated at 37°C for 10 minutes. The absorbance at 546 nm was then read using an Anthos Zenyth 200rt microplate reader (Anthos Labtech Instruments). The concentration of glycerol in the samples was then estimated by comparing the absorbance reading of the samples to the glycerol standard curve.

2.14 Measurement of Cell Number and Cell Size using a MOXI Z Mini Automated Cell Counter

Cell size was measured using a MOXI Z mini automated cell counter purchased from Orflo. Cultures of yeast cells were sampled at 24 hours and when necessary the culture was diluted in Orflo diluent to a maximum cell density of 2.5 × 10^6 cells/mL. The instrument was then turned on and when the home screen displayed, the tray was pressed down and a cassette was inserted until “Pipette 75 µL Sample” was displayed. The sample was then placed into the port of the cassette and cell size and cell number were measured. The measurement was initiated pressing the “Small Particle Mode” button. The results of the cell measurement were then automatically displayed on the screen.

2.15 Statistical Analysis

Raw data were initially compiled in Minitab® 15 for Windows®. This software package was then used to perform one-way analysis of variance (one-way ANOVA) to compare the variance of each parameter (e.g. GP, viability reduction) between the strains with the variance within each set of replicate experiments using the same strain. The statistical significance of any observed differences between the means obtained for data sets was determined based on the *p* value. When $p < 0.05$ the null hypothesis was rejected, i.e. there was considered to be a significant difference between the means of the data. When a significant difference was detected using the one-way ANOVA, this test was followed by use of Tukey’s HSD (honestly significant difference) post-hoc test to further determine which data set differed significantly from one another.

CHAPTER THREE: ASSESSMENT OF YEAST FERMENTATION PERFORMANCE AND CELLULAR MEMBRANE FLUIDITY IN CHEMICALLY-DEFINED FERMENTATION MEDIA

3.1 Introduction

3.1.1 General Introduction

The fermentation performance of the yeast *S. cerevisiae* during ethanol production is influenced by many factors. In addition to the choice of fermentable sugar, the availability of key nutrients is one of the most important factors for fermentation performance (Batistote, da Cruz & Ernandes 2006; Walker 1998). For an effective and efficient fermentation, a nutritionally-rich medium is required (Bafrncová *et al.* 1999; da Cruz, Cilli & Ernandes 2002). Nitrogen is one of the main elements that can be found in many macromolecules of living organisms and it plays crucial roles in the structure and function of these macromolecules. Therefore, many cellular activities are dependent on nitrogen uptake and assimilation (da Cruz, Cilli & Ernandes 2002; Walker 1998).

Media with structurally complex nitrogen sources were found to promote fermentation efficiency (da Cruz, Cilli & Ernandes 2002; Júnior *et al.* 2009). It was found that media with ammonium salts as the only nitrogen source (termed poor media) led to poor yeast fermentation performance. Fermentation performance was improved with the use of media containing peptides (peptone) and further improved with the use of media containing casamino acids (da Cruz, Cilli & Ernandes 2002).

When cultured in complex media, such as those using yeast extract as the nitrogen source, yeast cells can utilize high initial sugar concentrations (e.g. in the case of very high gravity fermentations with ≥ 27% w/v sugar) and efficiently

convert the sugar into ethanol (Bafrncová *et al.* 1999; Chan-u-tit *et al.* 2013; Deesuth *et al.* 2012; Deesuth *et al.* 2015). However, if the aim is to conduct certain experimental investigations of yeast physiology during fermentation, e.g. study of proteins or membranes using fluorescence spectroscopy, yeast extract or other complex sources of nitrogen cannot be used as their high autofluorescence results in an unacceptably high background fluorescence that interferes with measurement of the fluorescence of the experimental probe (Learmonth 2012). Such spectrofluorimetry studies must utilize “poor” nitrogen media (i.e. those that contain simple ammonium salts, such as yeast nitrogen base). Furthermore, the media must be sterilized by filtration rather than by autoclaving to minimize the contribution of the media to background fluorescence (Butcher 2008; Ishmayana, Learmonth & Kennedy 2011; Learmonth 2012; Learmonth & Gratton 2002).

When such poor nitrogen media are used, very low glucose concentrations are usually used in the initial stage of fermentation (Butcher 2008; Ishmayana, Kennedy & Learmonth 2011; Ishmayana, Learmonth & Kennedy 2011; Learmonth & Gratton 2002; Matsuura & Takagi 2005; Poole *et al.* 2009). While the use of poor nitrogen media does enable yeast to be studied under standardized conditions without the potential issues caused by the commonly observed batch-to-batch variations in complex nitrogen medium ingredients, the use of poor nitrogen media can lead to lower tolerance to stresses, including high osmotic pressure, which limits the initial glucose concentration that can be used in fermentation studies (Ishmayana, Kennedy & Learmonth 2011). In the context of the present study, a suitable initial sugar concentration must be chosen that enables one to observe any effect of inositol supplementation on the ability of yeast to consume sugar and also on other cellular functions. If the concentration is too low, the sugar will be exhausted too rapidly and therefore it will be difficult to observe the effect of inositol supplementation on the glucose consumption. However, if the concentration is too

high, there may be a lot of residual sugar remaining at the end of the fermentation and the cells will also be exposed to an osmotic stress. This may mean that any effect of inositol supplementation on the glucose consumption or other cellular functions may not be clearly observed. Therefore, it is very important to determine the suitable initial sugar concentration such that the effects of inositol supplementation can be observed easily. Finally, given that other nutrients are not as plentiful as in rich media, it is important to determine the maximal amount of sugar that can be metabolized before these other nutrients (e.g. yeast available nitrogen) become limiting.

3.1.2 Yeasts

Three *S. cerevisiae* strains, A12, A15 (ATCC 38554) and K7 (ATCC6422) were used in the present study. A12 is an ethanol tolerant baker's yeast according to a previous study (Lewis *et al.* 1997). A15 is a thermotolerant wild yeast strain isolated from fruit juice (Bell, Higgins & Attfield 2001; Lewis *et al.* 1993). K7 is a sake yeast strain (ATCC 26422) that can produce up to 17.5% ethanol (Bell, Higgins & Attfield 2001; Lewis *et al.* 1993).

3.1.3 Specific growth conditions and experimental design

Cells were grown in the defined medium YNB (Yeast Nitrogen Base, Difco) broth containing 0.67% (w/v) YNB with 5% (w/v) glucose. Note that for these experiments "standard" YNB was used, containing 2000 µg/L (0.002 g/L) inositol. Later experiments to assess the effects of inositol supplementation utilized a variant of YNB without inositol (Formedium). Starter cultures were inoculated from slopes and grown overnight (~18 h) at 30°C and 180 opm in an orbital shaker (Paton).

Experimental cultures were prepared in YNB with either 5, 10 or 15% (w/v) glucose. Aerobic cultures were prepared by aseptically adding media to sterile Erlenmeyer flasks, each sealed with an oxygen-permeable cotton wool bung, and then inoculating with one of the three yeast strains to give an initial viable cell number of ~10^6 cells/mL. The ratio of flask size to culture volume was 4:1 to ensure adequate oxygen mixing. Culture samples were aseptically removed by drawing off with a sterile micropipette every 6 h from 0 to 30 h, followed by 12 h intervals from 48 – 168 h. Examination of the samples included measuring cell density by optical density (Section 2.4), viable cell numbers (Section 2.5) and both glucose (Section 2.11) and ethanol concentrations (Section 2.12).

GP (Section 2.7) was measured at 6 and 24 hours in these experiments, representing early and mid- to late-respiro-fermentative (exponential) phases of cell growth, respectively.

All data presented in this report were obtained from three independent experiments.

When appropriate, the experimental data were analysed for statistical significance using a one-way ANOVA with *post-hoc* comparison using Tukey's HSD (honestly significant difference) *post-hoc* test to determine whether the observed differences between the means of different experimental data sets were statistically significant (Section 2.15). Differences were considered statistically significant when $p < 0.05$.

3.2 Results

3.2.1 Cell growth in chemically-defined media with different initial sugar concentrations

Cell growth (as assessed by OD_{600nm}) of the three strains as a function of initial glucose concentration is presented in Figure 3.1. Figure 3.1 shows that the three

yeast strains have different growth patterns. The strains can be clearly differentiated, with K7 showing the highest final cell density and A15 the lowest. At the plateaux of cell density, the OD responses of the strains are separated from each other by more than the differences of responses of each individual strain to the initial glucose concentration.

To refine the analysis, the maximum growth rate of each yeast strain was assessed. From a plot of the natural logarithm of the $OD_{600\ nm}$ against time one can note that the highest growth rate occurred during the first 12 hours of the fermentation experiment (Figure 3.2), and therefore the maximum growth rate was calculated based on the $OD_{600\ nm}$ value change from 0-12 hours (Table 3.1). In terms of maximum growth rate (μ_{max}), K7 seems to be the fastest while A15 has the slowest maximum growth rate. However, the growth rates of A12 were not significantly different from either K7 or A15 (Table 3.1). Even though the maximum growth rate of K7 tended to be higher than those of A12 and A15, at 12 hours there was no difference in the $OD_{600\ nm}$ value of the three strains. Interestingly, at later stages of the fermentation, K7 had a significantly higher $OD_{600\ nm}$ value than A15 or A12, as can be seen in Figure 3.1 and Table 3.1 (where the OD data for 36 h are presented as an indication of the difference in cell density between the three strains later in the culture).

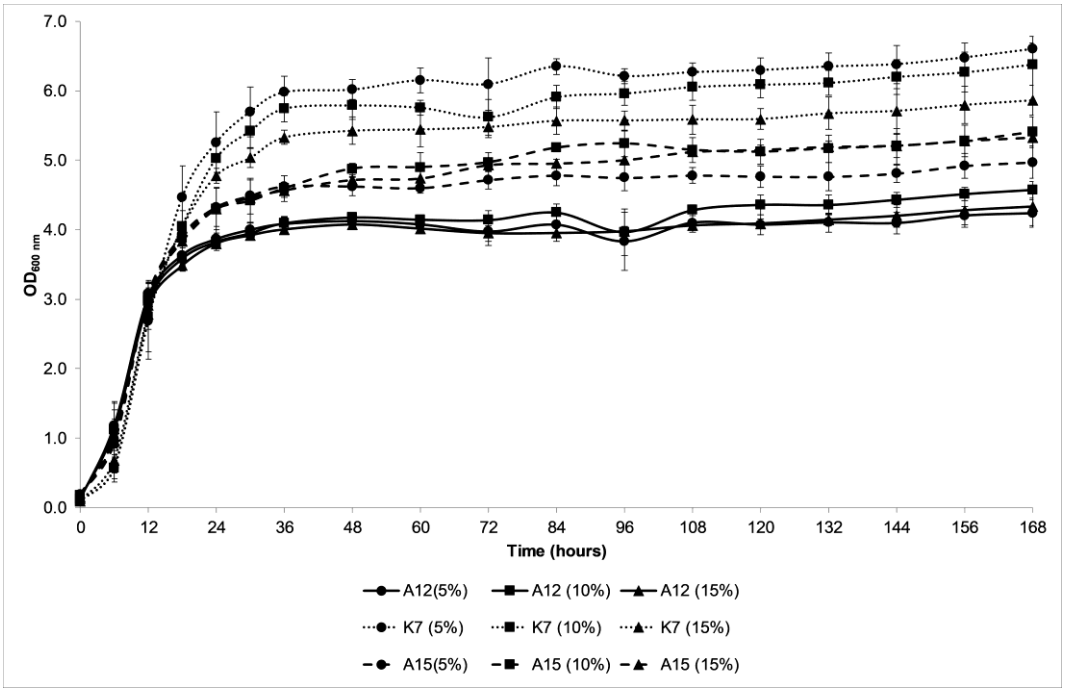


Figure 3.1 Growth curves of A12, K7 and A15 yeast strains in YNB media with different initial glucose concentrations. Error bars indicate the standard deviation (SD) of three independent experiments.

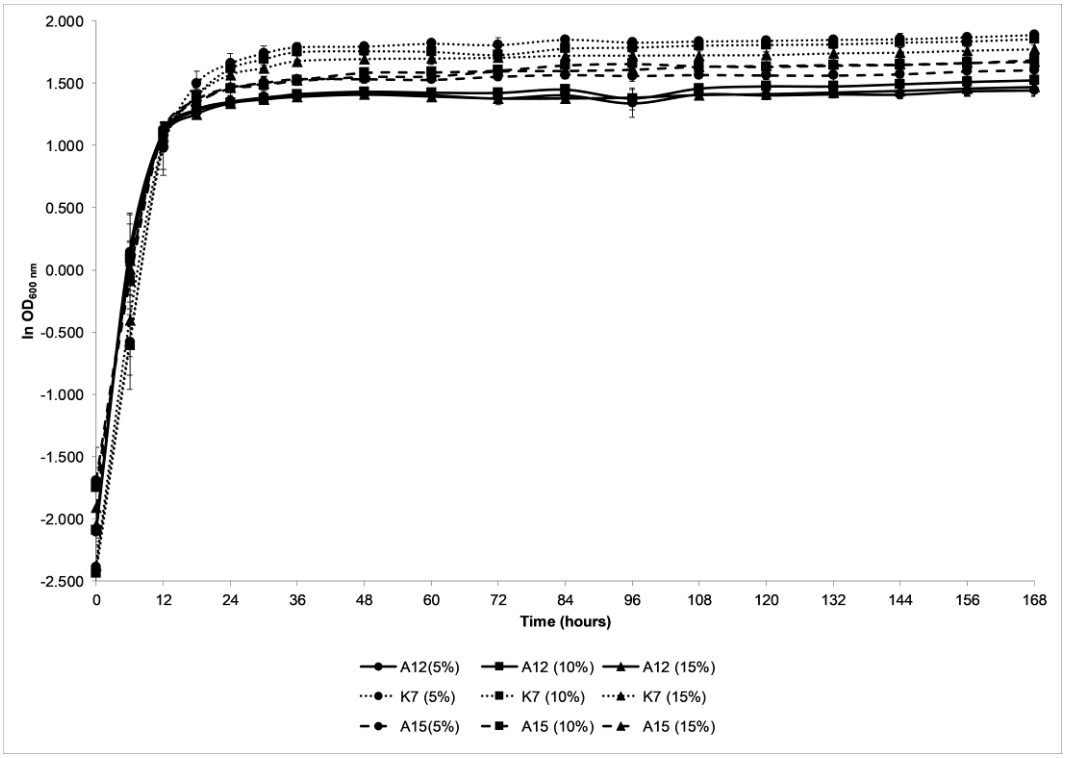


Figure 3.2 Plot of the ln of the $OD_{600\ nm}$ of yeast strains A12, K7 and A15. Each data point is the average of three biological replicates (n=3).

Table 3.1 Growth parameters of yeast strains A12, K7 and A15 in YNB media with different initial glucose concentrations. Values are the means of three independent growth curves ±SD. The values in the column with the same letter are not significantly different according to Tukey's *post-hoc* test.

Strain	Initial Sugar Concentration (% w/v)	μ_{max} (h^{-1})	$OD_{600\ nm(12\ h)}$	$OD_{600\ nm\ (36\ h)}$
A12	5	0.271 ± 0.005^{abc}	3.036 ± 0.217^{a}	4.081 ± 0.094^{d}
	10	0.268 ± 0.008^{abc}	2.967 ± 0.202^{a}	4.093 ± 0.100^{d}
	15	0.263 ± 0.006^{abc}	2.918 ± 0.161^{a}	4.002 ± 0.056^{d}
K7	5	0.275 ± 0.010^{ab}	2.687 ± 0.548^{a}	5.977 ± 0.235^{a}
	10	0.281 ± 0.010^{a}	2.743 ± 0.501^{a}	5.744 ± 0.187^{a}
	15	0.287 ± 0.024^{a}	2.785 ± 0.217^{a}	5.327 ± 0.101^{b}
A15	5	0.238 ± 0.028^{c}	3.075 ± 0.192^{a}	4.623 ± 0.152^{c}
	10	0.244 ± 0.019^{bc}	3.013 ± 0.213^{a}	4.591 ± 0.187^{c}
	15	0.248 ± 0.008^{abc}	3.052 ± 0.114^{a}	4.566 ± 0.054^{c}

3.2.2 Cell viability

Differences in cell viability were noted for the three yeast strains shown in Figure 3.3. The cultures of A12 at all initial glucose concentrations trialled in the present experiment started with moderately high viability (~80%). This increased to ~ 96% at 6 hours and gradually decreased to almost zero after 168 h. It was observed that when the A12 strain was grown in 15% w/v initial glucose, the viability was substantially lower at around 72-96 hours. For K7, the viability generally started from the highest point (~95%) and remained high compared to the other strains until 48 hours. K7 cells grown in the lowest glucose concentration had the lowest viability at about 96 h while those grown at the higher glucose concentration maintained higher viability up to about 132 h. The viability of A15 was initially the lowest, although it rapidly increased to ~90% at 6 hours. This was followed by a rapid decrease to ~60% when grown in 5 and 10% w/v glucose, while for cells grown in 15% w/v glucose the viability dropped to ~50%. Interestingly, the viability of this strain did not decrease gradually as observed for the other strains,

but reached a plateau at about 40-50% viability which was maintained for up to 84 hours.

The total viable cell counts of all strains were similar at the early stage of fermentation (0-12 hours) as presented in Figure 3.4. K7 began to have higher total viable cell counts at 30 h and maintained the highest cell counts up to ~60 hours. Compared to the other strains, A12 tended to have lower total viable cell counts after 48 h, followed by a gradual decrease to almost zero at the end of fermentation (168 h). K7 and A15 showed slightly different patterns, in that total viable cell counts decreased sharply after a particular time point (~72 h for K7 and 84 h for A15), when the highest ethanol concentrations in the media were reached (see Figure 3.6). Although the viability of A12 was higher than A15 over 12-24 hours (Figure 3.3), no statistically significant differences were observed in the total viable cell counts.

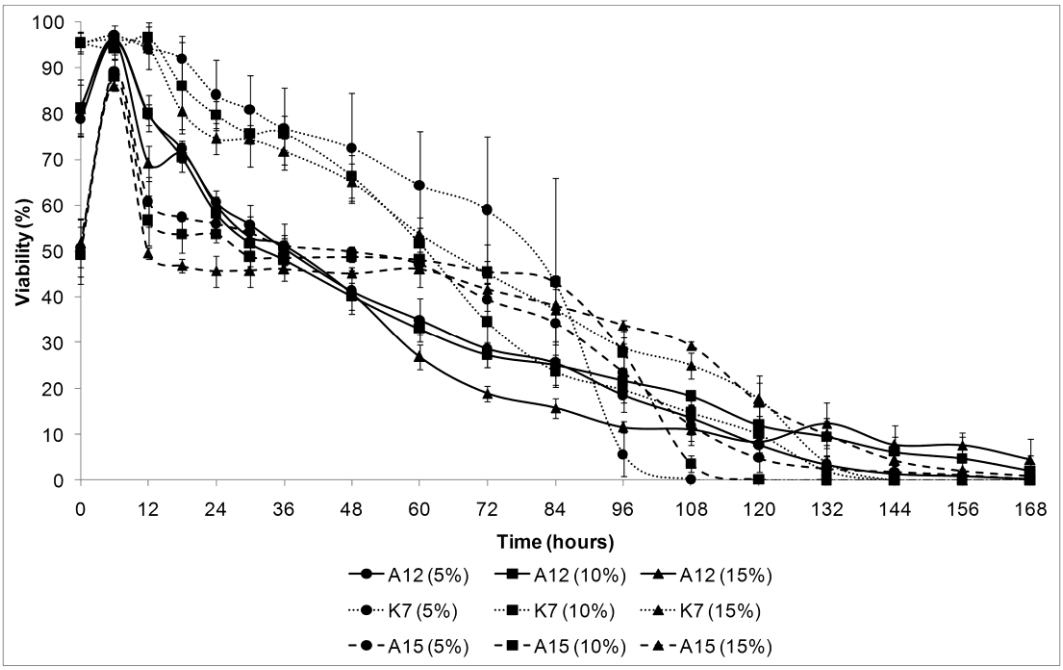


Figure 3.3 Viability of yeast strains A12, K7 and A15 in YNB media as a function of culture time and initial glucose concentration. Error bars indicate the SD of the means of data obtained from three independent growth curves.

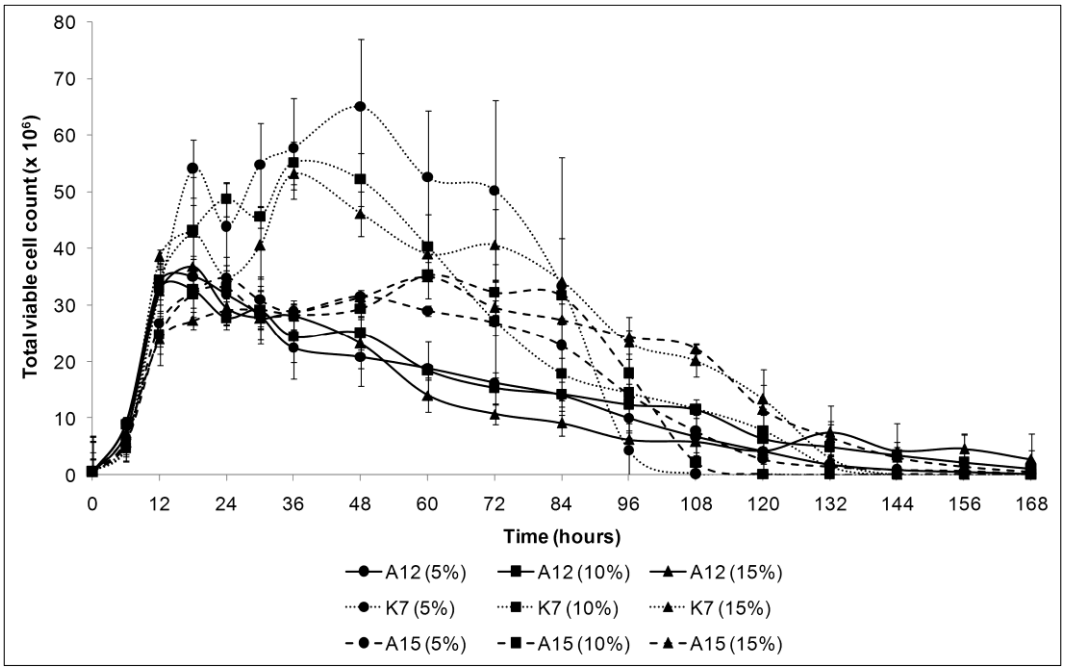


Figure 3.4 Total viable cell counts of yeast strains A12, K7 and A15 in YNB as a function of culture time and initial glucose concentration. Error bars indicate the SD of the means of data obtained from three independent growth curves.

3.2.3 Glucose consumption and ethanol production

Most studies using YNB as the basal medium have used 2% (w/v) initial glucose as the carbon source, a relatively low concentration as compared to industrial fermentations. As noted above, in this study three initial glucose concentrations were used to test inositol effects at more industrially relevant sugar concentrations, i.e. 5, 10 and 15% (w/v). The glucose consumption data obtained is presented in Figure 3.5. It is clear that under the conditions of these experiments (nutritional value of media, standard cell counts for inoculation), when the initial glucose concentration was higher, more time was required to consume the sugar. As can be seen from the cultures with 15% (w/v) initial glucose, when too high sugar concentration is used, stuck fermentation is commonly observed (Ishmayana, Learmonth & Kennedy 2011; Panchal & Stewart 1980; Reddy & Reddy 2006).

In industrial processes, which use complex nitrogen sources in the basal media, stuck fermentation generally occurs when sugar exceeds 27% (w/v) (Thomas, Hynes & Ingledew 1994; Thomas *et al.* 1993). In the present study, stuck fermentation was observed at 10% (w/v) initial glucose for yeast strain K7, leaving ~3% (w/v) of residual glucose at the end of the fermentation. With an 15% (w/v) initial glucose concentration, all strains displayed stuck fermentation after about 96 hours. While no significantly different fermentation performance was detected with 5% (w/v) initial glucose, where all strains consumed the glucose within about 36 h, differences in the performance of the different yeast strains were observed when 10% (w/v) initial glucose concentration was used. At this level of initial glucose, yeast strain A15 was the fastest strain to consume almost all the glucose at 84 h followed by yeast strains A12 and K7 showed stuck fermentation. Thus, it seems that 10% (w/v) initial glucose concentration would be optimal to discriminate

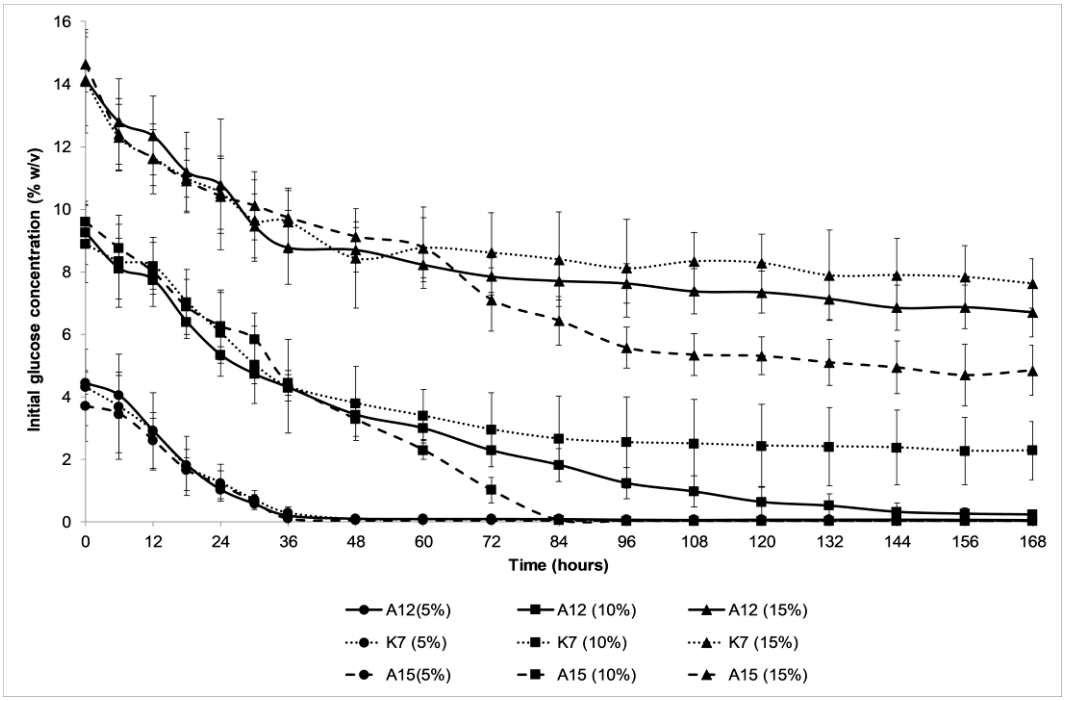


Figure 3.5 Glucose consumption displayed by yeast strains A12, K7 and A15 in YNB media as a function of culture time and initial glucose concentrations. The error bars indicate the SD of the means of data obtained from three independent growth curves.

between the three strains. However, to assess the fermentation one must also evaluate ethanol production, as discussed below.

In agreement with glucose consumption, ethanol production showed similar trends, as presented in Figure 3.6. Yeast strain A15 with 10% (w/v) initial glucose concentration produced the highest ethanol concentration at around 96 hours (~2.2% w/v), while yeast strains A12 and K7 produced similar levels of ethanol. When initial glucose concentration increased to 15% (w/v), the ethanol production decreased. The lower ethanol production in media with higher initial glucose concentration may be related to the diversion of carbon to glycerol to combat osmotic stress and this may be related to the stuck fermentation previously noted. The lowest initial glucose concentration led to the fastest ethanol production with the ethanol concentration peak at 36 hours for yeast strain A15 and 48 hours for yeast strains K7 and A12.

A decline in ethanol concentration was observed after the ethanol peak for all strains and conditions. For the lowest initial glucose concentration, which resulted in the glucose being exhausted at around 36 hours, the ethanol concentration began to decrease at 48 hours, indicating a diauxic shift, after which under aerobic conditions the yeast start to use the ethanol as a carbon source. For 10% (w/v) initial glucose, the diauxic shift seemed to start later at around 96 hours for yeast strain A15 with exhaustion of glucose occurring at 84 hours. It is difficult to explain the disappearance of ethanol in the cultures of yeast strains A12 and K7 with 10% (w/v) initial glucose concentration, as these cultures maintained glucose levels higher than the repression threshold (0.2% i.e. 2 g/L) for most of the culture period. Interestingly, with 15% (w/v) initial glucose the decline of ethanol was observed after 36, 72 and 96 hours for yeast strains A12, K7 and A15, respectively. Generally, diauxic shift occurs when the glucose concentration decreases to below the repression threshold, therefore the yeast start to use ethanol. For 15% (w/v)

initial sugar concentration, even though glucose was not exhausted, ethanol started to decline. It seems that the cells may have in fact started to use the ethanol as carbon source (Piškur *et al.* 2006), the ethanol was oxidized to acetic acid (Albers *et al.* 1996) or some ethanol may have evaporated, resulting in the decreased ethanol concentrations detected at the later time points.

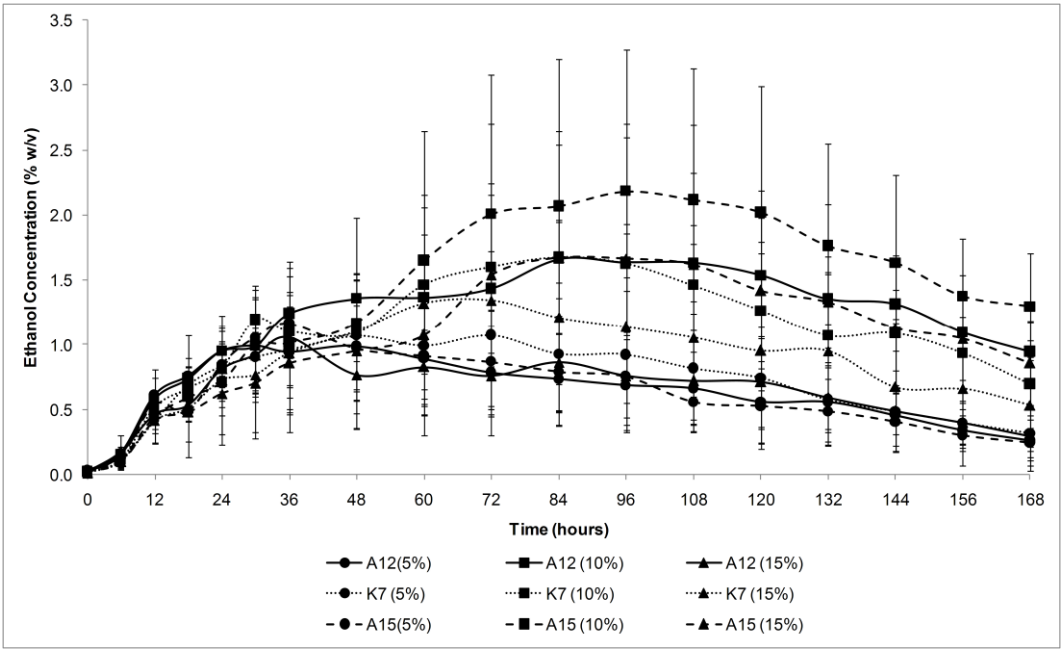


Figure 3.6 Ethanol production displayed by yeast strains A12, K7 and A15 in YNB media as a function of culture time and initial glucose concentration. The error bars indicate the SD of means of data obtained from three independent growth curves.

The amount of ethanol detected in the fermentation media was actually much lower than the theoretical amount. When yeast cells are exposed to osmotic stress, they synthesize glycerol as a stress protector (Albers *et al.* 1996; Myers, Lawlor & Attfield 1997). Under osmotic stress conditions, glucose in the media is not only used for ethanol production, but is also used to synthesize glycerol. Therefore, the amount of ethanol cannot reach the theoretical amount due to diversion of carbon from ethanol production to glycerol synthesis for protecting the cell. To check this hypothesis, the concentration of glycerol was determined at 96 hours (Figure 3.7).

It is clear that the glycerol concentration increases with increasing initial glucose concentration. It was also observed that yeast strain A12 synthesizes the most glycerol, followed in order by yeast strains A15 and K7. This observation supports the ethanol production findings, in that yeast strain A12 produced the least ethanol of the three strains. This demonstrates that yeast strain A12 converts more sugar to glycerol, decreasing the amount of ethanol produced. However, while yeast strain K7 synthesized a lower level of glycerol, cell growth was higher, effectively diverting carbon to cell growth thereby leading to lower ethanol production compared to the other strains. This also led to a higher glucose concentration remaining in the medium at the end of the fermentation, as described above, which may be due to exhaustion of a nutritional factor such as available nitrogen. Yeast strain A15 seemed to have the best fermentation performance as indicated by a higher ethanol concentration and a lower glycerol concentration and also a lower

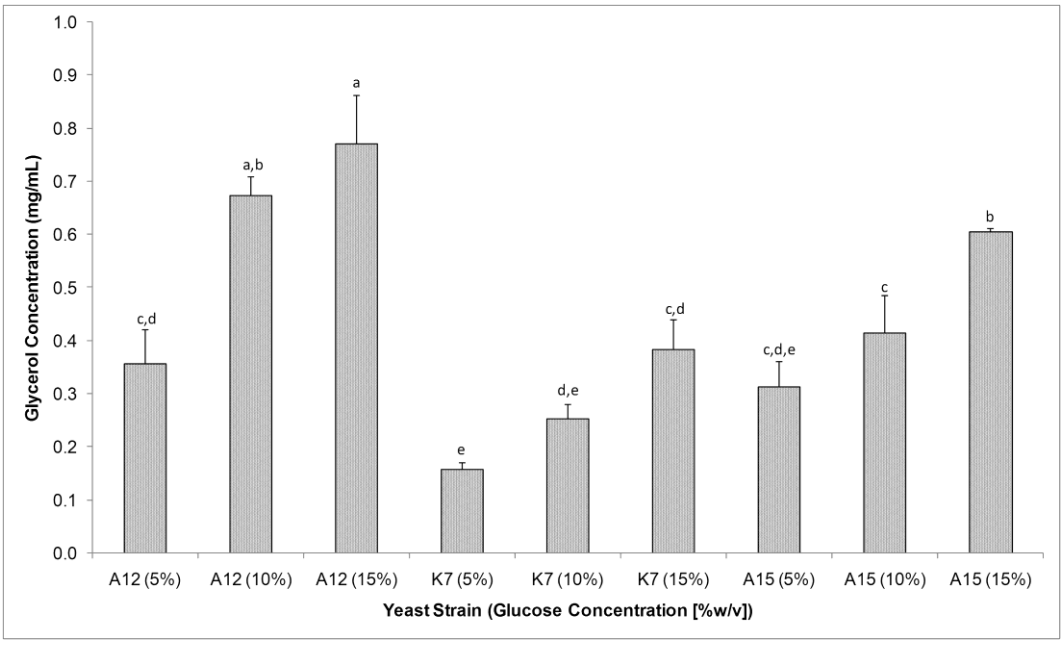


Figure 3.7 Glycerol concentrations in cultures of yeast strains A12, K7 and A15 in YNB media as a function of initial glucose concentration at 96 hours. The error bars indicate the SD of the means of data obtained from three independent experiments. Bars with the same letters indicate that the differences between the means are not statistically significant.

residual sugar concentration in the medium (Figure 3.5 and Table 3.2). Other than that, lower ethanol detected in the media could be due to evaporation of ethanol since the plug used was porous, allowing some ethanol to evaporate. However, previous control checks in this laboratory have assessed the evaporation of ethanol under each new condition of culture (for example, see Lewis *et al.* 1993). While evaporation of ethanol is possible, it has been shown that there is no significant loss of ethanol by evaporation, leading to the conclusion that decreases in ethanol level were due to metabolic activity and not simply due to evaporation.

Fermentation kinetic parameters were evaluated to study whether there were any effects of different initial sugar concentration on the fermentation performance (Table 3.2). While in general the glucose consumption rate (Qs) values were not significantly different, the highest Qs observed for yeast strain A12 at 5% (w/v) initial glucose was significantly different to the lowest Qs for yeast strain K7 at 15%

Table 3.2 Fermentation kinetic parameters displayed by yeast strains A12, K7 and A15 in YNB media with different initial glucose concentrations. Qs, Qp and Yp/s were determined from 0-30 h data (where the highest rate of glucose consumption and ethanol production were detected), while glucose consumption was determined at the end of the fermentation (168 hours). The values shown are the means of data obtained from three independent experiments ±SD. The values with the same superscript letter in the same columns (for different strains) are not significantly different according to a one-way Anova with Tukey's *post-hoc* test.

Strain	Initial Sugar Concentration (% w/v)	Qs ($g.L^{-1}.h^{-1}$)	Qp ($g.L^{-1}.h^{-1}$)	Yp/s ($g.g^{-1}$)	Glucose Consumption (%)
A12	5	1.285 ± 0.128^{a}	0.322 ± 0.145^{a}	0.246 ± 0.085^{a}	98.34 ± 0.41^{a}
	10	0.883 ± 0.181^{ab}	0.196 ± 0.036^{a}	0.224 ± 0.031^{a}	97.21 ± 1.88^{a}
	15	0.767 ± 0.158^{ab}	0.101 ± 0.044^{a}	0.131 ± 0.053^{a}	52.52 ± 3.46^{c}
K7	5	1.199 ± 0.340^{ab}	0.297 ± 0.066^{a}	0.250 ± 0.022^{a}	98.74 ± 0.22^{a}
	10	0.740 ± 0.232^{ab}	0.198 ± 0.105^{a}	0.256 ± 0.078^{a}	73.71 ± 11.29^{b}
	15	0.678 ± 0.059^{b}	0.143 ± 0.088^{a}	0.205 ± 0.108^{a}	45.61 ± 5.35^{c}
A15	5	1.020 ± 0.296^{ab}	0.342 ± 0.135^{a}	0.329 ± 0.035^{a}	98.53 ± 0.29^{a}
	10	1.134 ± 0.064^{ab}	0.244 ± 0.134^{a}	0.214 ± 0.112^{a}	99.55 ± 0.02^{a}
	15	0.975 ± 0.025^{ab}	0.197 ± 0.115^{a}	0.200 ± 0.112^{a}	66.82 ± 5.17^{b}

(w/v) initial glucose. No significant differences were observed for ethanol production rate (Qp) and ethanol productivity (Yp/s). Significant differences were noticed in terms of percentage of glucose consumed; the cultures with 5% (w/v) initial glucose displayed the highest percentage of glucose consumption for all strains. For 10% (w/v) initial glucose, yeast strains A15 and A12 did not differ significantly in percentage of glucose consumption but yeast strain K7 had a significantly lower percentage of glucose consumption. Finally, for 15% (w/v) initial glucose, yeast strains A12 and K7 were not significantly different in percentage of glucose consumption, while yeast strain A15 had a significantly higher percentage of glucose consumption.

The kinetic parameter data indicate that even though the Qs, Qp and Yp/s values do not differ significantly, the total amount of ethanol produced or glucose consumed (see Table 3.3) may differ significantly. In these experiments the Yp/s values were substantially lower than the theoretical value for production of ethanol from glucose (assuming 100% efficiency and no diversion of sugar to other processes) of 0.511. As seen in Table 3.2, generally only about half of the theoretical value was achieved. In all cases one has to consider that a certain amount of the carbon from glucose will end up as cell biomass (note that from an inoculum of about one million cells per mL (Section 2.3), viable cell counts reached 40 to 60 million cells per mL (Figure 3.4)) and under aerobic conditions a certain amount of the glucose may have gone through respiration to CO_2 to provide energy for growth. However, as well as these uses of glucose, the differences between the observed and expected values for ethanol productivity were most likely also caused by the diversion of glucose from ethanol production to glycerol production in order to overcome osmotic stress. As described above, glycerol was detected under all conditions for all strains in this study. Furthermore, in media with a higher initial glucose concentration, more glycerol was produced, leading to no

improvement in ethanol production. However, at the end it is most likely that glycerol reached a “saturation point” where the cells stopped producing it, or a “flagfall level” of glucose had been reached at which the osmotic pressure was no longer at stressful levels so the remaining sugar could be converted to ethanol instead of glycerol.

To check the inferences about glycerol metabolism, key data from Figures 3.5, 3.6 and 3.7 were utilized to calculate the total amount glucose consumed in mol/L and also the total production of ethanol and glycerol expressed in the same unit and the values obtained are presented in Table 3.3.

Table 3.3 The total glucose consumption and the total production of ethanol and glycerol expressed in units of mol/L as calculated from the mean data from Figures 3.5, 3.6 and 3.7.

Yeast strain and medium	Glucose consumed (mol/L)	Ethanol produced (mol/L)	Glycerol produced (mol/L)	Remainder of glucose consumed for other purposes (mol/L)
K7, 5% (w/v) initial glucose	0.236	0.197	0.002	0.037
K7, 10% (w/v) initial glucose	0.352	0.349	0.003	<0.001
K7, 15% (w/v) initial glucose	0.332	0.245	0.004	0.083
A12, 5% (w/v) initial glucose	0.242	0.144	0.004	0.094
A12, 10% (w/v) initial glucose	0.444	0.352	0.007	0.085
A12, 15% (w/v) initial glucose	0.362	0.161	0.008	0.193
A15, 5% (w/v) initial glucose	0.203	0.158	0.003	0.042
A15, 10% (w/v) initial glucose	0.530	0.469	0.005	0.056
A15, 15% (w/v) initial glucose	0.503	0.358	0.007	0.138

Considering these data one can see that while neither the ethanol nor glycerol production were at the theoretical values (2 moles of ethanol or 2 moles of glycerol per mole of glucose), it can be observed that most of the glucose is accounted for, except in the case of 15% (w/v) glucose initial concentration for strains A12 and A15. As discussed earlier, the other uses of glucose could include cell growth and

respiratory metabolism. Notably, according to these figures, glycerol production does not account for a large proportion of the "missing" ethanol production. This is also consistent with the previously published finding that glycerol production occurs at the expense of biomass accumulation rather than ethanolic fermentation (Petelenz-Kurdziel *et al.* 2013).

3.2.4 Membrane fluidity

In this study the fluidity of cellular membranes was assessed using fluorescence spectroscopy of laurdan-labelled cells to determine the generalized polarization (GP) parameter. Higher GP values indicate lower membrane fluidity (Butcher 2008; Learmonth 2012). In addition to the factors discussed above, GP values were determined at 6 and 24 hours of culture for the three yeast strains grown in 5, 10 and 15% (w/v) initial glucose concentration. The results are presented in Figure 3.8, where it can be seen that the GP values for 6 hours of culture were generally lower than the GP values for 24 hours of culture, except for yeast strain A12 for 10 and 15% (w/v) initial glucose concentration (although these differences were not statistically significant). This indicates that the cellular membranes become less fluid with longer times of culture. At 6 hours of culture, yeast strain K7 had the lowest and yeast strain A12 had the highest GP value, while that of yeast strain A15 was not significantly different from that of either yeast strains K7 or A12, except in the case of 15% (w/v) initial glucose concentration for which yeast strain A12 had a higher GP value than yeast strain A15. Thus, in general, yeast strain A12 has the lowest membrane fluidity, while yeast strain K7 has the highest.

The GP results were analysed by ANOVA for three factors (i.e. strain, initial glucose concentration and culture time) which were chosen as the independent variables. Statistical analysis of the main factors and the interactions between the

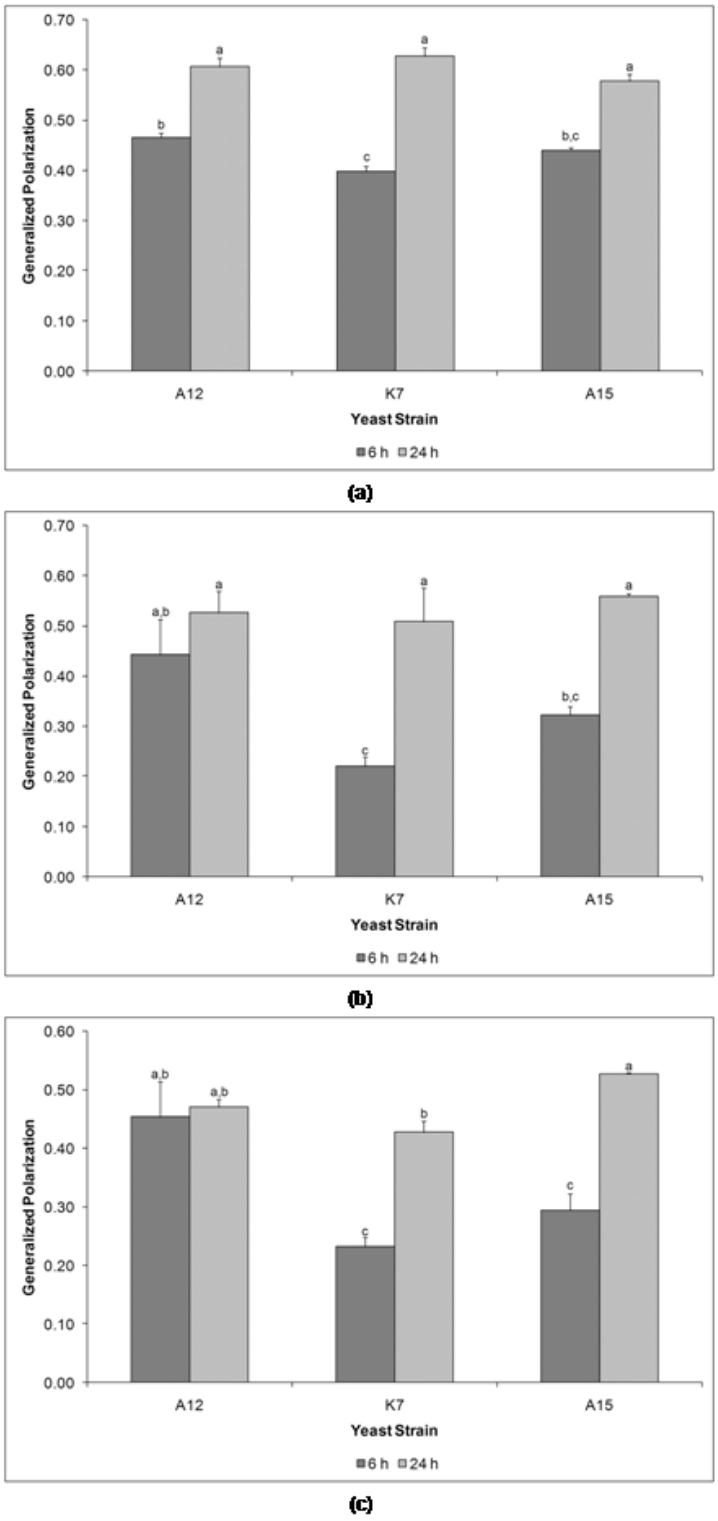


Figure 3.8 Fluidity of the yeast cellular membranes as assessed by generalized polarization of yeast strains A12, K7 and A15 grown in YNB media with (a) 5 (b) 10 or (c) 15% (w/v) initial glucose concentration at 6 and 24 hours of culture. The error bars indicate the SD of the means of data obtained from three independent experiments. Columns with different letters indicate that differences between the means of those columns are statistically significant at $\alpha = 0.05$ according to Tukey's post hoc test.

main factors was performed to determine if and how these main factors affect GP. All three factors showed statistically significant effects on GP ($p < 0.001$). For the interaction effect of the main factors, strain vs glucose concentration ($p = 0.007$) and strain vs time ($p < 0.001$) exhibited statistically significant differences, but interactions between initial glucose concentration and time were not statistically significant ($p = 0.101$). The main factor plot and interaction plot are presented in Figures 3.9 and 3.10, respectively. For the main factors, it is clear that yeast strain A12 has the highest GP, followed by yeast strains A15 and K7. Thus, as noted above, in general yeast strain A12 has the least fluid membranes, while yeast strain K7 has the most fluid membranes, with membranes of yeast strain A15 having relatively intermediate fluidity. The relationship between membrane fluidity and stress tolerance will be further investigated below.

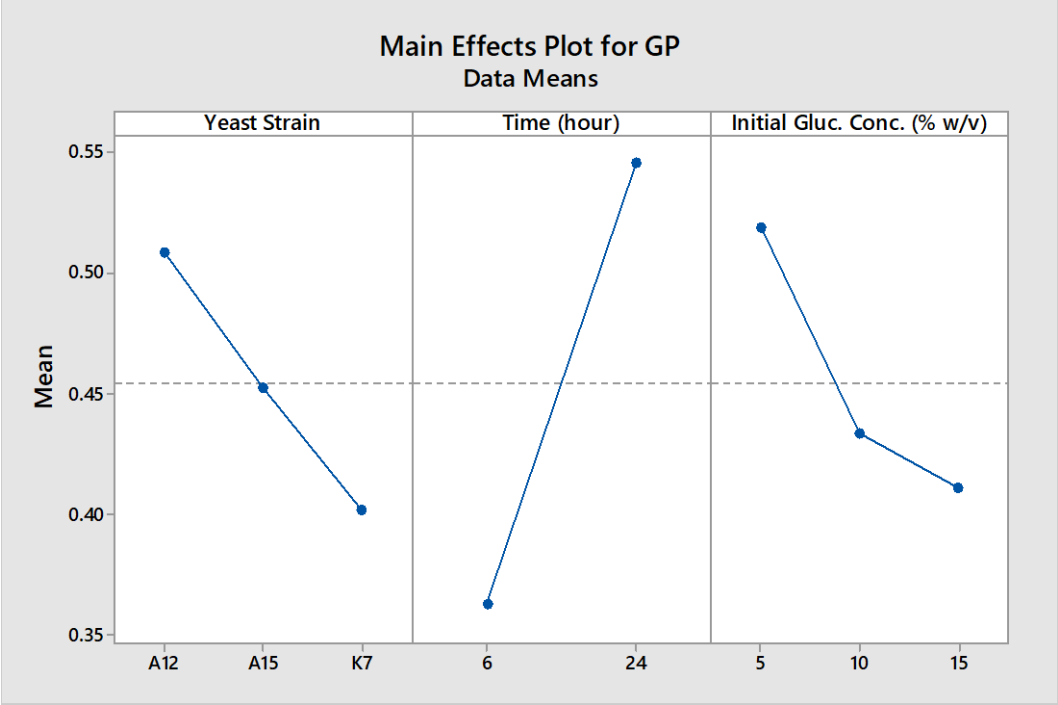


Figure 3.9 Main effect plot of independent variables used in assessment of membrane fluidity.

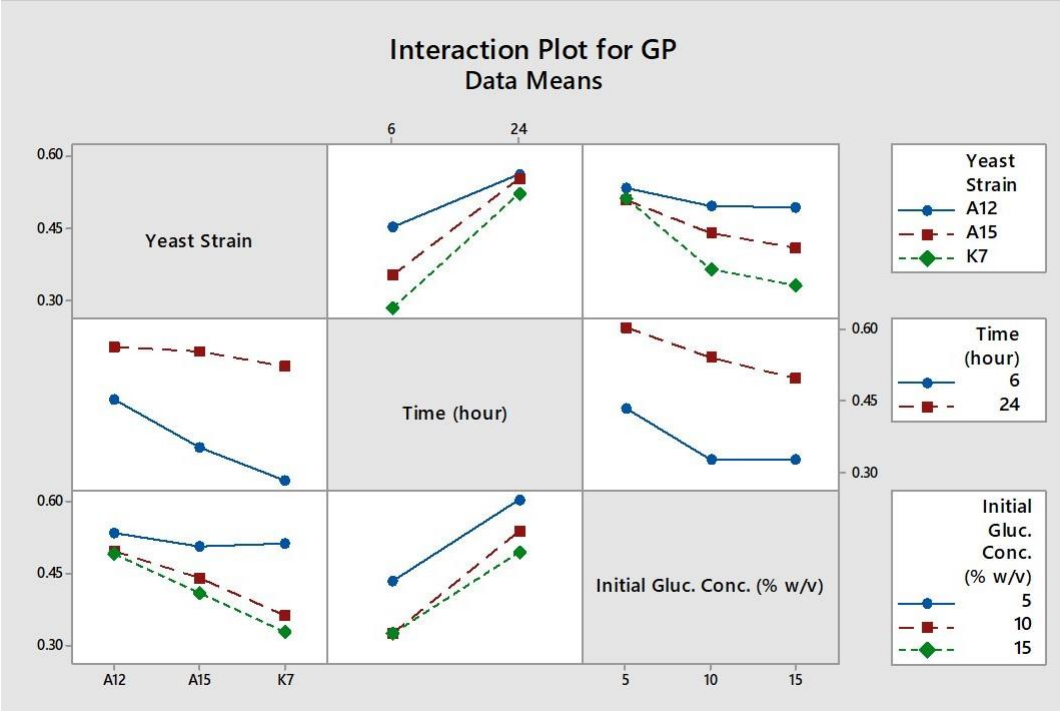


Figure 3.10 Interaction effects of independent variables used in assessment of membrane fluidity.

When considering the effect of initial glucose concentration, the GP values for the cellular membranes of cells grown in 5% (w/v) initial glucose concentration were the highest, while the GP values for the cellular membranes of cells grown in 10 and 15% (w/v) initial glucose concentration were not significantly different to each other. Thus, it seems that the yeast cell have higher membrane fluidity when subjected to the higher osmotic stress associated with 10 or 15% (w/v) initial glucose concentration in the media. Note that at 24 hours the residual glucose concentrations were about 1, 6 and 11% (w/v) in the cultures starting with 5, 10 and 15% (w/v) glucose, respectively. Finally, with respect to time, it is clear that the GP values at 6 hours of culture were significantly lower than those at 24 h of culture of each strain. As noted above, this means that the membrane fluidity is higher in the early respiro-fermentative growth phase of the cultures and decreases either as the cultures initially approach the later mid-respiro-fermentative growth phase (cultures with 10 or 15% (w/v) initial glucose concentration) or towards the

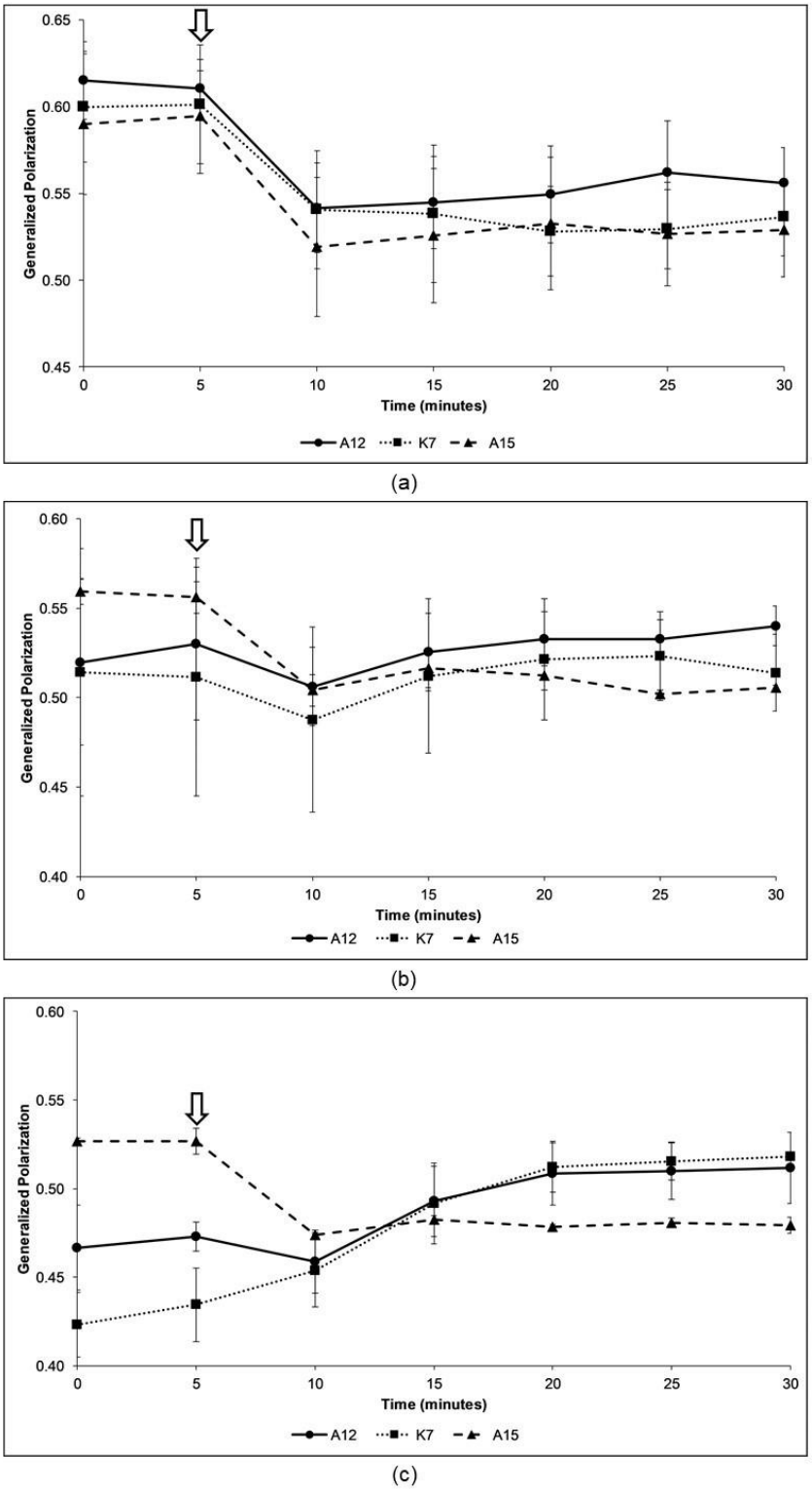


Figure 3.11 Fluidity of the cellular membranes of yeast strains A12, K7 and A15 grown in YNB media with (a) 5, (b) 10 or (c) 15% (w/v) initial glucose concentration before and after ethanol exposure as assessed by generalized polarization. The arrow sign indicates the time of addition of absolute ethanol to give a final concentration of 18% (v/v). Error bars indicate the SD of the means of data obtained from three independent experiments.

end of the respiro-fermentative growth phase (cultures with 5 % (w/v) initial glucose concentration; see Figure 3.5).

Ethanol is known for its ability to increase fluidity when cellular membranes are exposed to this compound (Jones & Greenfield 1987). The extent to which fluidity is affected may differ between one yeast strain and another. To investigate the effect of ethanol on the cellular membrane fluidity of the strains used in this study, the GP value was determined before and after exposure of the cells to 18% v/v ethanol (Figure 3.11). It was observed that the GP value dropped immediately after ethanol exposure. This indicates that the fluidity of the cellular membranes increases immediately following ethanol exposure, except for K7 with 15% (w/v) initial glucose concentration, which showed GP increase after ethanol was added. All strains showed the same trend, with a slight recovery of GP values in response to the fluidization, however no statistically significant differences between the three strains were detected.

3.2.5 Tolerance to ethanol stress

The ethanol stress tolerance of the three yeast stains used in the present study was investigated only for cultures with 10% (w/v) initial glucose as this seemed the most promising initial concentration of glucose to utilize for subsequent experiments. The results are presented in Figure 3.12. There was no statistically significant differences in % survival between yeast strains A12 and K7, while yeast strain A15 showed a statistically significantly lower % survival compared to the other two strains (p = 0.023). The results of the present study confirm previously published results. A12 and K7 are known to have good tolerance to ethanol (Ishmayana 2011; Lewis *et al.* 1997), while A15 has good thermotolerance (Lewis *et al.* 1993). Analysis of the correlation between GP and survival data indicates a

strong positive correlation between the two parameters (R = 0.754; p = 0.019). This indicates that cells with a higher GP value will have higher survival, or, in other words, cells with lower membrane fluidity will have better tolerance when exposed to the membrane fluidization caused by ethanol stress.

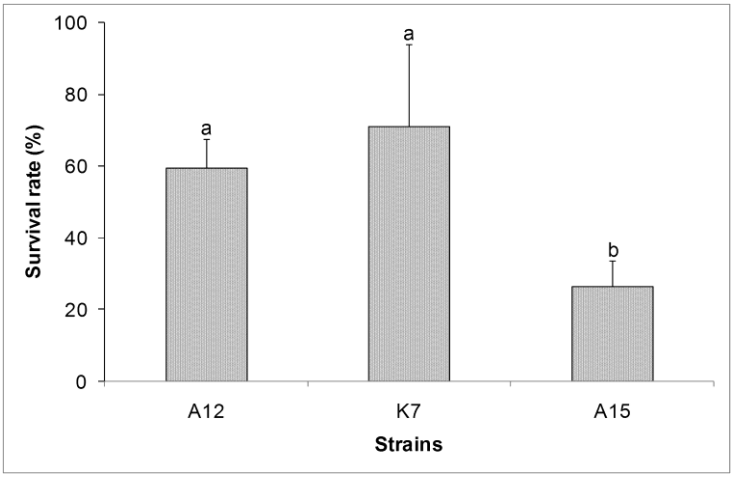


Figure 3.12 Survival of the three yeast strains after exposure to 18% (v/v) ethanol for 1 hour followed by growing on agar plates. The cells were grown in YNB with 10% (w/v) initial glucose concentration and tested after 24 hours of culture. Error bars indicate the SD of the means of data obtained from three independent replicates. Columns with the same letter indicate that differences between the means of the columns are not statistically significant according to Tukey's post-hoc test.

3.3 Discussion

3.3.1 Comparison of growth parameters

The $OD_{600\ nm}$ value was used to monitor cell growth in the present study. The maximum growth rate, based on $OD_{600\ nm}$ value, indicated that yeast strain K7 tended to have a relatively higher growth rate, followed in decreasing order by yeast strains A12 and A15. It is interesting to note that even though the maximum growth rates (calculated over 0-12 h in Section 3.2.1) for some experimental conditions showed differences for different strains, the measured $OD_{600\ nm}$ values, as an indication of cell number, at 12 hours of culture did not show statistically significant differences. This result indicates that even though the cultures may have

had different maximum growth rates, they may plateau at similar final cell densities (as assessed by $OD_{600\ nm}$). However, further examinations of the $OD_{600\ nm}$ values indicated that at 36 hours of culture the $OD_{600\ nm}$ values of all strains tested showed statistically significant differences.

Charoenchai, Fleet & Henschke (1998) found that high initial sugar concentrations tended to reduce growth rates. However, the present study did not find this. Neither the maximum growth rates nor the plateau $OD_{600\ nm}$ values show statistically significant differences between the different initial glucose concentrations, although the three strains had different plateau $OD_{600\ nm}$ values. The differences in observations between different studies may be due to differences in the growth media used or that the initial sugar concentration used was higher in the Charoenchai, Fleet & Henschke (1998) study. Yeast strain K7 consistently had the highest $OD_{600\ nm}$ value, followed by yeast strains A15 and A12. With respect to cell viability, it is clear that yeast strain K7 has a significantly higher viability compared to yeast strains A15 and A12 and this difference is maintained up to 60 hours of culture. $OD_{600\ nm}$ may not be the best indicator of growth performance as it includes contributions from both live and dead cells.

The three yeast strains used in the present study had distinct viability profiles during culture. Even though yeast strain A15 started with the lowest viability, it could actually maintain comparatively higher viability throughout the fermentation period. This higher cell viability may be contribute to in the higher ethanol levels produced by this strain. Even though yeast strain K7 had the highest viability during the initial stage of the fermentation, its viability decreased gradually and became substantially lower than that of yeast strain A15 during the later stage of the fermentation. It is most likely that yeast strain A15 entered a state in which it could maintain the maximum number of cells actively fermenting sugar to ethanol.

However, for the other strains the number of viable cells decreased more rapidly, leading to less actively fermenting cells and thus lower ethanol production.

Stuck fermentation is commonly observed when using culture media with very high gravity (VHG), i.e. very high concentrations of sugar (> 27% w/v). Under these conditions, a high amount of sugar may remain unutilized after the fermentation has ended and all the yeast cells have died. In the present study, stuck fermentation was observed when a 15% (w/v) initial glucose concentration was used. This concentration is actually lower than the definition given by Thomas, Hynes & Ingledew (1994) and Thomas *et al.* (1993), where VHG was defined as media with more than 27% (w/v) initial glucose. Notwithstanding, the accepted definition is determined in nutritionally-rich media. In contrast, the present study used relatively poor media, so it is perhaps not surprising that stuck fermentation was observed at a lower initial glucose concentration. Additionally, stuck fermentations are not uncommon in the wine industry with initial sugar levels of 18% (w/v) or less. Therefore, we propose that for nutritionally-poor media (such as YNB), a 15% (w/v) initial glucose concentration could be considered as a “very high gravity” condition, based on the occurrence of stuck fermentation at this initial concentration level. For K7, stuck fermentation was even observed at a 10% (w/v) initial glucose concentration, leaving about one fifth of the initial glucose remaining in the media at the end of the fermentation. High sugar concentration correlates with high osmotic stress. Based on this result, it is most likely that K7 has the lowest tolerance against osmotic stress. This possibility will be further explored in Chapter 4.

Ethanol production, for all three strains, was in agreement with glucose consumption patterns. A15, which consumed more glucose, produced more ethanol. In the very high gravity condition (15% (w/v) initial glucose concentration), less ethanol was produced. It is well known that when yeast cells are exposed to

high osmotic stress, they will produce glycerol as a protection agent against the stress. Therefore, under VHG conditions, more sugar is converted to glycerol to protect the cell and as a consequence, less ethanol is produced.

It is interesting to note that among the three yeast strains used in the present study, K7 produced the least glycerol, followed by A15 and A12. Considering this and the previously described results, it is most likely that K7 has the lowest osmotic tolerance due to its inability to adapt to increasing osmotic stress through enhanced glycerol synthesis. A12 showed lower ethanol production and higher glycerol production compared to A15. It seems that in A12 the main protection against hyperosmotic stress is by directing metabolism to glycerol production at the expense of ethanol production. The results also indicate that A15 may have tolerance mechanisms other than glycerol synthesis, which protect the cell without sacrificing ethanol production.

3.3.2 Membrane fluidity and ethanol tolerance

As also found in previous studies (Ishmayana 2011; Ishmayana, Kennedy & Learmonth 2017), the present study found that membrane fluidity during the initial stage of the fermentation was higher compared to later stages. The membrane fluidity was determined at two time points, 6 and 24 hours. The 6 h time point represents the early part of the respiro-fermentative growth phase, not long after the initial lag phase, in which the cells adapt to the new environment. In comparison, the 24 h time point represents the later part of the respiro-fermentative growth phase, in which the cells actively ferment sugar. In the cultures with a 5% (w/v) initial glucose concentration, the 24 h timepoint was close to the end of the respiro-fermentative growth phase, with about 1% (w/v) glucose still remaining in the cultures. In contrast, in the cultures with a higher initial glucose concentration the 24 h timepoint represented a point in mid-respiro-fermentative growth phase,

with much more sugar still remaining to be fermented. At the 6 h timepoint, the yeast cells in all cultures were actively growing and dividing as this is during the period of maximum growth rate. However, by the 24 h timepoint cell growth and division had slowed, with the $OD_{600\ nm}$ and viable cell counts plateauing. When the cells are growing and dividing, they most likely require the cellular membrane components to be freely mobile (Singer 1975) and therefore the cells maintain high membrane fluidity during this growth phase. However, when the growth rate of the cells is markedly reduced, the membranes can stabilise with a lower fluidity.

The results of the present study indicate that cells grown in higher initial glucose concentrations tended to have lower GP values, which suggests higher membrane fluidity. However other membrane fluidity studies found that when cells are exposed to high osmotic stress, the fluidity of the membrane decreases, for example studies by Laroche *et al*. (2001) who examined *S. cerevisiae* exposed to glycerol; Hosono (1992) who examined *Zygosaccharomyces rouxii* exposed to NaCl; Beney, Mille & Gervais (2004) who exposed *Escherichia coli* to high glycerol; López, Garda & Rivas (2002) who exposed *Bacillus subtilis* to high NaCl concentrations; Tymczyszyn, Gómez-Zavaglia & Disalvo (2005) who exposed *Lactobacillus bulgaricus* to polyethylene glycol and Machado *et al.* (2004) who exposed *Lactobacillus casei* to high NaCl concentrations. However, a study by Khaware, Koul & Prasad (1995) also had similar conclusions to the present study. This study found that membranes prepared from spheroplasts of *Candida membranefaciens* following exposure of the cells to hyperosmotic stress (high NaCl concentrations had higher membrane fluidity. Given the much simpler membranes and stress response signalling systems of bacteria compared to yeast, one could reasonably exclude the bacterial studies and consider only the studies on yeast species. This leaves two studies reporting that osmotic pressure stress decreases membrane fluidity and two studies (the present study included)

reporting the opposite. Thus, our findings are somewhat controversial and clearly more studies need to be performed in the future to clarify the situation.

Ethanol is known for its membrane fluidizing effect. After the yeast strains were exposed to 18% (v/v) ethanol the GP values decreased rapidly, except for yeast strain K7 grown in 15% (w/v) initial glucose concentration that showed an increase of GP value. This result indicates that, as expected, ethanol exposure increases cellular membrane fluidity. However, it was found that for yeast strains A12 and K7 when cultured with relatively high initial sugar concentrations, the reduction of GP was lower and the cells appeared to be able to respond and restore the initial membrane fluidity level. The effect of ethanol on membrane fluidity appeared to correlate with the ability of the strain to tolerate exposure to 18% (v/v) ethanol. It was found that yeast strain A12 and K7 have higher ethanol tolerance than yeast strain A15. This result indicates that even though the yeast strain A15 produces the highest ethanol concentration, it is more susceptible to high ethanol stress. GP data also suggests that yeast strain A15 tends to maintain a lower GP after ethanol exposure than the other two strains tested. This suggests that the membrane of A15 cells is already more fluid and that upon ethanol exposure the "over fluidization" interferes with the membrane's function as a semi-permeabile barrier, leading to cell leakiness and ultimately, cell death.

3.4 Conclusions

All yeast strains used in this study showed different performances when grown in poor media (YNB) with various initial glucose concentrations ranging from 5-15% (w/v). No statistically significant differences of glucose consumption were detected when 5% (w/v) initial glucose was used for fermentation. Yeast strain A15 showed the highest total glucose consumption, followed by yeast strain A12 and then by yeast strain K7 when 10 or 15% (w/v) initial glucose concentration were used.

Furthermore, a stuck fermentation was observed for all strain used in the present study when a 15% (w/v) initial glucose concentration was used. Statistically significant increases were observed between the GP values of the cellular membranes of the cells at 6 and 24 hours of culture, indicating that the cellular membrane fluidity decreases as the cultures progress. An increase in cellular membrane fluidity was also observed when the yeast cells were exposed to 18% (v/v) ethanol, except for yeast strain K7 with 15% (w/v) initial glucose concentration.

CHAPTER FOUR: EFFECTS OF INOSITOL ON FERMENTATION PERFORMANCE AND CELLULAR MEMBRANE PHYSIOLOGY OF YEAST GROWN IN CHEMICALLY-DEFINED FERMENTATION MEDIA

4.1 Introduction

4.1.1 General Introduction

Supplementation of media with various agents has been found to improve yeast performance in fermenting sugar and consequently to increase ethanol yield. Such agents include yeast extract, catechin, dry spent yeast, glycerol, metal ions (Zn^{2+}, Mg^{2+}, Mn^{2+}) and inositol (Caridi 2002; Chi, Kohlwein & Paltauf 1999; Deesuth *et al.* 2012). Inositol is known to have important roles in ethanol tolerance and to improve the fermentation performance of the yeast *S. cerevisiae*. One of the possible mechanisms by which inositol may protect yeast cells against ethanol stress is by stabilizing the plasma and other cellular membranes and thus protecting the cell from permeabilization and leakage of cytoplasmic content when yeast are exposed to high ethanol concentrations. Another possible mechanism of ethanol resistance is through the ability of inositol to increase the ability of the cell to pump excess protons out of the cell by activation of the plasma membrane H^+-ATPase (Furukawa *et al.* 2004). When yeast cells are exposed to high ethanol concentrations, protons may enter the cell via passive diffusion due to disruption of the plasma membrane. Therefore, increasing plasma membrane H^+-ATPase activity will increase the ability of the cell to maintain homeostasis and therefore increase cell survival when exposed to ethanol stress.

Besides its positive roles in increasing tolerance to ethanol stress, inositol supplementation was also found to increase fermentation performance, resulting in higher ethanol production by the end of the fermentation. The concentration of

inositol used in inositol supplementation experiments varies, ranging from 1 mg/L (Yao, Chi & He 2006) to 1 g/L (Nikolić *et al.* 2009a).

Inositol supplementation of fermentation media was reported to change the phospholipid composition of the plasma membrane of the yeast cell. It led to an increase in the proportion of phosphatidylinositol and this was accompanied by a rapid decrease in the level of phosphatidylcholine and phosphatidylethanolamine, while phosphatidylserine and phosphatidic acid levels were relatively constant (Chi, Kohlwein & Paltauf 1999). The change in phospholipid composition of the plasma membrane was found to affect its fluidity (Jurešić, Blagović & Rupčić 2009). Jurešić, Blagović & Rupčić (2009) used unsaturation index to monitor alteration in membrane fluidity. However, Alexandre, Berlot & Charpentier (1994) suggested that the results obtained using indirect assessment of membrane fluidity based on the fatty acid unsaturation index should be used with caution, since many factors contribute to membrane fluidity, including protein components embedded in the membrane (Learmonth 2012). It has been proposed that direct measurement of membrane fluidity using fluorescence spectrometry provides a better understanding of membrane alterations, since this method takes into account all the factors affecting the membrane fluidity (Alexandre, Rousseaux & Charpentier 1994; Learmonth 2012).

There are no data published that indicate an optimal concentration of inositol to provide positive effects on ethanol production or ethanol tolerance by *Saccharomyces* species. However, a study by Ji *et al*. (2008) using a different yeast species, *Paschycolen tannophillus*, for ethanol production showed that even though inositol supplementation improved ethanol production and cell growth, beyond a certain concentration it imparted negative effects, reducing cell growth and also ethanol production. They found that the maximal ethanol yield was achieved when the medium was supplemented with 0.1 g/L inositol. When the

medium was supplemented with a higher concentration of inositol, ethanol production decreased.

In the present study, two stages of experimentation were conducted. The initial sugar concentration used in this study was selected based on the experiments in Chapter 3, which showed that fermentation of media with 10 and 15% (w/v) initial sugar resulted in residual sugar in the fermentation media. The initial experiment was conducted using a modified YNB medium which contained no inositol, contained a 15% (w/v) initial glucose concentration and was supplemented with 0, 0.1 or 0.4 g/L inositol. However, under these conditions the residual sugar in the fermentation media was too high and therefore it was hard to distinguish differences in yeast fermentation performance between inositol-supplemented and unsupplemented media. Based on these findings, it was decided to decrease the initial glucose concentration to 10% (w/v), and also to test more inositol concentrations (i.e. 0.05, 0.1, 0.2, 0.4 and 0.8 g/L). The level of inositol supplementation was varied over this range to more precisely define any effects of inositol and also to investigate whether excess inositol can negatively affect ethanol production when *S. cerevisiae* is used in the fermentation process, as previously reported for *P. tannophillus*. Furthermore, direct measurement using steady-state fluorescence spectroscopy was also conducted to investigate the effect of inositol supplementation on cellular membrane fluidity. Since the level of inositol supplementation directly correlates with ethanol stress tolerance, the effect of inositol supplementation on membrane fluidity after ethanol exposure was also investigated in order to gain more insight into how inositol supplementation may affect the changes in membrane fluidity that occur in response to ethanol stress.

4.1.2 Yeast strain and culture conditions

The work in this chapter was divided into two sections. The first section describes an investigation using only one yeast strain (A15, ATCC 38554, originally isolated from canned cherries). This section describes a preliminary experiment performed to investigate the suitability of a relatively high initial sugar concentration (15% w/v) as the experimental condition. However, as noted above it was found that at this concentration so much sugar was unconsumed at the end of fermentation that it was very hard to discriminate effects of inositol supplementation on the fermentation performance. Therefore, the main experiment (second section) was performed with a lower initial sugar concentration (10% w/v) and more strains were employed, i.e. the three *S. cerevisiae* strains A15, A12 and K7.

4.1.3 Specific growth conditions and experimental design

The cells were grown in a chemically-defined fermentation medium containing 0.69% (w/v) YNB without inositol (inositol free) and amino acid (Formedium^{TM}), 0.005% (w/v) amino acid mixture (Sunrise Science, containing L-histidine, DL-methionine, and DL-tryptophan with ratio of 10:20:20) (Section 2.2), and a 15% (w/v) initial glucose concentration for the preliminary experiment. However, for the main experiment a 10% (w/v) initial glucose concentration was employed. Starter cultures were inoculated from slopes and grown overnight (~18 h) at 30°C and 180 opm in an orbital shaker (Paton).

Experimental cultures were prepared using the above medium supplemented with inositol to achieve 0, 0.1 and 0.4 g/L inositol for the preliminary experiment and 0, 0.05, 0.1, 0.2, 0.4 and 0.8 g/L inositol for the main experiment. Each culture was prepared aseptically by adding the fermentation media to a sterile Erlenmeyer flask, each sealed with an oxygen-permeable cotton wool bung, and then

inoculated with yeast strain A15 for the preliminary experiment or one of the three strains (A12, K7 and A15) for the main experiment. The amount of inoculum was set to give an initial viable cell density of ~10^6 cell/mL. The ratio of flask size to culture volume was 4:1 to ensure adequate oxygen mixing. Samples of the culture were aseptically removed by drawing off with a sterile micropipette every 6 h from 0 to 30 h, followed by sampling at 12 h intervals from 48 to 168 h. Monitoring of the cutures included measurement of growth rate by optical density (Section 2.4), viable cell count (Section 2.5), glucose concentration (Section 2.13) and ethanol concentration (Section 2.12).

The GP of laurdan-labelled yeast cells (Section 2.7) was measured at 24 hours, as in Chapter 3.

All data presented in this report were obtained from three independent experiments.

Where appropriate, differences between the means of different set of experimental data were analysed for statistical significance using a one-way ANOVA, with *post-hoc* comparison using Tukey's post-hoc test to identify any statistically significant differences (Section 2.15). Differences were considered significant if $p < 0.05$.

4.2 Results

4.2.1 Preliminary experiment: cell growth and fermentation performance in medium containing a 15% w/v initial glucose concentration

The growth of yeast cultures is influenced by the composition of both macro- and micro-nutrients in the fermentation media. Inositol, as one of the vitamins, is essential for yeast cell growth and is required at the micro-nutrient level (Begea *et al.* 2010; Nikolić *et al.* 2009a; Nikolić *et al.* 2009b). In the present study, the yeast growth rate was determined by measuring the optical density at 600 nm. It was

found that inositol supplementation promoted yeast cell growth as indicated by the decreased growth rate when the cells were cultured in a medium that was not supplemented with inositol, as shown in Figure 4.1. This conclusion was also supported by the values for maximum growth rate presented in Table 4.1. The maximum growth rate in medium with inositol supplementation was substantially higher than that in medium without inositol supplementation. However, even though two levels of inositol supplementation were tested, no substantial differences in maximum yeast cell growth rate were observed between the media with two different levels of inositol-supplementation.

With respect to yeast cell viability, it was observed that cells cultured in media without inositol supplementation displayed higher viability throughout the fermentation, as presented in Figure 4.2. This effectively resulted in similar viable cell count, as even though the total cell number in media without inositol supplementation was lower, the viability was higher.

Table 4.1 Kinetic parameters of yeast grown in inositol-free YNB media without or with supplementation with 0.1 g/L or 0.4 g/L inositol and with a 15% w/v initial glucose concentration. The data presented are the means of data obtained from two independent experiments ± SD.

Level of Inositol Supplementation (g/L)	μ_{max} (h^{-1})	Qs ($mg.mL^{-1}.h^{-1}$)	Qp ($mg.mL^{-1}.h^{-1}$)	Yp/s ($mg.mg^{-1}$)
0.0	0.296 ± 0.012	0.787 ± 0.409	0.210 ± 0.086	0.276 ± 0.034
0.1	0.421 ± 0.027	0.657 ± 0.534	0.129 ± 0.088	0.211 ± 0.039
0.4	0.430 ± 0.032	0.792 ± 0.428	0.171 ± 0.151	0.192 ± 0.086

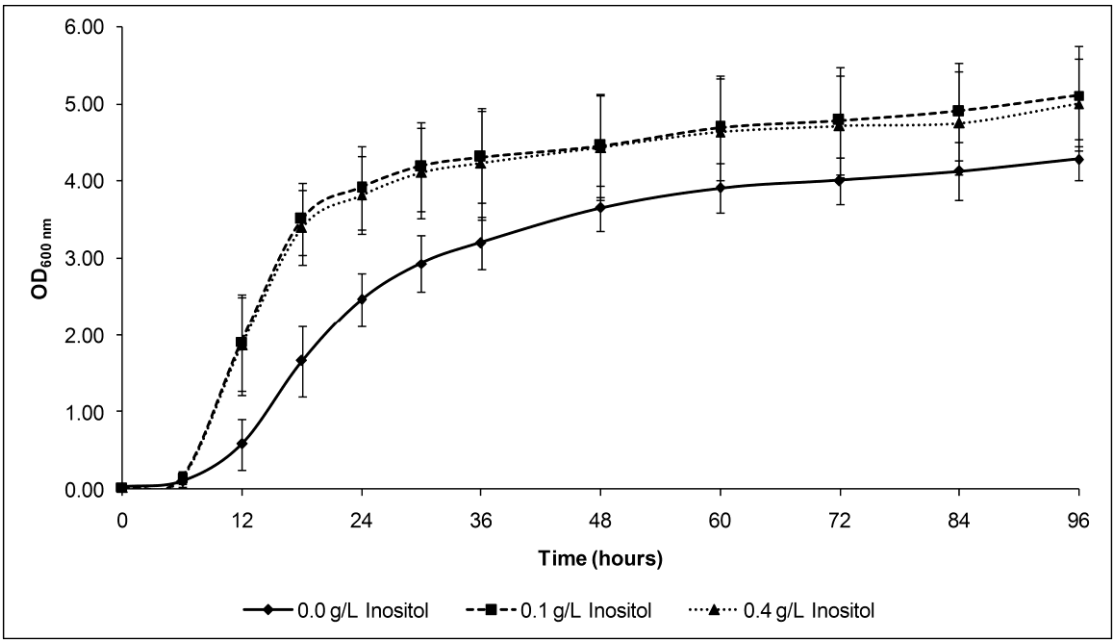


Figure 4.1 Growth curve of yeast strain A15 grown on inositol-free YNB media without supplementation or with supplementation with 0.1 g/L or 0.4 g/L inositol. The initial glucose concentration used in this experiment was 15% (w/v). The data presented are the means of data obtained from two independent experiments and the error bars indicate ± SD.

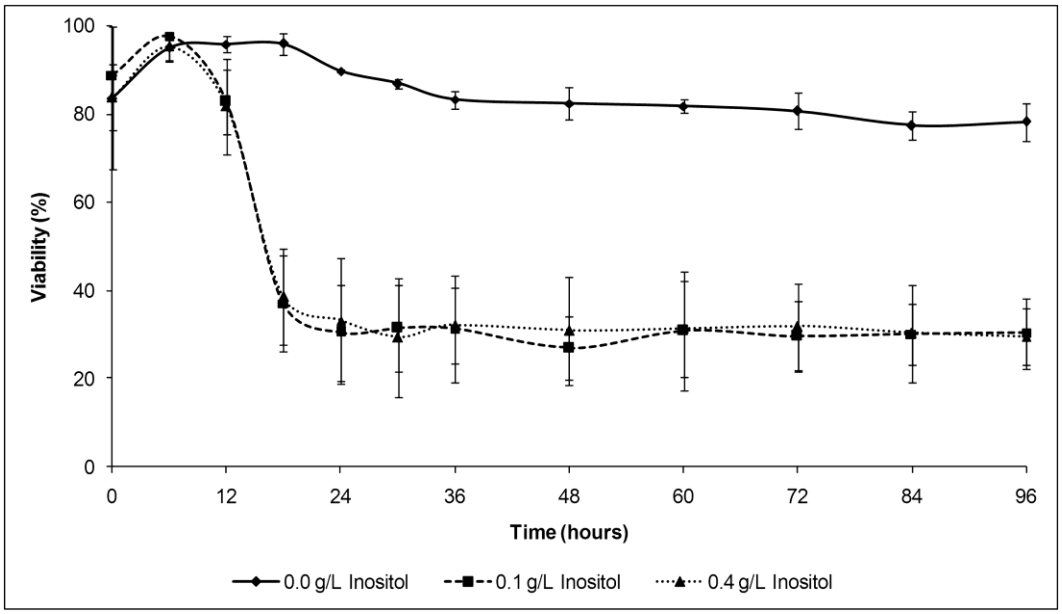


Figure 4.2 The viability of yeast strain A15 grown in inositol-free YNB media without supplementation or with supplementation with 0.1 g/L or 0.4 g/L inositol. The initial glucose concentration used in this experiment was 15% (w/v). The values graphed are the means of data obtained from two independent experiments and the error bars indicate ± SD.

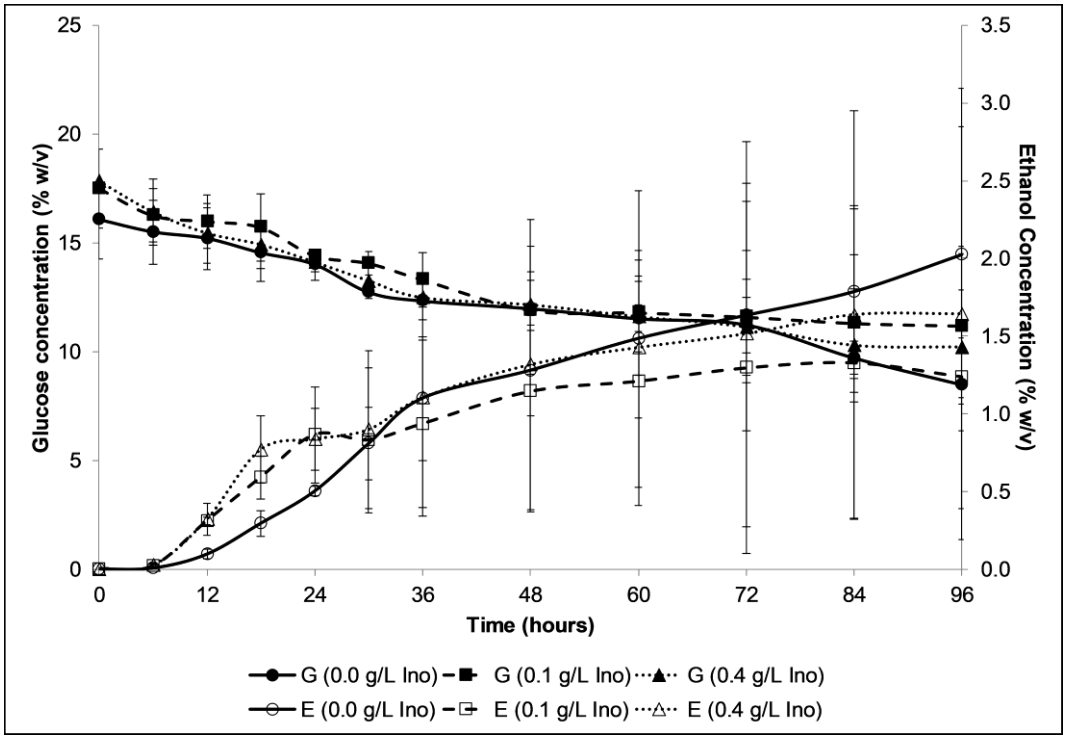


Figure 4.3 The Glucose consumption and ethanol production of yeast strain A15 grown in inositol-free YNB media without supplementation or with supplementation with 0.1 g/L or 0.4 g/L inositol. The letters G and E in the legend indicate glucose and ethanol concentration, respectively. The values graphed are the means of data obtained from two independent experiments and the error bars indicate ± SD.

With respect to glucose consumption and ethanol production, there were no substantial differences between cultures grown with or without inositol supplementation, however inositol-supplemented cultures tended to have glucose consumption rate (Qs), lower ethanol production rate (Qp) and ethanol productivity (Yp/s) (Figure 4.3, Table 4.1). The glucose consumption results showed that when a 15% (w/v) initial glucose was used, only ~60% of the glucose could be consumed by the cells, leading to the conclusion that for further experimentation, a lower initial glucose concentration should be used.

4.2.2 Preliminary Experiment: Membrane fluidity

The fluidity of the yeast cellular membranes was assessed using fluorescence spectroscopy of laurdan-labelled cells to determine the GP parameter. The fluidity was monitored at 24 hours (Figure 4.4) and the effect of inositol supplementation was investigated. It was found that inositol supplementation led to a substantial decrease in GP value, which indicates that when inositol is present in the fermentation media, the cellular membranes are more fluid.

The effect of exposing the cells to 18% v/v ethanol on membrane fluidity was investigated by the addition of absolute ethanol to the cell culture and monitoring the GP value before and after the addition of absolute ethanol. It can clearly be seen in Figure 4.5 that the GP was markedly decreased immediately after the addition of ethanol. As in Chapter 3 (Figure 3.11), this indicates that ethanol fluidizes the membranes. It was also found that the GP values tend to increase over time after the initial decrease. The initial decrease in GP value was less for

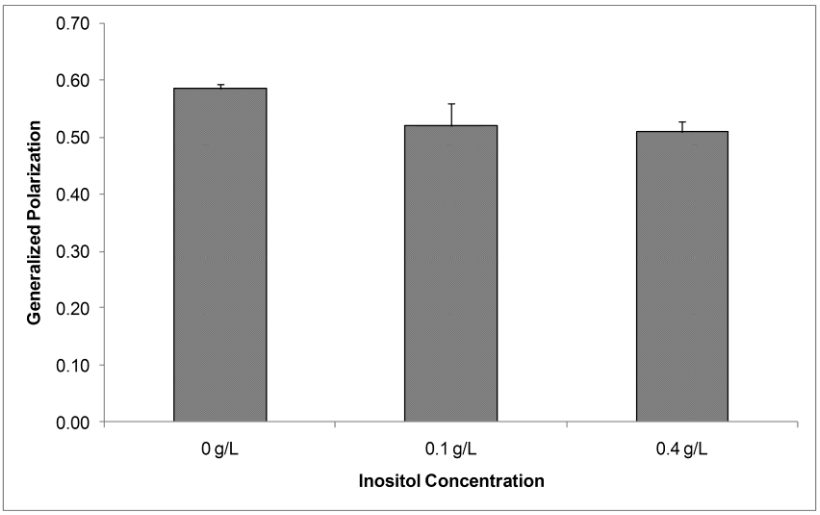


Figure 4.4 Generalized polarization of cellular membranes of cells at 24 h of inositol-free culture of yeast strain A15 grown in YNB medium without supplementation or with supplementation with 0.1 g/L or 0.4 g/L inositol. The initial glucose concentration used in this experiment was 15% (w/v). The data graphed are the means of the data obtained from two independent experiments and the error bars indicate ± SD.

those cells that were grown without inositol supplementation compared to those cells that were grown with inositol supplementation (Table 4.2), although the latter cells tended to recover their membrane fluidity somewhat better after the initial decrease.

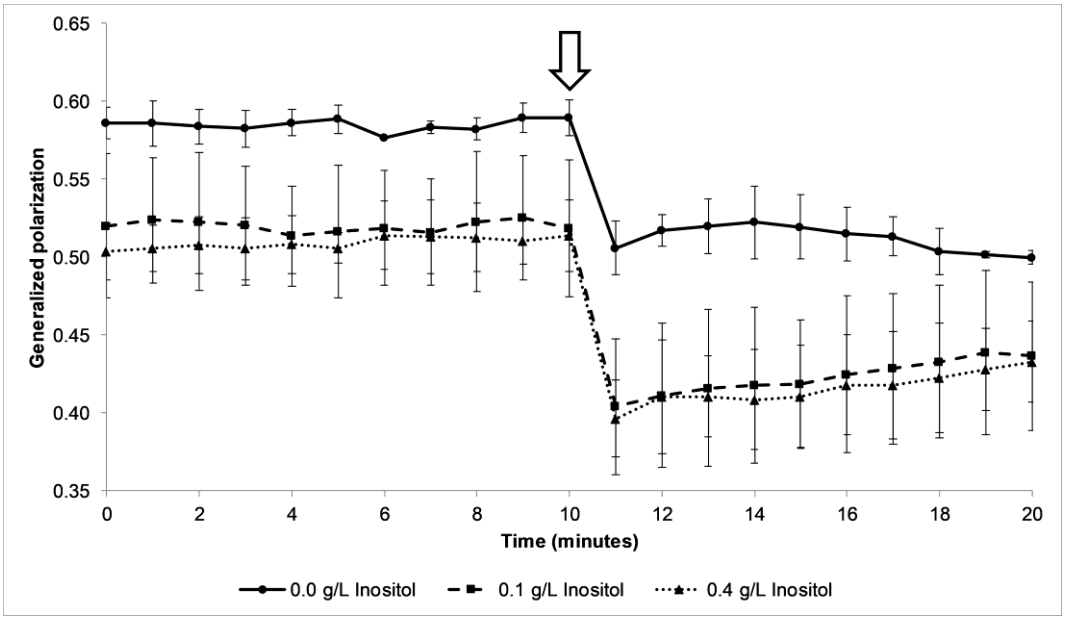


Figure 4.5 Changes in the cellular membrane fluidity of yeast strain A15 as monitored by changes in GP following exposure to 18% (v/v) ethanol. The cells were grown on inositol-free YNB media without supplementation or with supplementation with 0.1 g/L or 0.4 g/L inositol for 24 hours. The arrow indicates addition of absolute ethanol to give an 18% (v/v) final concentration. The values graphed are the means of the data obtained from two independent experiments and the error bars indicate ± SD.

Table 4.2 The initial decrease in generalized polarization of laurdan-labelled cellular membranes after ethanol addition to yeast strain A15 cells grown in inositol-free YNB media without supplementation or with supplementation with 0.1 g/L or 0.4 g/L (C) inositol. The initial glucose concentration used in this experiment was 15% (w/v). The values presented are the means of the experimental data obtained from two independent experiments ± SD.

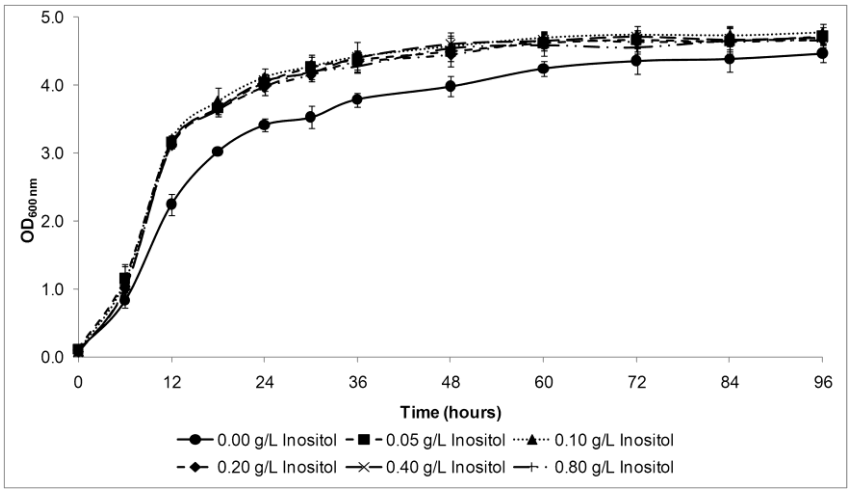
Level of Inositol Supplementation (g/L)	Initial decrease in GP value (%)
0.0	13.53 ± 1.66
0.1	22.37 ± 2.35
0.4	22.20 ± 2.02

4.2.3 Main experiment: cell growth and fermentation performance in medium containing a 10% w/v initial glucose concentration

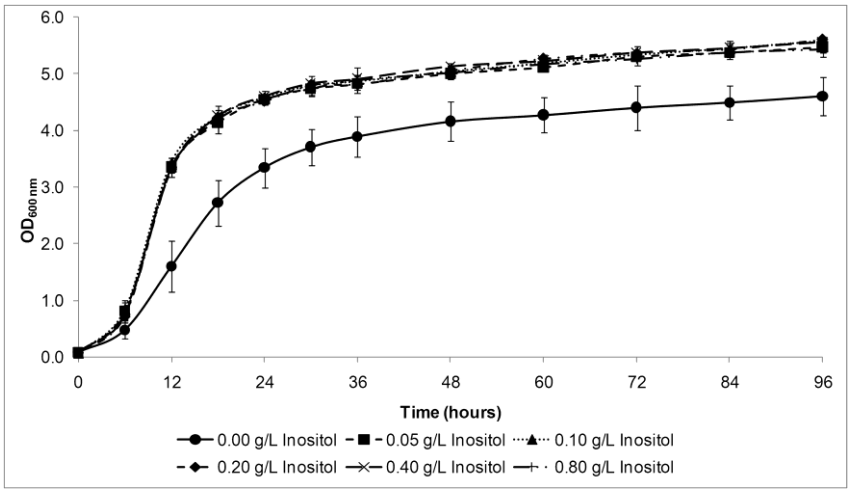
The preliminary experiments in this chapter were performed only in duplicate and therefore the statistical analysis could not be performed. Although one does not normally calculate the standard deviation of only two data points, this was presented in the figures above to give the reader an idea of the variability of the data. For better and more robust comparisons, the main experiments of this chapter were performed in triplicate, allowing statistical analysis. The concentration of glucose used in the main experiment was as described above (10% w/v). All three yeast strains were used in the main experiment so that we could distinguish between strain-dependent and general yeast responses.

The results of the growth measurements as monitored using OD_{600nm} are presented in Figure 4.6. It can be noted that A12 ($p < 0.001$) and A15 ($p < 0.001$) had significantly higher OD_{600nm} values when the growth medium was supplemented with inositol, while K7 did not show any difference in OD_{600nm} values between cells grown in the presence or absence of inositol supplementation ($p = 0.788$). This result indicates that for A12 and A15, but not for K7, inositol supplementation tends to promote cell growth.

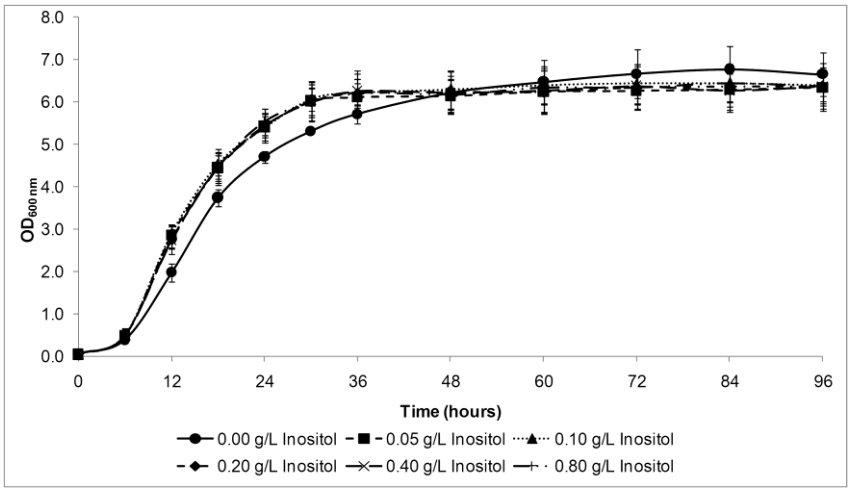
Cell viability was monitored by differential staining of viable and non-viable cells using methylene violet (Section 2.5). The viability results are presented in Figure 4.7. It is apparent that yeast cells grown without inositol supplementation tend to have higher viability throughout the fermentation, except for K7 which shows higher viability without inositol supplementation only after 60 h of fermentation ($p < 0.001$ for all strains).



(A)

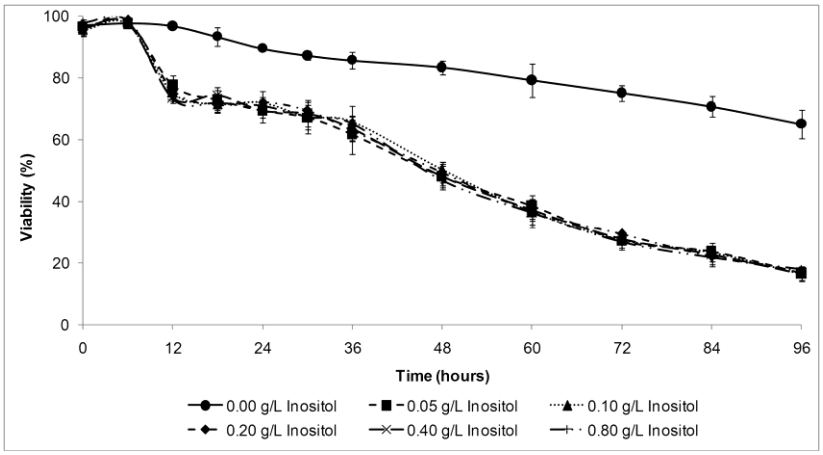


(B)

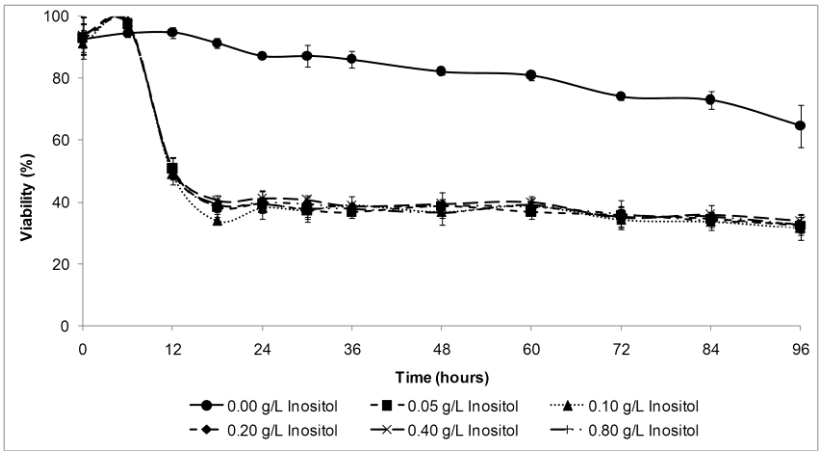


(C)

Figure 4.6 Growth curves of yeast strains (A) A12, (B) A15 and (C) K7 grown in inositol-free YNB media without supplementation or with supplementation with the concentration of inositol indicated in the figure. The initial glucose concentration used in this experiment was 10% (w/v). The values graphed are the means of data obtained from three independent experiments and the error bars indicate ± SD.



(A)



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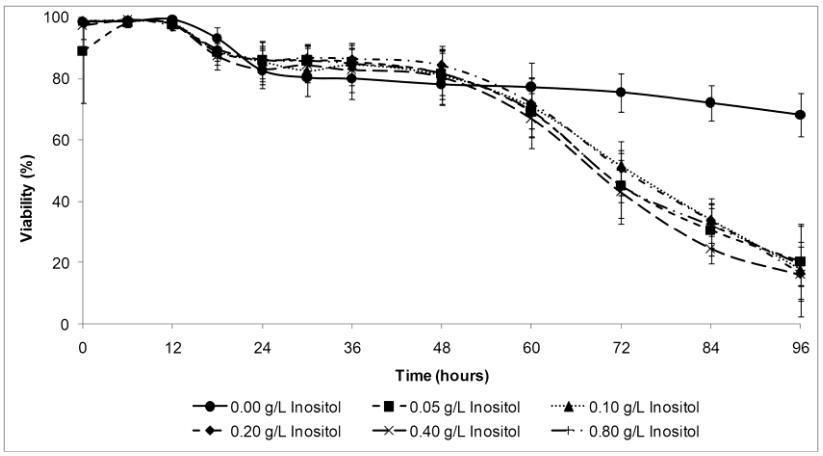
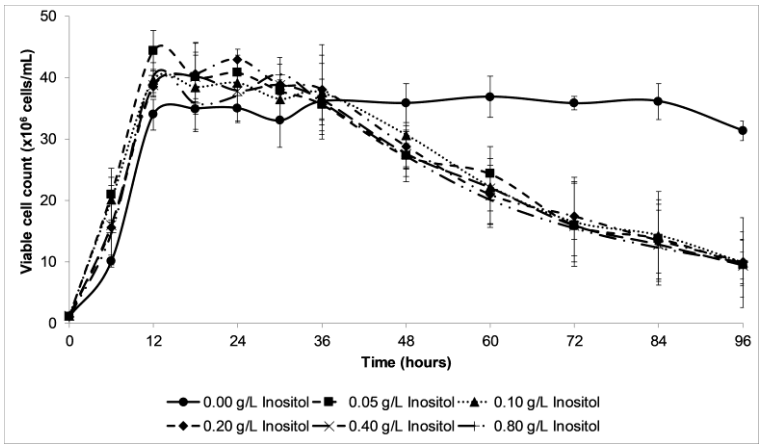


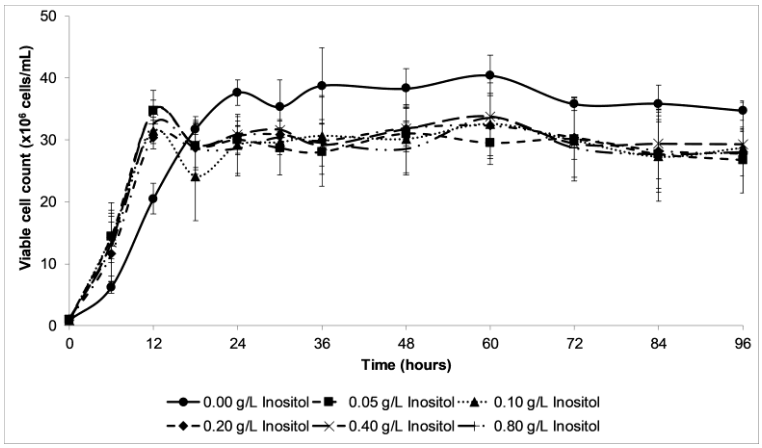
(C)

Figure 4.7 Cell Viability of yeast strains (A) A12, (B) A15 and (C) K7 grown in inositol-free YNB media without supplementation or with supplementation with inositol at the concentrations indicated in the figure. The initial glucose concentration used in this experiment was 10% (w/v). The value graphed are the means of data obtained from three independent experiments and the error bars indicate ± SD.

In relation to cell viability, viable cell counts were also monitored in order to gain a better understanding of the potential of the various yeast strains for fermentation, i.e. whether higher viability but lower total cell counts results in to similar viable cell counts as described in Chapter 3. The results of the viable cell count determination are presented in Figure 4.8. Significant differences in viable cell counts were observed between all strains tested in the experiment (A12, $p < 0.001$; A15, $p = 0.008$; K7, $p < 0.001$). Even though the cell viabilities of A12 and A15 were higher in inositol unsupplemented media throughout the fermentation experiment, interestingly the time points at which the differences between inositol-supplemented and -unsupplemented media became statistically significant were different. For the first 48 h, the viable cell counts for yeast strain A12 showed no statistically significant differences between inositol supplementation and no supplementation treatments, however after 60 h the A12 culture grown in medium without inositol supplementation had significantly higher viable cell counts. For yeast strain A15, there were no statistically significant differences at most time points between cells grown in the presence or absence of inositol supplementation. A low *p*-value was obtained since at 12 h, the culture grown in inositol unsupplemented media had a significantly lower number of viable cells. Statistically significant differences between inositol supplemented and unsupplemented media for yeast strain K7 were detected after 72 h, while prior to that time point, no statistically significant differences in viable cell counts between these conditions were detected.



(A)



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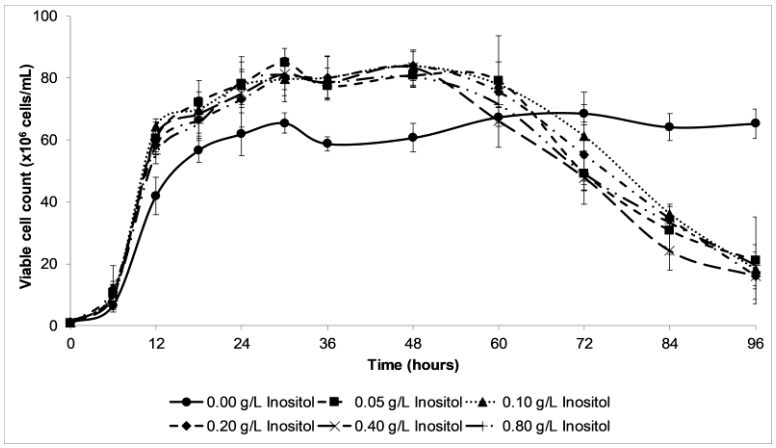


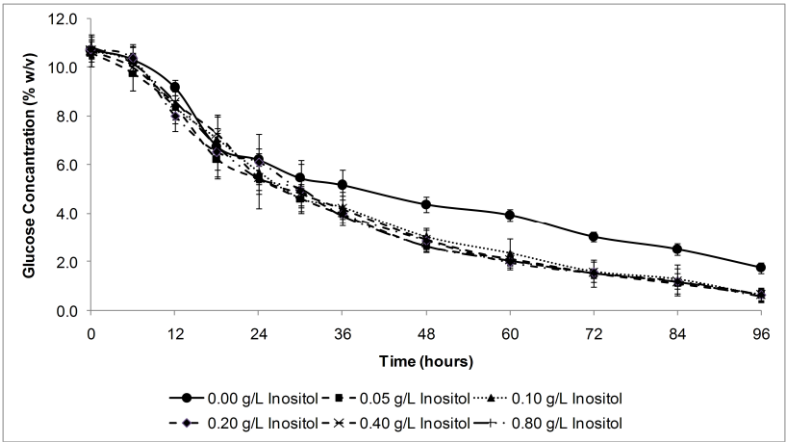
(C)

Figure 4.8 Viable cell counts of yeast strain (A) A12, (B) A15 and (C) K7 grown in inositol-free YNB media without supplementation or with supplementation with inositol at the concentrations indicated in the figure. The initial glucose concentration used in this experiment was 10% (w/v). The value graphed are the means of data obtained from three independent experiments and the error bars indicate ± SD.

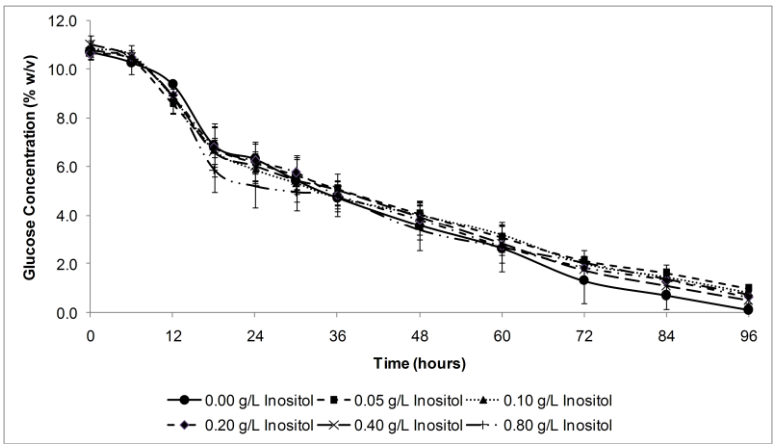
The glucose consumption by yeast strains A12 and K7 increased significantly when the fermentation medium was supplemented with inositol as shown in Figure 4.9(A) and (C). In contrast, the glucose consumption by A15 seemed to not be affected by supplementation of the medium with inositol (Figure 4.9(B)). This result is in agreement with the ethanol production by the three yeast strains used in the present study. Ethanol production by A15 seems to not have been affected by supplementation of the medium with inositol, while A12 and K7 showed an increase in ethanol production when the fermentation medium was supplemented with inositol (Figure 4.10).

In terms of fermentation kinetic parameters, the maximum growth rates of yeast cells grown in unsupplemented media were generally lower than those of cells grown in inositol-supplemented media (Tables 4.3, 4.4. and 4.5). For all strains, significantly higher maximum growth rates were observed for 0.10-0.20 g/L inositol supplementation than control unsupplemented cultures. When inositol was supplemented at higher concentrations, each strain had a a unique pattern of responses. A12 tended to have a higher growth rate at 0.40 to 0.80 g/L inositol supplementation, A15 maintained relatively similar growth rates from 0.05 up to 0.80 g/L inositol supplementation, while for K7 supplementation of inositol exceeding 0.20 g/L tended to decrease the growth rate.

As shown in Tables 4.3, 4.4 and 4.5, the substrate consumption rate (p = 0.017) and ethanol productivity (p = 0.011) of A12 grown in inositol unsupplemented media tended to have lower values compared to the same cells grown in inositol-supplemented media. For K7, ethanol productivity was significantly different between cells grown in medium with or without inositol supplementation (p = 0.003); cells grown with inositol supplementation exhibited significantly higher ethanol productivity. However, A15 did not exhibit any statistically significant differences for substrate consumption rate or ethanol



(A)



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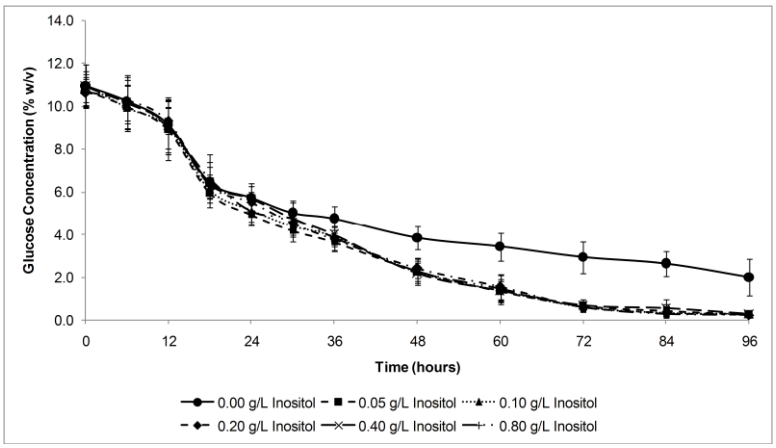
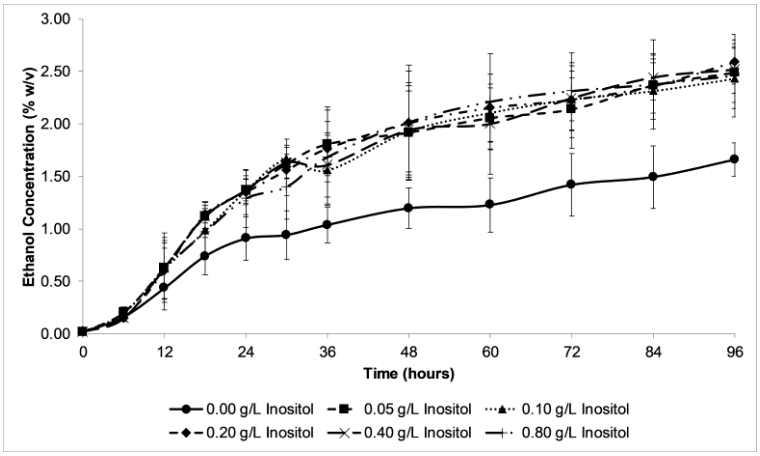
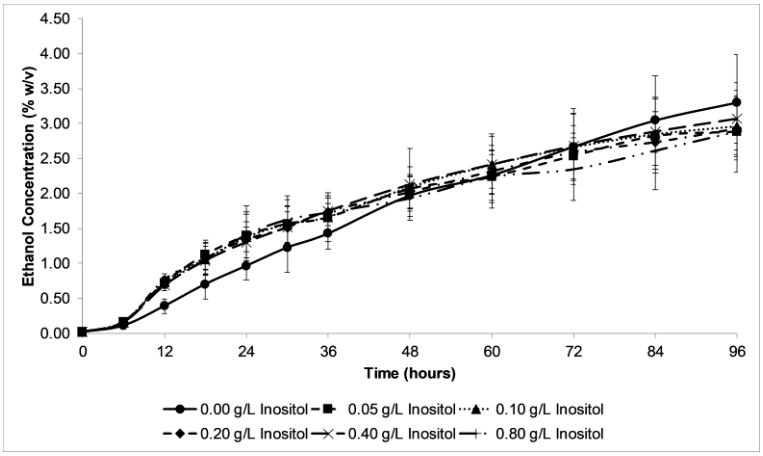


(C)

Figure 4.9 Glucose consumption by yeast strain (A) A12, (B) A15 and (C) K7 grown in inositol-free YNB media without supplementation or with supplementation with inositol at the concentrations indicated in the figure. The initial glucose concentration used in this experiment was 10% (w/v). The values graphed are the means of data obtained from three independent experiments and the error bars indicate ± SD.



(A)



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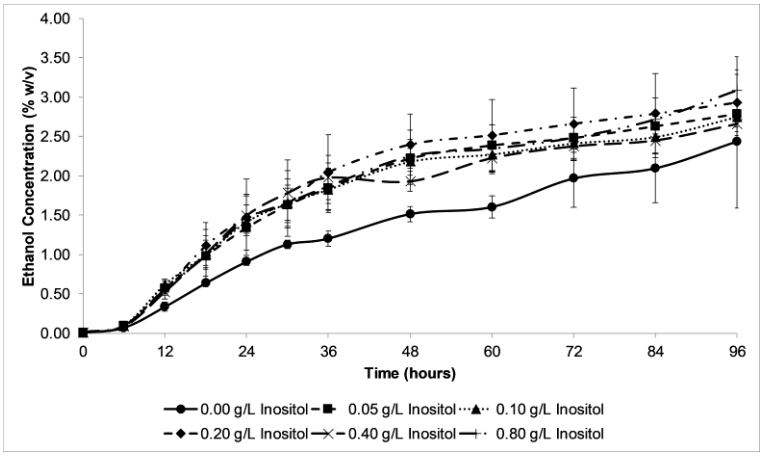


(C)

Figure 4.10 Ethanol production by yeast strain (A) A12, (B) A15 and (C) K7 grown in inositol-free YNB media without supplementation or with supplementation with inositol at the concentrations indicated in the figure. The initial glucose concentration used in this experiment was 10% (w/v). The values graphed are the means of data obtained from three independent experiments and the error bars indicate ± SD.

productivity between cells grown in inositol-supplemented or unsupplemented media. When cells were grown in media with or without inositol supplementation no statistically significant differences were observed in terms of the ethanol yield (*Yp/s*) for any strain used in the present study ($p > 0.05$).

Table 4.3 Kinetic parameters of yeast strain A12 grown in inositol-free YNB media without or with inositol supplementation. The values presented are the means of three independent experiments ± SD. The means in the same column with different superscript letters are significantly different at α = 0.05 as assessed using Tukey's post-hoc test.

Level of Inositol Supplementation (g/L)	μ_{max} (h^{-1}) p=0.001	*Qs* ($mg.mL^{-1}.h^{-1}$) p=0.017	*Qp* ($mg.mL^{-1}.h^{-1}$) p=0.011	*Yp/s* ($mg.mg^{-1}$) p=0.082
0.00	0.260±0.006 ^{c}	0.934±0.016 ^{b}	0.171±0.017 ^{b}	0.183±0.021 ^{a}
0.05	0.279±0.014 ^{bc}	1.035±0.022 ^{ab}	0.257±0.029 ^{a}	0.248±0.028 ^{a}
0.10	0.299±0.028 ^{ab}	1.047±0.060 ^{a}	0.251±0.030 ^{a}	0.240±0.023 ^{a}
0.20	0.311±0.010 ^{ab}	1.050±0.019 ^{a}	0.268±0.021 ^{a}	0.255±0.016 ^{a}
0.40	0.321±0.006 ^{a}	1.047±0.046 ^{a}	0.260±0.023 ^{a}	0.249±0.022 ^{a}
0.80	0.324±0.005 ^{a}	1.067±0.053 ^{a}	0.254±0.041 ^{a}	0.240±0.050 ^{a}

Note: μ_{max} = maximum growth rate, *Qs* = glucose consumption rate, *Qp* = ethanol productivity, *Yp/s* = ethanol yield

Table 4.4 Kinetic parameters of yeast strain A15 grown in inositol-free YNB media without or with inositol supplementation. The values presented are the means of three independent experiments ± SD. The means in the same column with different superscript letters are significantly different at α = 0.05 as assessed using Tukey's post-hoc test.

Level of Inositol Supplementation (g/L)	μ_{max} (h^{-1}) p=0.014	*Qs* ($mg.mL^{-1}.h^{-1}$) p=0.116	*Qp* ($mg.mL^{-1}.h^{-1}$) p=0.891	*Yp/s* ($mg.mg^{-1}$) p=0.760
0.00	0.247±0.014 ^{b}	1.106±0.023 ^{a}	0.342±0.071 ^{a}	0.302±0.055 ^{a}
0.05	0.320±0.022 ^{a}	1.008±0.049 ^{a}	0.299±0.017 ^{a}	0.305±0.033 ^{a}
0.10	0.323±0.021 ^{a}	1.045±0.038 ^{a}	0.307±0.045 ^{a}	0.271±0.034 ^{a}
0.20	0.321±0.022 ^{a}	1.046±0.032 ^{a}	0.303±0.046 ^{a}	0.283±0.042 ^{a}
0.40	0.310±0.027 ^{ab}	1.097±0.018 ^{a}	0.318±0.053 ^{a}	0.271±0.058 ^{a}
0.80	0.319±0.033 ^{a}	1.047±0.071 ^{a}	0.299±0.060 ^{a}	0.262±0.027 ^{a}

Note: μ_{max} = maximum growth rate, *Qs* = glucose consumption rate, *Qp* = ethanol productivity, *Yp/s* = ethanol yield

Table 4.5 Kinetic parameters of yeast strain K7 grown in inositol-free YNB media without or with inositol supplementation. The values presented are the means of three independent experiments ± SD. The means in the same column with different superscript letters are significantly different at α = 0.05 as assessed using Tukey's post-hoc test.

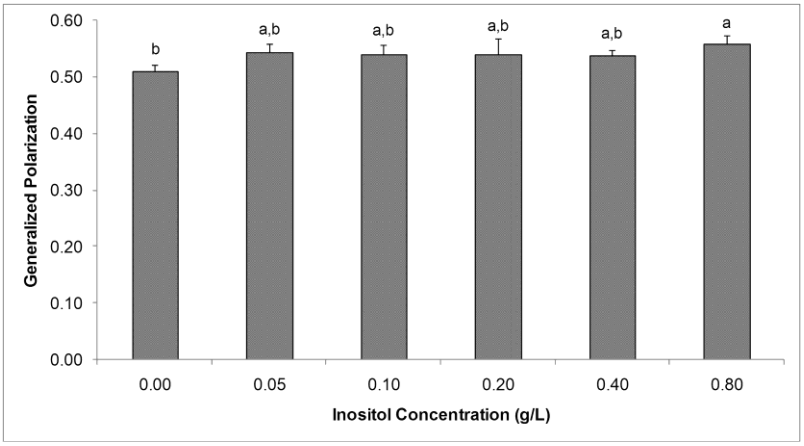
Level of Inositol Supplementation (g/L)	μ_{max} (h^{-1}) p=0.002	*Qs* ($mg.mL^{-1}.h^{-1}$) p=0.272	*Qp* ($mg.mL^{-1}.h^{-1}$) p=0.003	*Yp/s* ($mg.mg^{-1}$) p=0.172
0.00	0.291±0.016 ^{b}	1.021±0.102 ^{a}	0.200±0.004 ^{b}	0.229±0.038 ^{a}
0.05	0.342±0.008 ^{a}	1.091±0.086 ^{a}	0.319±0.033 ^{a}	0.329±0.072 ^{a}
0.10	0.338±0.015 ^{a}	1.163±0.063 ^{a}	0.316±0.034 ^{a}	0.293±0.023 ^{a}
0.20	0.334±0.002 ^{a}	1.112±0.025 ^{a}	0.338±0.044 ^{a}	0.316±0.053 ^{a}
0.40	0.316±0.003 ^{ab}	1.124±0.062 ^{a}	0.300±0.048 ^{a}	0.281±0.027 ^{a}
0.80	0.315±0.016 ^{ab}	1.115±0.034 ^{a}	0.338±0.018 ^{a}	0.316±0.045 ^{a}

Note: μ_{max} = maximum growth rate, *Qs* = glucose consumption rate, *Qp* = ethanol productivity, *Yp/s* = ethanol yield

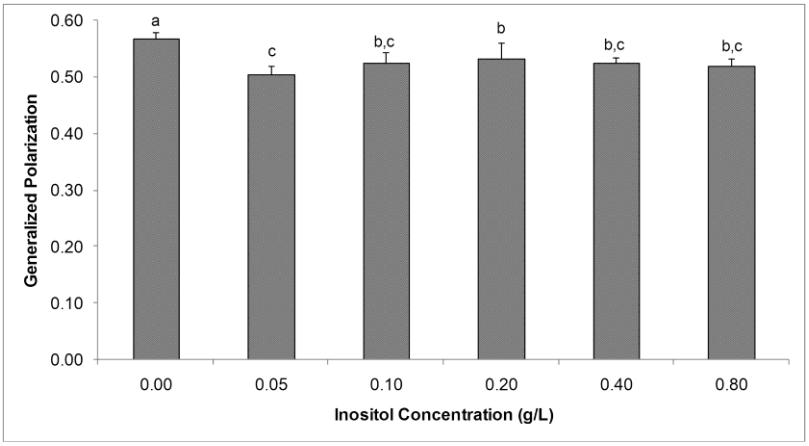
4.2.4 Main Experiment: Membrane Fluidity

As previously described, membrane fluidity was monitored by measuring the GP of laurdan (Figure 4.11). Each strain had different trends for the effect of inositol supplementation on membrane fluidity. When the medium was supplemented with inositol, the cellular membranes of the yeast strains A12 and K7 had significantly higher GP values ($p < 0.05$), except for the case of A12 supplemented with 0.05 g/L inositol where the GP was not significantly different to that of the same cells without inositol supplementation ($p = 0.074$). These results indicate that inositol supplementation leads to a decrease cellular membrane fluidity of the A12 and K7 strains. However, in contrast, in the case of yeast strain A15 the GP value of the cellular membranes at all levels of inositol supplementation of the medium was significantly lower than that of cellular membranes of the same cells grown in unsupplemented media, indicating a higher fluidity of membranes of A15 when grown in medium with inositol supplementation.

Further investigation of the effect of inositol supplementation of the medium on cellular membrane fluidity after ethanol exposure was performed by measuring generalized polarization after addition of absolute ethanol to the media to give an 18% (v/v) final concentration (Figure 4.12). The initial decreases in GP values are presented in Table 4.6. A rapid decrease in generalized polarization value when the cells were exposed to 18% (v/v) ethanol was detected for all strains tested. However, each stain



(A)



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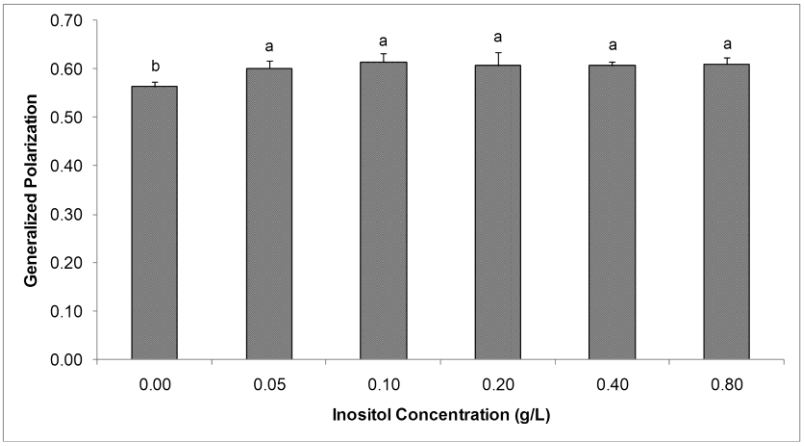
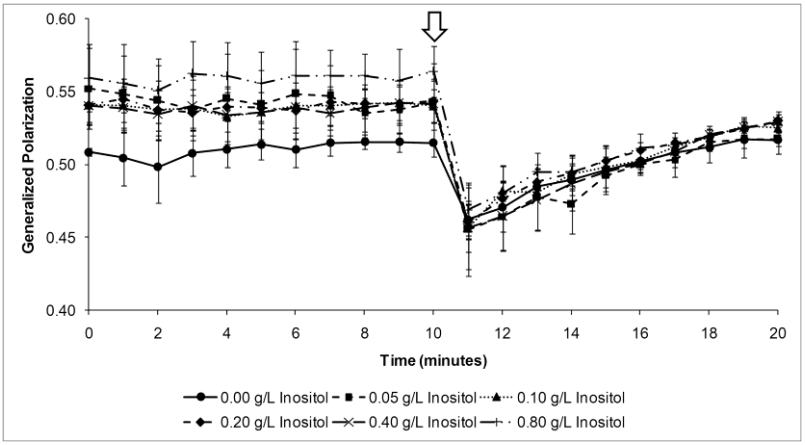
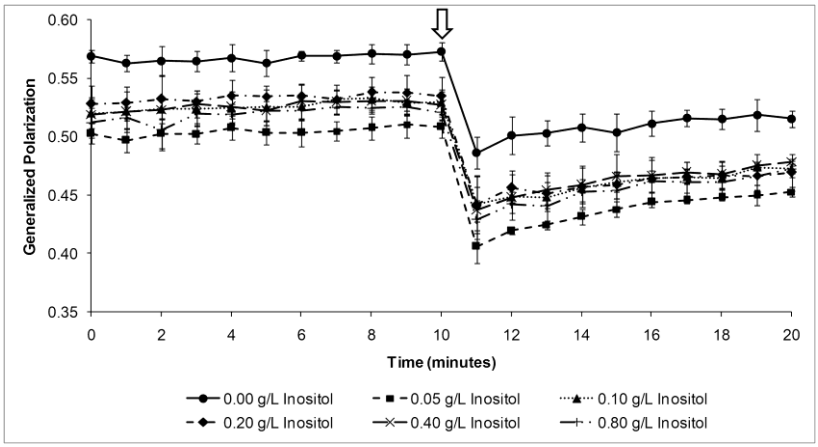


(C)

Figure 4.11 The generalized polarization of laurdan-labelled cellular membranes of yeast strains (A) A12, (B) A15 and (C) K7 grown in inositol-free YNB media without supplementation and with supplementation with inositol at the concentrations indicated in the figure. The initial glucose concentration used in this experiment was 10% (w/v). The values graphed are the means of data obtained from three independent experiments and the error bars indicate ± SD. Bars with different letters exhibit differences that are statistically significant at $\alpha = 0.05$ as assessed using Tukey's post-hoc test.



(A)



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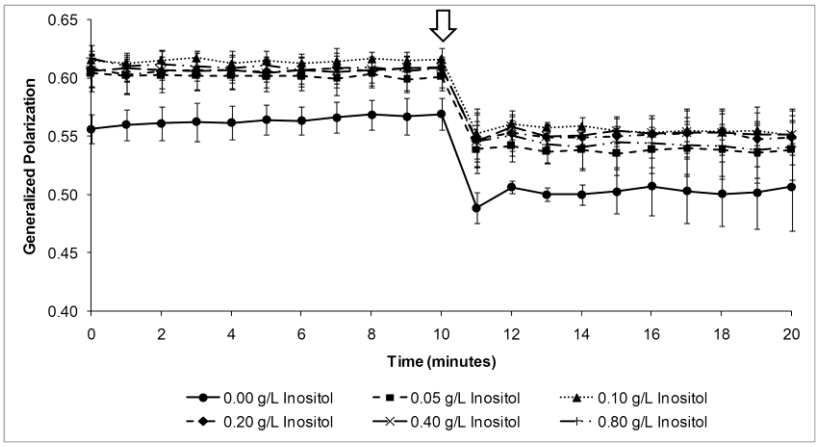


(C)

Figure 4.12 The changes in GP values of cellular membranes of yeast strains (A) A12, (B) A15 and (C) K7 grown in inositol-free YNB media without supplementation and with supplementation with inositol at the concentrations indicated in the figure. The initial glucose concentration used in this experiment was 10% (w/v). Arrows indicate the time of addition of absolute ethanol to give a final concentration in the medium of 18% v/v. The values graphed are the means of data obtained from three independent experiments and the error bars indicate ± SD.

Table 4.6 The initial decrease in cellular membrane generalized polarization after ethanol addition to yeast cells grown in inositol-free YNB media without supplementation and with supplementation with 0.05, 0.10, 0.20, 0.4 and 0.8 g/L inositol. The initial glucose concentration used in this experiment was 10% (w/v). The values presented are the means of data obtained from three independent experiments ± SD. The means in the same column followed by different superscript letters exhibit differences that are statistically significant at α=0.05 as assessed using Tukey's post-hoc test.

Level of Inositol Supplementation (g/L)	Percentage initial decrease in GP value after ethanol addition (%)		
	A12 (p = 0.005)	A15 (p = 0.416)	K7 (p = 0.538)
0.00	10.18±1.67[b]	15.15±1.68[a]	14.05±3.39[a]
0.05	16.07±1.90[a]	20.11±1.37[a]	10.34±2.27[a]
0.10	15.51±1.55[a]	16.45±2.95[a]	10.29±3.15[a]
0.20	15.23±0.27[a]	17.38±2.64[a]	10.08±2.83[a]
0.40	15.78±2.70[a]	17.17±4.09[a]	10.33±3.21[a]
0.80	16.87±0.79[a]	17.60±2.53[a]	10.38±2.30[a]

showed different trends. As previously mentioned, during the 10 minutes before ethanol exposure, similar trends in the GP values were observed. Yeast strain A12 and K7 tended to have cellular membranes with higher generalized polarization values when grown in media with inositol supplementation, while yeast strain A15 showed the opposite result, where the cellular membranes of cells grown in media with inositol supplementation had lower GP values. After ethanol exposure, no difference in GP value was observed for the cellular membranes of strain A12 grown with or without inositol supplementation. However, yeast strain A15 and K7 exhibited similar trends as before exposure to ethanol, in that the cellular membranes of yeast strain A15 tended to have higher GP values without inositol supplementation, while those of yeast strain K7 tended to have lower GP values when grown in medium without inositol supplementation. The initial decrease in GP value as presented in Table 4.6 shows that only in the case of yeast strain A12 were significant differences observed between the GP values of cellular membranes of cells grown in media with and without inositol supplementation. The cellular membranes of strains A15 and K7 showed no significant

difference in the magnitude of the decrease in GP value between cells grown in media with and without inositol supplementation.

4.3 Discussion

The results of the preliminary study using the A15 strain showed that inositol supplementation of the medium improves cell growth and biomass accumulation as indicated by the higher OD_{600nm} values achieved by yeast cells grown in media supplemented with inositol (Figure 4.1). This result confirms previous studies which suggested that inositol supplementation of the medium stimulates yeast growth (Becker & Lester 1977; Krause *et al.* 2007). The specific maximum growth rate achieved in the respiro-fermentative growth phase was higher for yeast grown in media with inositol supplementation than for the same yeast grown in media without inositol supplementation (Table 4.1). Interestingly, even though the specific maximum growth rates achieved were higher, the cell viability was actually substantially lower when the cells were grown in media supplemented with inositol (Figure 4.2). This offers a likely explanation for the observation that cultures grown on media with and without inositol supplementation had similar viable cell counts and thus likely had similar numbers of fermentation-active cells. This finding is in contrast to the results of previous published studies which indicated that besides improving cell growth, inositol supplementation also promotes high cell viability during the phase of active cell growth (Hanson & Lester 1980; Lewin 1965; Ridgway & Douglas 1958).

Further investigation in the main experiment confirmed the finding that inositol supplementation promoted growth of A12 and A15. However, K7 only showed a significant increase in cell growth rate during the respiro-fermentative growth phase (i.e. up to 12 hours), with no significant improvement in the growth rate of culture after that time. This result indicates that the activity of inositol in promotion

of growth is strain-dependent, with the growth rate of some strains of *S. cerevisiae* not greatly affected. It would seem that lack of a growth response to inositol is rare, since most previous studies reported that inositol supplementation of fermentation media tended to promote yeast cell growth (Almaguer *et al.* 2003; Krause *et al.* 2007; Yao, Chi & He 2006).

The maximum growth rate of yeast strain A12 and A15 when grown in medium without inositol supplementation was substantially lower compared to the same cells when grown in medium with inositol supplementation (Tables 4.3 and 4.4, respectively), which is in agreement with the cell density data. Yeast strains A12 and A15 showed similar trends as have been previously published (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Krause *et al.* 2007) which confirms that inositol, besides increasing growth rate, also increases the final cell density. However, it was found that even though the cell density of yeast strain K7 for most of the growth curve did not seem to be affected by inositol supplementation, the main experiment results presented in Table 4.5 indicate that the maximum growth rate of K7 was significantly higher in medium with inositol supplementation. This result indicates that each strain responds differently to inositol supplementation.

As shown in Figure 4.7, the cell viability of each of the three yeast strains used in the present study was significantly lower when the fermentation media were supplemented with inositol. Interestingly, while yeast strain A12 and A15 maintained a significantly lower percentage cell viability throughout the fermentation in inositol-supplemented media compared to unsupplemented media, yeast strain K7 grown in medium without inositol supplementation only showed higher cell viability than the same cells grown in inositol-supplemented media only after 60 hours of fermentation. This seems to indicate that while inositol does not seem to promote growth of K7, it preserves viability up to 60 hours.

When viable cell counts are considered, each yeast strain was observed to show a different pattern. The viable cell counts of yeast strain A12 were similar for cells grown in media with or without inositol supplementation during the lag, respiro-fermentative and early diauxic lag phases as shown in Figure 4.8(A). After 48 hours, the cells grown without inositol supplementation tended to have higher viable cell counts. For yeast strain A15, no significant differences in viable cell count were observed. Interestingly, for yeast strain K7, during the initial stage of fermentation the viable cell count was lower for cultures grown in media without inositol supplementation than culture grown in media with inositol supplementation, but after 72 hours the viable cell counts were significantly higher than those for culture grown in media with inositol supplementation due to the viable cell count of the later cultures declining rapidly. This indicates, yet again that the effects of inositol on cell growth and cell viability are strain-specific, since of the three strains used in the present study no two strains exhibited the exact same pattern of responses to inositol supplementation of the medium.

Glucose consumption by yeast strains A12 and K7 was significantly increased by the presence of inositol in the fermentation media, while glucose consumption by yeast strain A15 was not affected. However, even for yeast strain A12 and K7 no differences were detected in glucose consumption between the different supplementation levels of inositol trialled in the present study. It is most likely that inositol supplementation of the medium can improve the glucose consumption of some yeast strains but not others. Furthermore, no additive effects of inositol were found when the concentration of inositol in the fermentation medium increased, which indicates that inositol is only required in relatively small amounts. The results of the glucose consumption analysis are consistent with the results of the ethanol production analysis, in which inositol supplementation of the medium significantly increased ethanol production, especially for yeast strain A12 which showed

significantly higher ethanol production compared to the same cells grown without supplementation until the last time point (96 hours). K7 also showed a significantly higher ethanol production when grown in media supplemented with inositol until the time point 60 hour, but the difference from the same cells grown on unsupplemented media was not significant after that time point. Similar to the glucose consumption data, yeast strain A15 did not show any difference in ethanol production with or without inositol supplementation of the media. An improvement in ethanol production when the medium was supplemented with inositol was also observed by Chi, Kohlwein & Paltauf (1999) and Nikolić *et al.* (2009a) who found that inositol supplementation of the growth medium improved ethanol production by *S. cerevisiae*. However, they only used one strain in their experiment and therefore could not compare the effect of inositol supplementation on different strains. Other than that, the authors did not provide glucose consumption data for their experiment. The present study indicates that different strains may respond differently when inositol is added to the fermentation media in term of ethanol production and glucose consumption.

The conditions under which the present experiments were performed were not exactly the same as in previously published studies. Those studies used lower inositol concentrations (0.01-0.04 g/L) and the glucose concentration also varied from 0.2% (w/v) (Ridgway & Douglas 1958), to 3% (w/v) (Hanson & Lester 1980) and to 20% (w/v) (Lewin 1965). Therefore, it is difficult to directly compare the present results with those of previous studies. Furthermore, while increasing the maximum growth rate, inositol supplementation led to lower ethanol yield (Yp/s) values in the preliminary experiment conducted with the A15 strain, seeming to promote greater biomass accumulation but not greater ethanol productivity at least under the conditions used in the preliminary experiment. However, statistical analysis of main the experiment revealed no significant differences in ethanol yield

between cells grown on unsupplemented of inositol-supplemented media for any of the three strains. Even though the differences in Yp/s values were not statistically significant, the ethanol productivity (Qp) values of yeast strain A12 and K7 were higher when the cells were grown in inositol-supplemented media than in unsupplemented media. In contrast, yeast strain A15 showed no statistically significant differences in Qp values when grown in inositol-supplemented and unsupplemented media. This result was is consistent with the viable cell counts in which yeast strain A12 and K7 showed higher viable cell counts during the late phase of the fermentation, which would be expected to lead to more actively fermenting cells and therefore potentially higher ethanol production by these two strains.

The findings of the main experiment described in this chapter are in agreement with the finding of previous studies, which led to conclusion that besides improving cell growth, inositol also improves fermentation performance (Caridi 2002; Chi, Kohlwein & Paltauf 1999; Nikolić *et al.* 2009a). Only the yeast strain A15 showed no difference in kinetic parameters, other than maximum growth rate (μ_{max}),when grown in media with or without inositol supplementation in the media in which the yeast were cultured for the experiments conducted in this study were different from the media used to culture the yeast in the previously published reports. Caridi (2002) used wine media with 40% (w/v) initial sugar, Nikolić *et al.* (2009a) used immobilized yeast cells with starch hydrolysate while Chi, Kohlwein & Paltauf (1999) used synthetic medium with 20% (w/v) sucrose. The present study used synthetic medium with a relatively high initial glucose concentration [15% (w/v) for the preliminary experiment and 10% (w/v) for the main experiment]. As previously mentioned, nutritional components of the fermentation media are very important factors in determining fermentation performance. Nutritional components other than inositol, in both previous studies and the present study, may also affect the

fermentation performance. Caridi (2002) and Nikolić *et al*. (2009a) used complex media, which had rich nutrition, while Chi, Kohlwein & Paltauf (1999) and the present study used defined synthetic media which are considered to provide relatively poor nutrition. Even though Chi, Kohlwein & Paltauf (1999) and the present study used similar media, different sugars were used (15 and 10% (w/v) glucose in this study, 20% (w/v) sucrose by Chi, Kohlwein & Paltauf (1999). These results further confirm our finding for other parameters in the present study that inositol effects on cell growth and fermentation performance are strain-specific.

The preliminary experiment using yeast strain A15 indicated that there were no substantial differences in ethanol productivity (Qp) for cells grown in media with or without inositol supplementation. The results of the main experiment confirmed the lack of effect of inositol supplementation of the medium on the ethanol productivity of yeast strain A15. However, for yeast strains A12 and K7 their ethanol productivity during fermentation was higher when grown in media with inositol supplementation. No precise level of inositol supplementation that provides positive effects on fermentation performance has been published. Chi, Kohlwein & Paltauf (1999) used 0 and 0.1 g/L inositol in their experiment while Furukawa *et al*. (2004) used much lower concentrations (10 or 90 μM with no zero level). Ji *et al*. (2008), who used a different species of yeast and supplementation with 0, 0.01, 0.05, 0.10, 0.15 and 0.20 g/L inositol, found that inositol supplementation of more than 0.1 g/L led to lowered ethanol productivity. In the present study, using inositol supplementation levels of 0, 0.05, 0.1, 0.2, 0.4 and 0.8 g/L, it would seem that for the strains studied, there are clear differences between zero and 0.05 g/L inositol, with little differences seen between 0.05 and 0.8 g/L inositol. Thus, we suggest that 0.05 g/L is sufficient to have effects and that these effects are relatively unchanged at up to 0.8 g/L inositol, with no evidence of “excess inositol” causing negative effects.

While direct measurement of the fluidity of the yeast membrane using generalized polarization of laurdan labelled membranes is believed to provide a more reliable indicator of membrane fluidity than determination of unsaturation index values, many studies continue to estimate fluidity simply by lipid content. To get a true measure of the fluidity, which can be affected by many factors in addition to lipid unsaturation, in the present study we measured membrane fluidity directly. It was found in the preliminary and main experiments described in this chapter that for yeast strain A15, inositol supplementation of the growth medium tended to decrease the generalized polarization values which led us to conclude that for yeast strain A15 inositol supplementation of the medium stimulated an increase in the fluidity of the cellular membranes. In contrast, the generalized polarization values of yeast strain A12 and K7 grown in inositol-supplemented media were higher, which indicated lower membrane fluidity (i.e. the membranes became more rigid). It is most likely that inositol supplementation increases the proportion of phosphatidylinositol in the membranes (Chi, Kohlwein & Paltauf 1999) and that this then leads to further changes in the phospholipid composition of the membranes. This may also lead to an increase in the unsaturation index of the membranes (Jurešić, Blagović & Rupčić 2009), which in turn would be expected to increase their fluidity. Follow up experiments to investigate possible changes in the fatty acid composition of the cellular membranes will be described in Chapter five.

To investigate the effect of inositol supplementation on the cellular membranes fluidity changes that occur when yeast is exposed to a high ethanol concentration, generalized polarization values of laurdan labelled yeast cellular membranes before and after exposure of the cells to 18% (v/v) ethanol was monitored. The results of the preliminary experiment using A15 (Figure 4.5) indicated that prior to ethanol addition yeast grown without inositol supplementation tended to maintain lower membrane fluidity as indicated by higher GP values. However, after ethanol

addition, the increase in fluidity of the cellular membranes of cells in the unsupplemented cultures was less than that of cells in the inositol-supplemented cultures. Indeed, the GP values of the membranes of cells in the unsupplemented cultures after exposure to ethanol were similar to those of the cells in the inositol-supplemented cultures prior to ethanol exposure. As presented in Table 4.2, the initial proportional decrease in GP values was much greater for cells grown in media with inositol supplementation, but the recovery of GP in these cells was much more rapid than that observed for cells grown in medium without inositol supplementation as shown in Figure 4.5.

In the main experiment no statistically significant differences in the magnitude of the decrease in GP value were observed for growth in media with or without inositol supplementation for yeast strains A15 and K7 when the cells were exposed to 18% (v/v) ethanol. Only cells of yeast strain A12 showed a statistically significant decrease in the magnitude of the GP value for cells grown in medium without inositol supplementation. Interestingly, it was observed that for yeast strain A12 the initial GP value is restored within 10 minutes of following exposure to 18% (v/v) ethanol. No statistically significant differences were observed between cells grown in inositol-supplemented and unsupplemented media with respect to their ability to restore the initial GP value following exposure to ethanol. This indicates that even though inositol supplementation of the media may trigger effects on the fluidity of the yeast cellular membranes, it does not seem to greatly affect the stress response mechanisms that restore normal membrane fluidity following ethanol exposure.

4.4 Conclusions

The results presented in this chapter indicate that inositol supplementation of the medium leads to effects on cell growth rate, fermentation kinetics and yeast

cellular membrane fluidity. However, the effects of inositol supplementation seem to be strain-specific, as indicated by the different responses observed in each strain of yeast studied. All strains exhibited an increase in cell growth rate when cultured in media supplemented with inositol. For yeast strains A12 and K7 improved ethanol productivity was observed when inositol-supplemented media were used, while for yeast strain A15 no effect of inositol supplementation of the growth medium was observed. For yeast strains A12 and K7 inositol supplementation of the fermentation media resulted in lower cellular membrane fluidity, while in contrast for yeast strain A15 inositol supplementation of the fermentation media increased themembrane fluidity.

CHAPTER FIVE: THE EFFECT OF INOSITOL ON THE YEAST CELLULAR MEMBRANES, GLYCEROL CONCENTRATION AND STRESS TOLERANCE

5.1 Introduction

5.1.1 General Introduction

Inositol has been reported to promote increased tolerance to ethanol stress in addition to its role in promoting cell growth. Supplementation of fermentation media with inositol was shown to result in a change in the phospholipid composition of the yeast plasma membrane (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Gaspar *et al.* 2006). This change in phospholipid composition has generally been considered to most likely reflect to altered fluidity of the membrane (Chi, Kohlwein & Paltauf 1999; Ishmayana, Kennedy & Learmonth 2015).

Our previous study (including reported in Chapter 4) indicated that when fermentation media are supplemented with inositol the cellular membrane fluidity increases, as indicated by a lower generalized polarization value (Ishmayana, Kennedy & Learmonth 2015). Consequently, it could reasonably be assumed that the unsaturation index (UI) value, which is a parameter commonly used as an indicator of membrane fluidity, would be higher for cells grown in media supplemented with inositol. However, Chi, Kohlwein & Paltauf (1999) found that yeast cells grown in media supplemented with inositol had lower UI values. These authors did not consider all unsaturated fatty acids in their analysis, but rather only the major constituents, C16:1 and C18:1. This limitation may affect their calculated UI. Therefore, in the present study a more comprehensive fatty acid analysis was undertaken to investigate the role of changes in UI upon supplementation of the medium with inositol in the observed changes in cellular membrane fluidity.

In terms of cell growth, some studies have found that inositol supplementation has positive effects on *S. cerevisiae* (Chi, Kohlwein & Paltauf 1999; Furukawa *et*

al. 2004; Krause *et al.* 2007; Navarro-Tapia, Querol & Pérez-Torrado 2018), *Schizosaccharomyces pombe* (Yao, Chi & He 2006) and *Pachysolen tannophilus* (Ji *et al.* 2008). In contrast, in agreement with the findings described in Chapter 4 of this study, other researchers found that supplementation of the medium with inositol does not have any effect on the growth rate of *S. cerevisiae* (Murray & Greenberg 2000). Krause *et al*. (2007) found that the positive effect of inositol supplementation of the medium was obvious when the medium contained a relatively low concentration of glucose (2% w/v), but not when the medium contained a higher glucose concentration (12% w/v). They found that the maximum growth rate of cells cultured in medium with the low glucose concentration was significantly higher when inositol was present in the fermentation media (0.153 ± 0.001 vs 0.102 ± 0.008 h^{-1}), while for cells cultured in medium with the higher glucose concentration no statistically significant difference in maximum growth rate was observed between cells grown in unsupplemented medium and cells grown in inositol supplemented media (0.102 ± 0.016 vs 0.100 ± 0.007 h^{-1}).

Ji *et al.* (2008) and Chi, Kohlwein & Paltauf (1999) reported that supplementation of the media with inositol had positive effects on fermentation performance, while another study by Furukawa *et al*. (2004) reported that supplementation of medium with inositol does not significantly affect fermentation performance. The only significant difference in the conditions used by the authors was that the former two groups compared the fermentation performance between cells cultured in media with no inositol to cells cultured in media supplemented with inositol, while the latter group compared cells cultured in media low and high levels of inositol. Even though Ji *et al*. (2008) used a yeast species other than *S. cerevisiae*, their results indicate that inositol promotes better fermentation performance. They reported that using *P. tannophilus*, ethanol production increased from about 30 up to about 45 g/L when the fermentation medium was

supplemented with 100 mg/L inositol, while Chi, Kohlwein & Paltauf (1999) reported that upon addition of 0.1 mg/L inositol to the fermentation medium, ethanol production increased from 15.5% (v/v) to 16.3% (v/v), i.e. 122 to 129 g/L. However, Furukawa *et al*. (2004) found that there is no difference in ethanol production rate when the cells are grown in fermentation media supplemented with 10 or 90 μM inositol (1.8 or 16.2 mg/L). We noted in Chapter 4 that the major differences in fermentation performance were between cultures grown on media lacking or containing inositol (e.g. a difference between 0 and 50 mg/L or more inositol) and not between cells grown on media with inositol but just with different levels of inositol supplementation. Thus, the conclusions of Fukurawa *et al*. (2004) were drawn from a different perspective, as they did not compare cell grown on media with “no inositol” with cells grown on media with “some inositol”.

Glycerol is one of the stress protectors that is synthesized when yeast cells are exposed to hyperosmotic stress. It protects the cell by acting as an osmoregulator when the cell starts losing water and therefore enable cellular processes to continue despite the low intracellular water activity (Nevoigt & Stahl 1997; Wojda *et al.* 2003). In the previous section (Chapter 4), evidence was presented that supplementation of the medium with inositol can increase the growth rate of the yeast strains studied in modified-YNB media with 10% w/v glucose. Such a glucose concentration is considered high under these conditions, since the YNB medium is considered a nutritionally-poor medium (Ishmayana, Learmonth & Kennedy 2011) and the cells are more susceptible to hyperosmotic stress when exposed to 10% (w/v) glucose under nutritionally poor conditions. Relatively high sugar concentration will induce the hyperosmotic stress response, which stimulates the synthesis of osmo-protectors such as glycerol. Since supplementation of the medium with inositol seems to increase cell growth rates (Chapter 4) at relatively high sugar concentrations, we predicted that it may also

affect the production of stress protectors such as glycerol, thereby enabling the cells to grow faster. Therefore, in the present chapter, we also examine whether supplementation of the medium with inositol affects the production of the stress protectant, glycerol.

5.1.2 Yeast strains and culture conditions

The experiments described in this chapter used the same three yeast strains described in the previous chapters (A12, A15 and K7). Yeast cells were grown in inositol-free YNB medium without supplementation and with inositol supplementation. Starter cultures were grown in YNB medium without inositol supplementation, so that no inositol would be introduced into the 0.00 g/L inositol medium when the strarter culture was used to inoculate the production medium.

5.1.3 Specific growth conditions and experimental design

Based on our previously described results showing that no significant effects were seen with concentrations of inositol concentrations higher than 0.10 g/L in the fermentation media, only two inositol supplementation levels were tested in the present experiment, v.i.z. 0.05 and 0.10 g/L. Therefore, in the present experiment, each strain was tested with three levels of supplementation, which were 0.00, 0.05 and 0.10 g/L inositol.

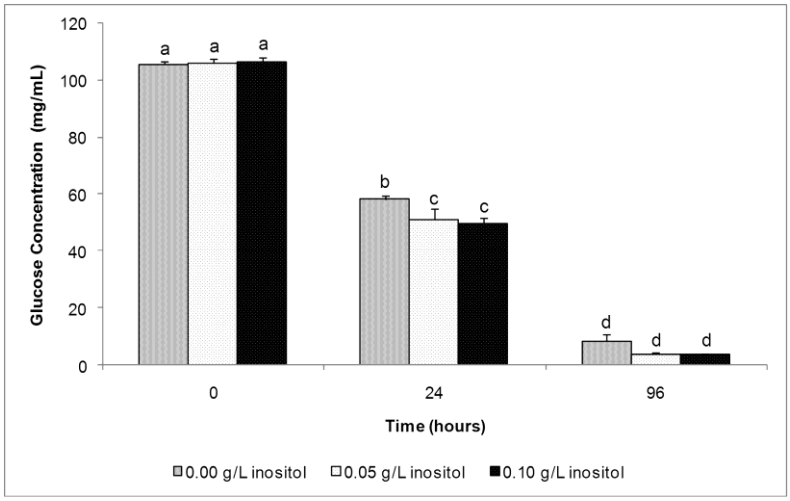
Cells were grown for 96 hours and glucose and ethanol production were measured at 0, 24 and 96 hours using the protocols described in Sections 2.11 and 2.12, respectively. In most cultures the time points corresponded to initial lag phase (0 h), respiro-fermentative growth on glucose (24 h) and, depending upon residual glucose levels, either during late respiro-fermentative metabolism or during the respiratory phase (96 h). The glycerol concentration was also monitored at 0, 24 and 96 hours using an enzymatic assay as described in Section 2.13. After

reaching respiro-fermentative phase (24 hours), the cultures were tested for their tolerance to ethanol, acetic acid and hyperosmotic stress using the methods described in Section 2.8.2. Fatty acid composition was determined after 24 hours of fermentation using GC-MS of lipid extracts prepared using *n*-hexane as solvent as described in Section 2.10. Yeast cell size was determined using a MOXI Z Mini Automated Cell Counter at the 24-hour time point (Section 2.14).

5.2 Results

5.2.1 Glucose consumption and ethanol production

Glucose consumption and ethanol production were measured at three time points representing lag, mid-respiro-fermentative and late respiro-fermentative or early-respiratory phases. The results of the glucose determination are presented in Figure 5.1. All strains showed similar trends in which the glucose concentration decreased over the duration of the culture. There were statistically significant differences in glucose consumption between cells grown in media without and with inositol supplementation. The differences were very clear in the respiro-fermentative phase (24 h) where glucose consumption of cells grown in inositol-supplemented media was higher than that of cells grown in unsupplemented media, leaving a significantly lower final concentration glucose in the inositol supplemented media. The greatest effect of inositol supplementation was observed for the K7 strain, where at 96 hours there was about 27 mg/mL residual glucose in the fermentation media without inositol supplementation, while in media with inositol supplementation there was only about 2 mg/L glucose remaining. As expected, the glucose concentrations at the zero time point were the same within experimental variability. At the 24 h time point, for yeast strain A12 and A15 the



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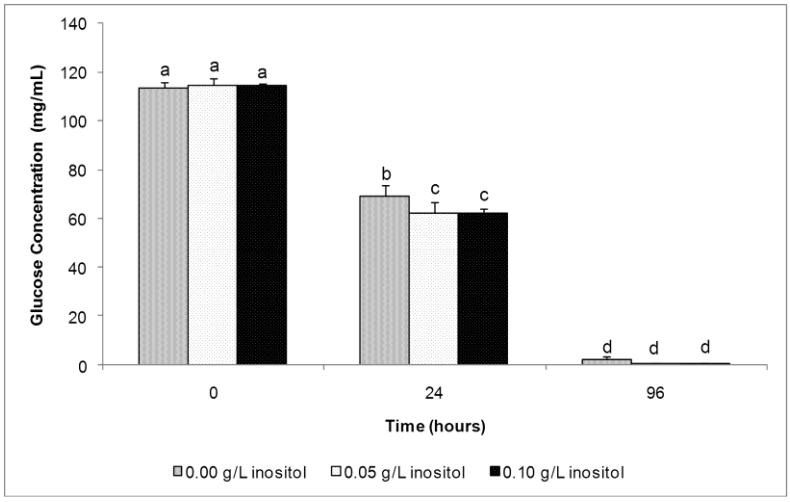

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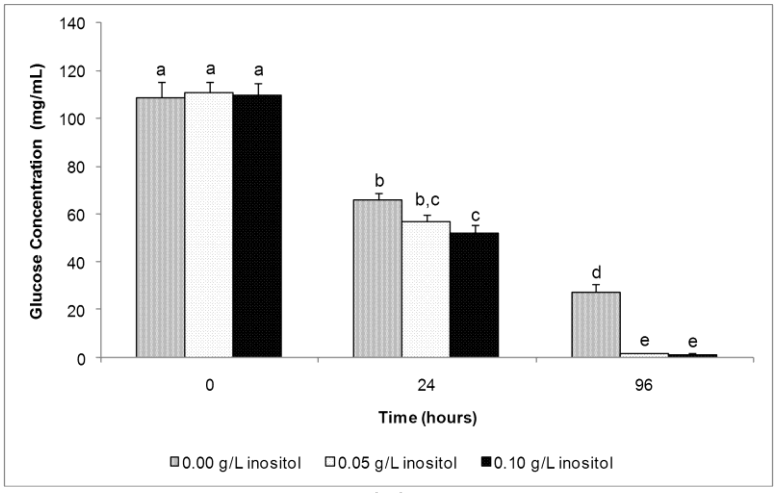
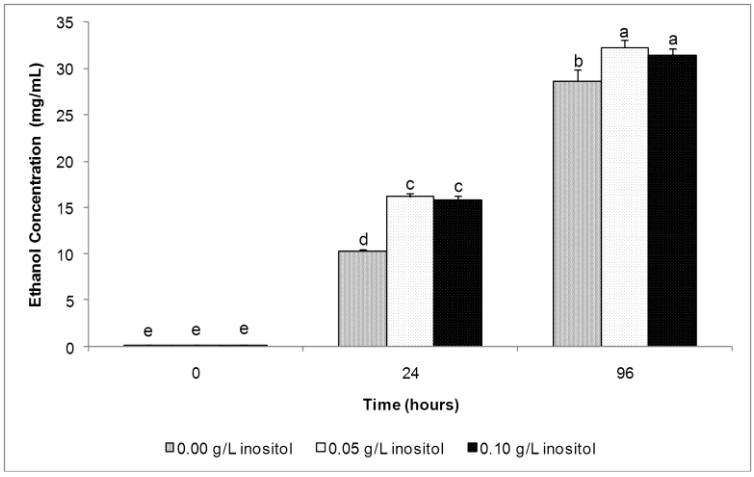
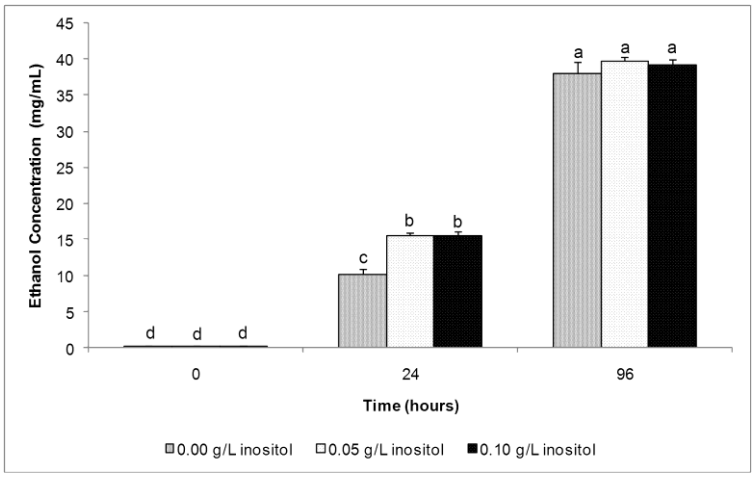


(C)

Figure 5.1 Glucose concentrations at three time points of fermentation of yeast strains (A) A12, (B) A15, and (C) K7. The values graphed are the means of data obtained from three independent experiments and the error bars represent SD. Different letters over bars indicate that the means have differences that are statistically significant at $\alpha = 0.05$ as assessed using Tukey's post-hoc test.



(A)



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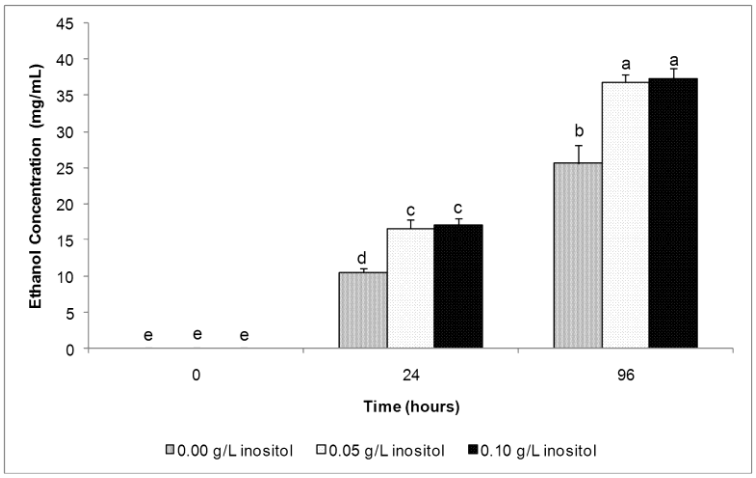


(C)

Figure 5.2 Ethanol concentrations at three time points of fermentation of yeast strains (A) A12, (B) A15, and (C) K7. The values graphed are the means of data obtained from three independent experiments and the error bars represent SD. Different letters over bars indicate that the means have differences that are statistically significant at $\alpha = 0.05$ as assessed using Tukey's post-hoc test.

inositol-supplemented media glucose levels were significantly lower than those in the unsupplemented media controls, although the two different levels of inositol supplementation could not be differentiated. The 24 h finding was similar for yeast strain K7, except that the final glucose level in the case of the lower supplementation level of 0.05 g/L inositol was intermediate between those of the 0.00 mg/L inositol control and the 0.10 mg/L inositol cultures supplementation. Nevertheless, these differences were not statistically significant. For yeast strain A12 and A15 most of the glucose had been consumed at 96 h, thus the residual glucose values were similar for unsupplemented and inositol-supplemented cultures of each strain, although for yeast strain K7 significantly more glucose remained in the medium of the unsupplemented control culture. Thus, again we observed an effect of inositol supplementation at the lowest concentration of inositol tested, with little difference observed with increasing concentrations.

The results of measuring ethanol concentration during the fermentation are presented in Figure 5.2. In agreement with the glucose consumption data, yeast strain A15 showed the highest level of ethanol production, followed by yeast strain K7 and A12. At the 96 h time point the ethanol concentration was so high (approximately 39 mg/mL) for yeast strain A15 that there were no significant differences between the unsupplemented control or inositol-supplemented cultures. In contrast for yeast strain K7 and A12 at 96 h and all strains at 24 h the inositol-supplemented cultures exhibited significantly higher ethanol concentrations than the unsupplemented controls. However, again, no significant difference between the two different levels of inositol supplementation were observed. At the 96 h time point, the ethanol concentration in the media fermented by yeast strain K7 without and with inositol supplementation were about 25 and 37 mg/mL, respectively, while for yeast strain A12, the ethanol concentrations were about 29 and 32 mg/mL, respectively.

Fermentation kinetic data for yeast strain A12, A15 and K7 with and without inositol supplementation are presented in Table 5.1, 5.2 and 5.3, respectively. It was found that inositol supplementation significantly increased the glucose consumption rate and ethanol productivity for yeast strains A12 and K7, while yeast strain A15 showed the same trend, but the increases were not statistically significant. No significant differences were seen for ethanol yield with or without inositol supplementation for any of the strains used in the present study, although yeast strains A12 and K7 tended towards higher yields with inositol supplementation. If we compare the glucose consumption rate, it seems that yeast strain A15 had the highest rate, which is consistent with it having the highest ethanol productivity. Interestingly, the glucose consumption rate for yeast strain A12 without inositol supplementation (about 1.009 $mg.mL^{-1}.h^{-1}$) was higher than that for yeast strain K7 without inositol supplementation (0.848 $mg.mL^{-1}.h^{-1}$). However, when the media were supplemented with inositol, the glucose consumption rate for yeast strain K7 dramatically increased to ~1.13 $mg.mL^{-1}.h^{-1}$, which is a significantly higher value compared to that for yeast strain A12 (1.07 $mg.mL^{-1}.h^{-1}$).

Table 5.1 Fermentation kinetic parameters of yeast strain A12 grown in media without or with inositol supplementation. Means in the same column with different superscript letters have differences that are statistically significant at α = 0.05 as assessed using Tukey's *post-hoc* test.

Level of Inositol Supplementation (g/L)	Qs ($mg.mL^{-1}.h^{-1}$)	Qp ($mg.mL^{-1}.h^{-1}$)	Yp/s ($mg.mg^{-1}$)
0.00	1.009 ± 0.030^{b}	0.297 ± 0.012^{b}	0.294 ± 0.003^{a}
0.05	1.065 ± 0.011^{a}	0.335 ± 0.007^{a}	0.315 ± 0.009^{a}
0.10	1.070 ± 0.015^{a}	0.326 ± 0.007^{a}	0.304 ± 0.011^{a}

Note: *Qs* = glucose consumption rate, *Qp* = ethanol productivity, *Yp/s* = ethanol yield

Table 5.2 Fermentation kineticsparameters of yeast strain A15 grown in media without or with inositol supplementation. Means in the same column with different superscript letters have differences that are statistically significant at α = 0.05 as assessed using Tukey's *post-hoc* test.

Level of Inositol Supplementation (g/L)	Qs ($mg.mL^{-1}.h^{-1}$)	Qp ($mg.mL^{-1}.h^{-1}$)	Yp/s ($mg.mg^{-1}$)
0.00	$1.158±0.029^{a}$	$0.394±0.017^{a}$	$0.340±0.015^{a}$
0.05	$1.184±0.026^{a}$	$0.412±0.006^{a}$	$0.348±0.011^{a}$
0.10	$1.185±0.008^{a}$	$0.407±0.008^{a}$	$0.344±0.006^{a}$

Note: *Qs* = glucose consumption rate, *Qp* = ethanol productivity, *Yp/s* = ethanol yield

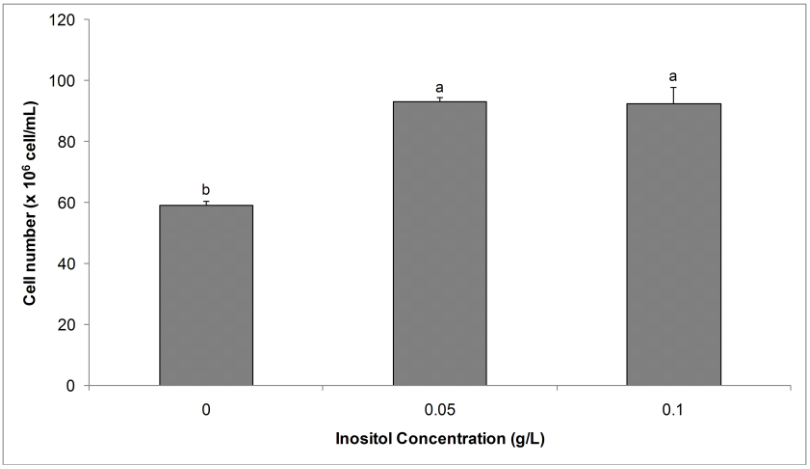
Table 5.3 Fermentation kinetic parameters of yeast strain K7 grown in media without or with inositol supplementation. Means in the same column with different superscript letters have differences that are statistically significant at α = 0.05 as assessed using Tukey's *post-hoc* test.

Level of Inositol Supplementation (g/L)	Qs ($mg.mL^{-1}.h^{-1}$)	Qp ($mg.mL^{-1}.h^{-1}$)	Yp/s ($mg.mg^{-1}$)
0.00	$0.848±0.094^{b}$	$0.267±0.027^{b}$	$0.315±0.009^{a}$
0.05	$1.135±0.049^{a}$	$0.383±0.011^{a}$	$0.338±0.023^{a}$
0.10	$1.126±0.053^{a}$	$0.388±0.015^{a}$	$0.345±0.026^{a}$

Note: *Qs* = glucose consumption rate, *Qp* = ethanol productivity, *Yp/s* = ethanol yield

5.2.2 Cell Number

Unlike in previous chapters, in this chapter, total cell number was determined using a MOXI Z automated cell counter. Cell size was also measured (Section 5.2.6). The results of total cell count performed after 24 hours of growth are presented in Figure 5.3. It is clear that inositol supplementation of the medium increased the cell count. Only yeast strain K7 showed no significant difference between growth on unsupplemented medium and growth on medium with 0.05 g/L inositol supplementation. However, in general, inositol supplementation of the growth medium did increase cell count, confirming the results previously described in Chapter 4 (Figure 4.6), which was measured using $OD_{600nm,}$ and in other studies



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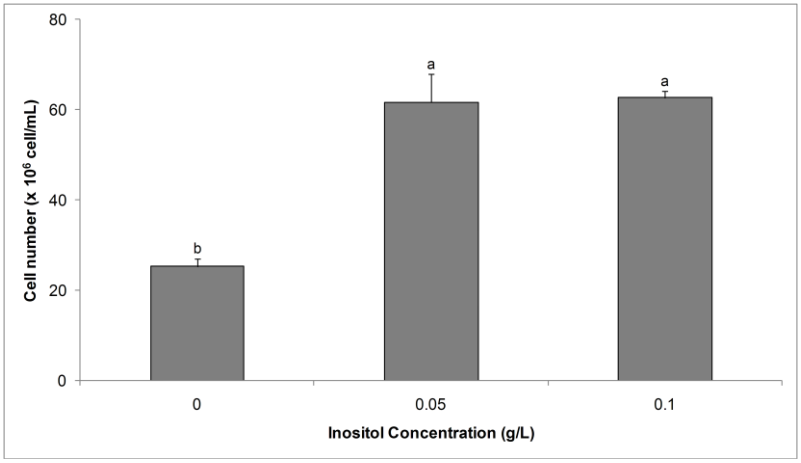

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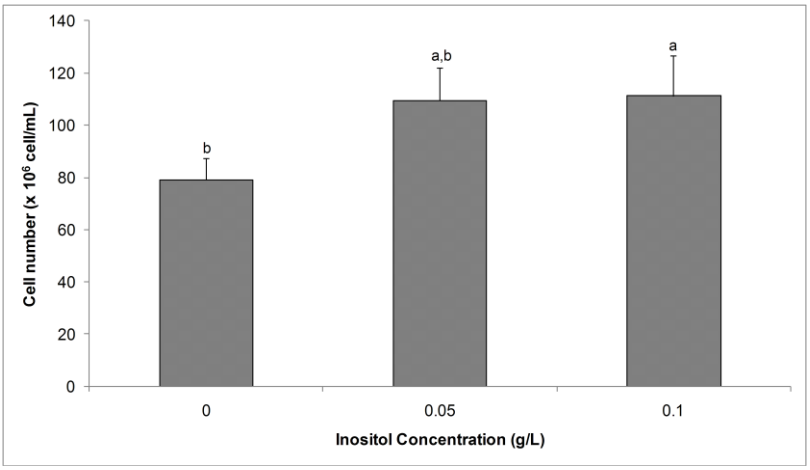


(C)

Figure 5.3 Total cell counts of yeast strains (A) A12, (B) A15 and (C) K7 grown in inositol-free YNB media with or without inositol supplementation with a 10% (w/v) initial glucose concentration at 24 hours. The values graphed are the means of data obtained from three independent experiments and the error bars represent SD. Different letters over bars indicate that the means have differences that are statistically significant at $\alpha = 0.05$ as assessed using Tukey's post-hoc test.

by Krause *et al.* (2007), who reported an increase in OD, and Chi, Kohlwein & Paltauf (1999), who reported an increase in cell dry weight.

The total cell count data also showed that yeast strain K7 had the highest total cell density followed, in order, by yeast strain A12 and A15. This trend was also observed when cell density was measured using $OD_{600nm.}$ As assessed by OD600nm, yeast strain K7 had the highest cell density followed by yeast strain A12 and A15. This result confirms that OD_{600nm} data exhibits a strong correlation with total cell count data even though it is affected by other factors besides cell number, such as cell size (Smit *et al.* 1992).

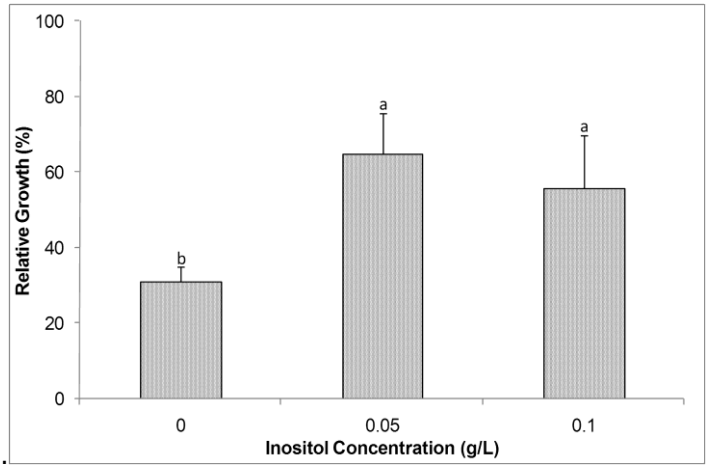
5.2.3 Yeast stress tolerance

Yeast tolerance to three stress factors was assessed in the present experiment, namely tolerance to ethanol stress (exposure to 7% v/v ethanol), hyperosmotic stress (exposure to 27% w/v sorbitol) and weak acid stress (exposure to 67 mM acetic acid). The concentration of ethanol used in this part of the study was different to that used in the experiments described in Chapter 3 which was chosen based on the results of preliminary study in which 18% (v/v) ethanol was used as the stress factor and the relative growth was practically 0% (data not shown). Therefore, a lower concentration of ethanol (7% v/v) was used for the stress tolerance study in this chapter based on the study of Zheng *et al.* (2011). It should be noted that the ethanol stress test protocol used in the experiments described in this chapter is different from the test used in the experiments described in Chapter 3, where in this chapter relative growth monitored by measuring $OD_{600\ nm}$ after 24 hour growth was used instead of TPC method (Garcia *et al*. 1997); the rationale for the use of an alternative test is explained in detail in Section 2.8.2.The three stress factors chosen for study are common stress experienced by yeast growing under industrial fermentation

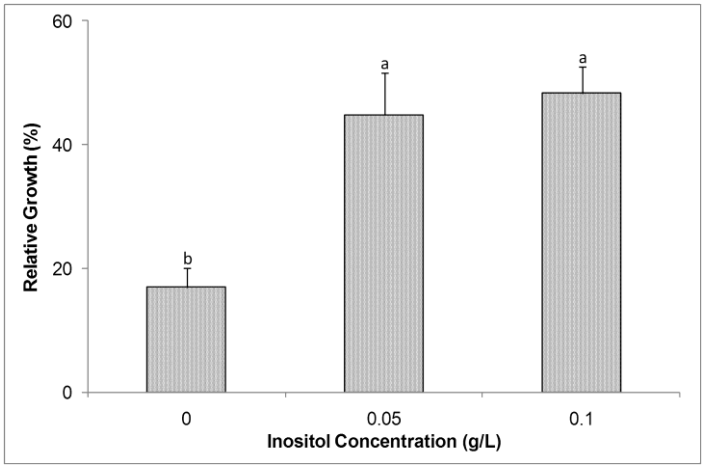
conditions (Basso, Rocha & Basso 2011). The protocol used to assess was that previously described by García *et al.* (1997).

The results of the experiments to test the responses of the yeast strains to ethanol, hyperosmotic and weak acid stresses are presented in Figure 5.4, Figure 5.5 and Figure 5.6, respectively.

It was found that supplementation of the medium with inositol affected the tolerance of yeast to all stresses trialled in this study. Additionally, the results reveal that each strain has different tolerance to the various stresses. For ethanol stress (Figure 5.4), it was found that yeast strain K7 is the most susceptible, followed in order by yeast strain A15 and A12. Addition of inositol to the medium improved the ethanol tolerance of yeast strain A12 significantly, from ~31% relative growth to ~64 and 56% relative growth in the presence of ethanol after addition of 0.05 and 0.10 g/L inositol, respectively. For yeast strain A15, inositol supplementation of the medium improved the ethanol tolerance as indicated by relative growth that increased from ~17% to ~45 and ~48% in the presence of ethanol after addition of 0.05 and 0.10 g/L inositol, respectively, while for yeast strain K7, ethanol tolerance was improved as indicated byrelative growth that increased from ~11% to ~38 and ~39% when the fermentation media was supplemented with 0.05 and 0.10 g/L inositol, respectively. As noted earlier for other factors, the presence of inositol in the medium significantly increased the ethanol tolerance of all three strains, however the differences in ethanol tolerance between the two levels of inositol supplementation of the medium were not statistically significant for any of the three strains.



(A)



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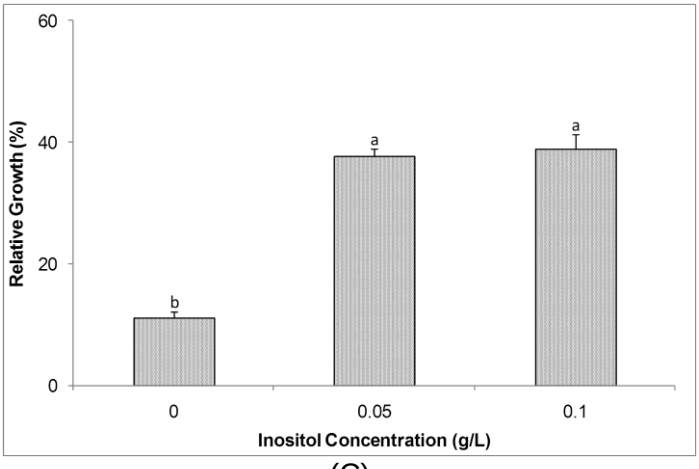
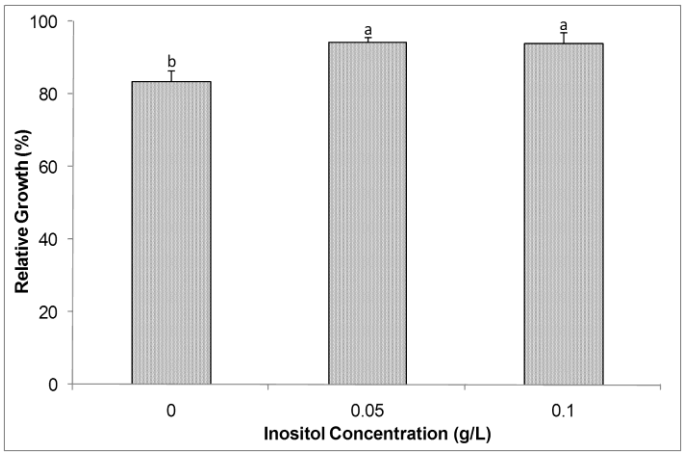
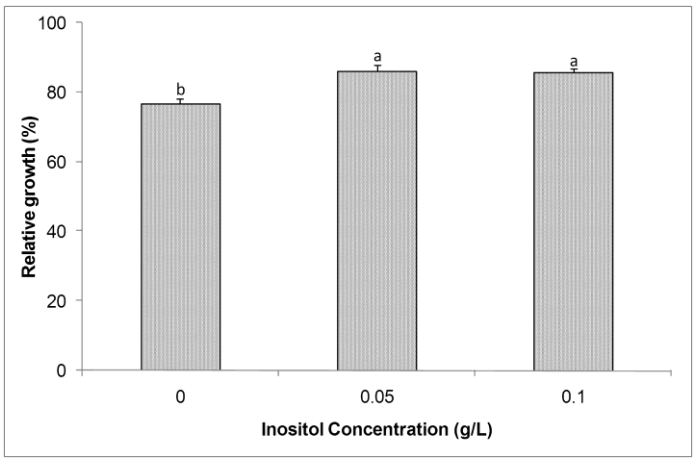


(C)

Figure 5.4 Relative growth of yeast strains (A) A12, (B) A15 and (C) K7 grown in the presence of 7% (v/v) ethanol. Yeast cells were grown in YNB media with or without inositol supplementation with a 2% (w/v) initial glucose concentration. The values graphed are the means of data obtained from the four independent experiments and the error bars represent SD. Different letters over bars indicate that the means have differences that are statistically significant at α = 0.05 as assessed using Tukey's post-hoc test.



(A)



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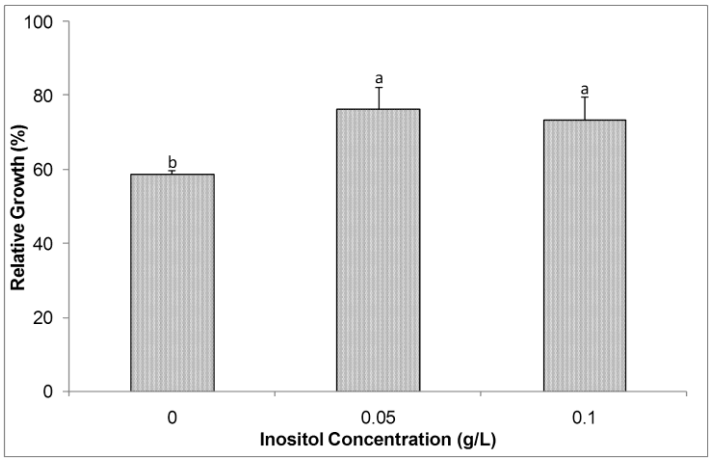
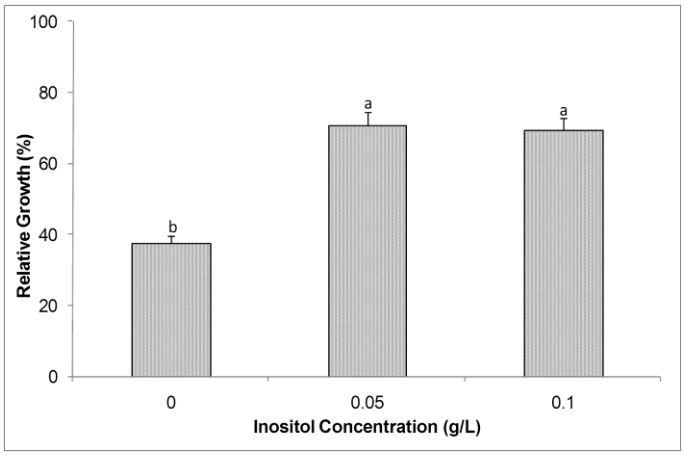
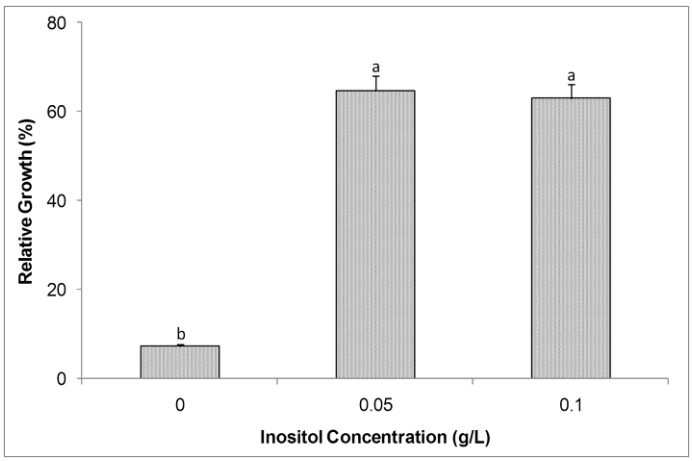


(C)

Figure 5.5 Relative growth of yeast strains (A) A12, (B) A15 and (C) K7 yeast strains grown in the presence of 27% (w/v sorbitol). Yeast cells were grown in YNB media with or without inositol supplementation with a 2% (w/v) initial glucose concentration. The values graphed are the means of data obtained from four independent experiments and the error bars represent SD. Different letters over bars indicate that the means have differences that are statistically significant at α = 0.05 as assessed using Tukey's post-hoc test.



(A)



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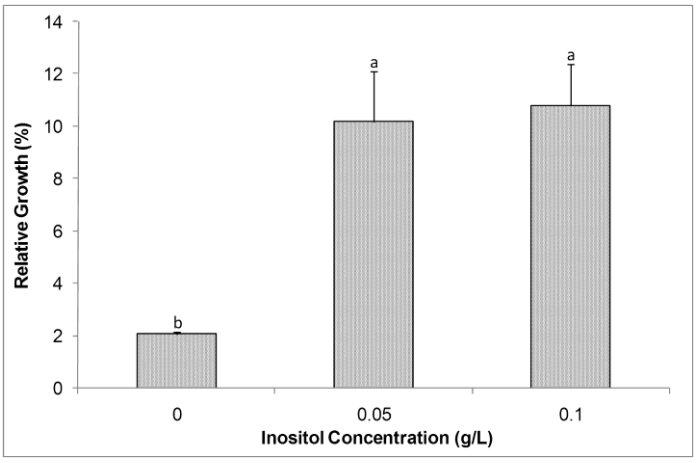


(C)

Figure 5.6 Relative growth of yeast strains (A) A12, (B) A15 and (C) K7 grown in the presence of 67 mM acetic acid. Yeast cells were grown in YNB media with or without inositol supplementation with a 2% w/v initial glucose concentration. The values graphed are the means of data obtained from three independent experiments and the error bars represent SD. Different letters over the bars indicate that the means have differences that are statistically significant at $\alpha = 0.05$ as assessed using Tukey's post-hoc test.

The relative growth values when the yeast strains were exposed to hyperosmotic stress were higher than the relative growth value when the yeast strains were exposed to either ethanol stress or acetic acid stress. For this stress, yeast strain K7 was the most susceptible, while the highest tolerance to hyperosmotic stress was observed for yeast strain A12. All yeast strains showed more than ~58% but less than ~84% relative growth in hyperosmotic medium when the cells were grown in medium without inositol supplementation. When inositol was supplemented into the fermentation medium, the relative growth values under osmotic stress condition increased significantly, by as much as ~18% for yeast strain K7 while for yeast strains A12 and A15 the relative growth increased by as much as ~10%. The relative growth were higher in the osmotically stressed samples when the medium was supplemented with inositol, no doubt a reflection of the relative intensity with which the stresses were experienced by the yeast and differences in the general capacity of the yeast to respond to and tolerate the different stresses. However, the same pattern was seen as for ethanol stress, i.e. supplementation of the growth medium with 0.05 g/L inositol increased tolerance and growth under the stress condition, while increasing the level of inositol supplementation of the medium to 0.10 g/L inositol did not provide any further increase in relative growth or tolerance.

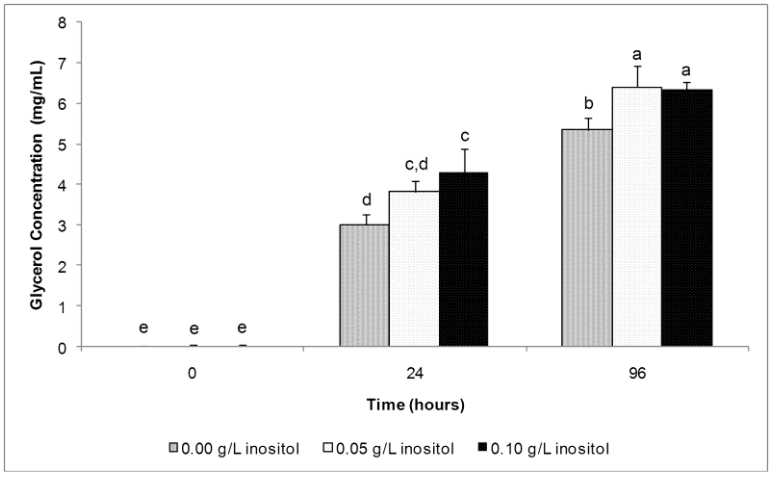
Acetic acid stress had the largest effect on relative cell growth. When the cells were grown in medium without inositol supplementation yeast strain A12 showed the highest tolerance with ~38% relative cell growth, while yeast strains A15 and K7 showed much lower relative cell growth, viz. ~7% and ~2%, respectively. When grown in medium with inositol supplementation the relative cell growth value increased dramatically to ~70%, ~65%, and ~10% for yeast strains A12, A15 and K7, respectively. The results showed that yeast strain K7 is the most susceptible to acetic acid stress. The same pattern was observed as for the other stresses,

with inositol supplementation of the medium significantly increasing the tolerance and relative growth under the stress condition, but no statistically significant differences in tolerance or relative growth were observed between the two different levels of inositol supplementation of the medium tested.

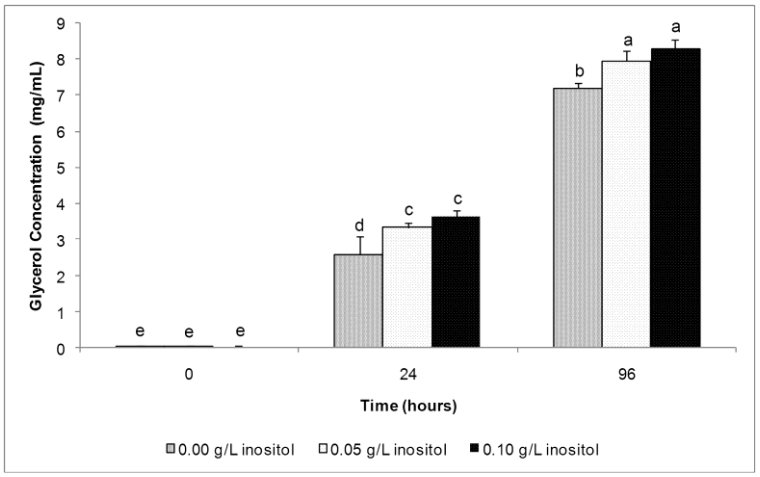
5.2.4 Glycerol concentration

The glycerol concentration was measured at three time points of the growth curve, viz. 0, 24 and 96 hours and the results are presented in Figure 5.7.

Only a low concentration of glycerol was detected at the beginning of fermentation. The glycerol concentration increased with increasing fermentation time. At 24 hours, yeast strain A12 showed significantly higher glycerol concentration when grown in medium supplemented with 0.10 g/L inositol than when grown in unsupplemented control medium, but this was not observed when the same cells were grown in medium supplemented with 0.05 g/L inositol. In contrast, yeast strain A15 showed significantly higher glycerol concentrations at both levels of inositol supplementation. No differences in glycerol concentration were observed between inositol-supplemented and unsupplemented control media for yeast strain K7. After 96 hours of fermentation, the glycerol concentration seems to increase further. For yeast strain A12 and A15, cells grown in medium supplemented with inositol had significantly higher glycerol concentrations than cells grown in unsupplemented medium. Only yeast strain K7 did not show any statistically significant difference in glycerol concentration between cells grown in medium supplemented with inositol and cells grown in unsupplemented control medium.



(A)



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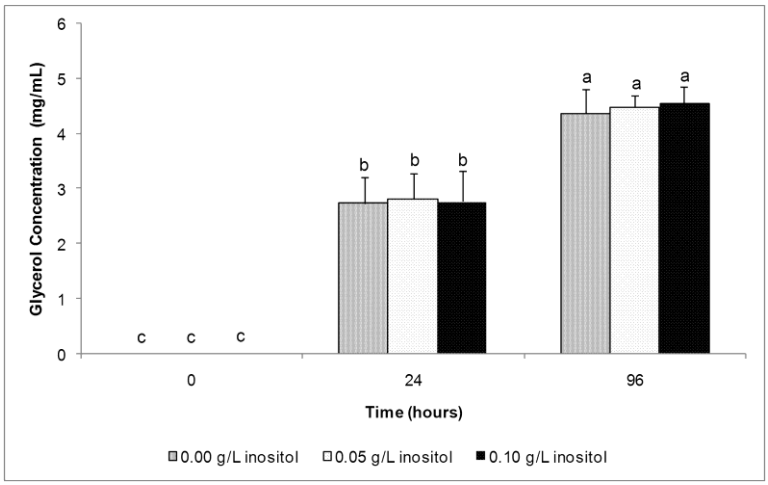


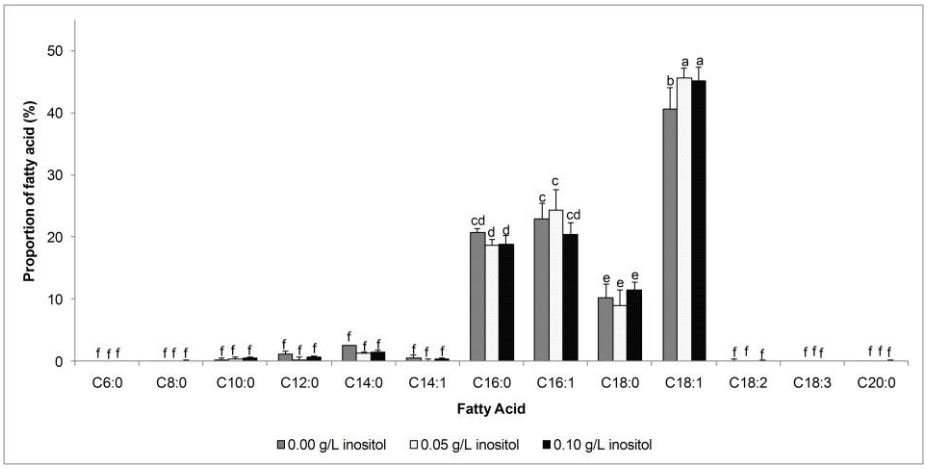
(C)

Figure 5.7 Glycerol concentrations at three time points of fermentation of yeast strain (A) A12, (B) A15, and (C) K7. The values graphed are the means of data obtained from three independent experiments and the error bars represent SD. Different letters over bars indicate that the means have differences that are statistically significant at α = 0.05 as assessed using Tukey's post-hoc test.

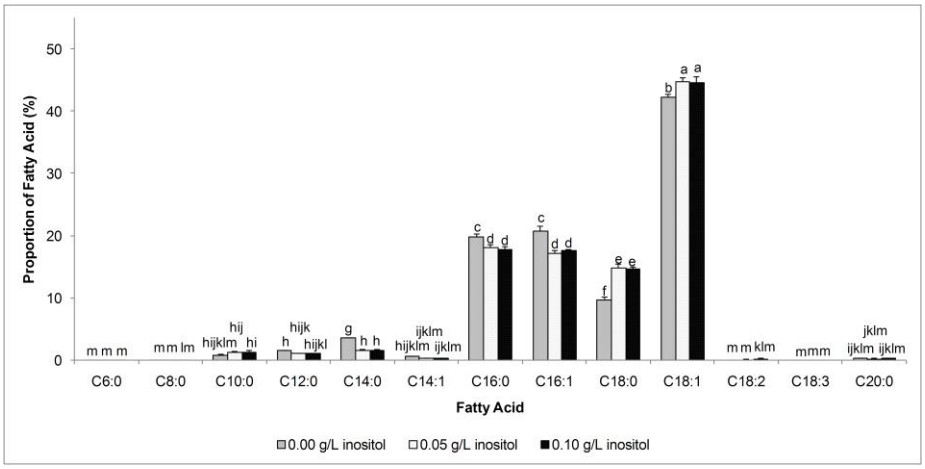
Among the three strains used in the present study, yeast strain K7 showed the lowest ability to synthesize glycerol. At 96 hours of fermentation, yeast strain K7 only achieved about 4 mg/mL glycerol, while yeast strains A12 and A15 reached about 6 and 8 mg/mL glycerol, respectively. Even though yeast strain A15 reached the highest glycerol concentration of the three strains at 96 hours, at the 24 hour time point, yeast strain A12 actually had synthesized more glycerol than yeast strain A15. When the fermentation medium was supplemented with inositol, yeast strain A15 produced about 3.5 mg/mL glycerol, while yeast strain A12 reached about 4 mg/mL glycerol. In terms of glycerol accumulation, it can be seen that in general, again, inositol supplementation of the medium stimulates a response in yeast cells even at the lower level of inositol supplementation of the medium and there is no significant increase in response at the higher level of inositol supplementation of the medium.

5.2.5 Fatty acid composition

The results of analysis of fatty acid composition presented in Figure 5.8 indicate that inositol supplementation of the medium seems to affect each of the three yeast strains differently. Growth of yeast strain in supplemented medium with inositol seems to decrease the proportion of saturated fatty acids, except for C18:0. No significant difference in the proportion of C18:0 was detected between cells grown in media without or with inositol supplementation in the case of yeast strain A12 and K7, while for yeast strain A15 cells grown in medium with inositol supplementation had a significantly higher C18:0 content than cells grown in unsupplemented control medium. The unsaturated fatty acid content of yeast strain A12 was not significantly different between cells grown in media with or without inositol supplementation. Yeast strains K7 and A15 showed a higher proportion of C18:1 when the cells were grown in medium with inositol supplementation, while



(A)



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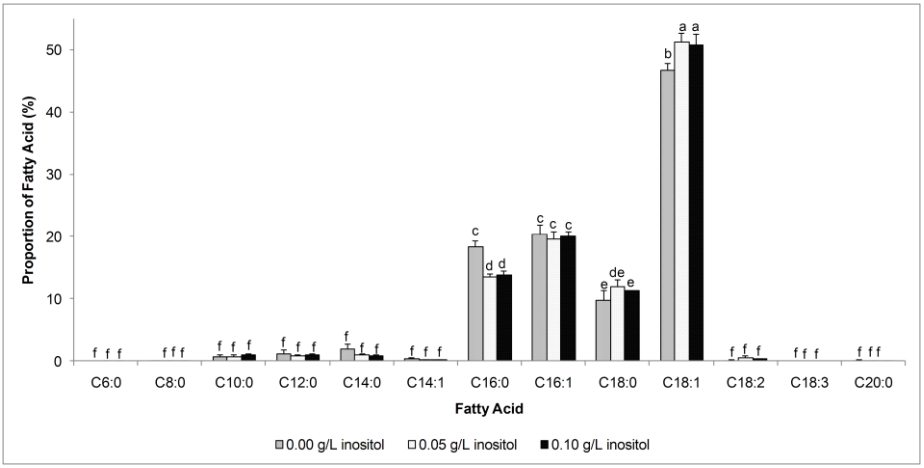


(C)

Figure 5.8 Fatty acid compositions of yeast strains (A) A12, (B) A15, and (C) K7 grown in media without and with inositol supplementation. Cells were harvested at 24 hours. The values presented are the means of data obtained from three independent experiments and the error bars represents SD. Different letters over the bars indicate that the means have differences that are statistically significant at $\alpha = 0.05$ as assessed using Tukey's post-hoc test.

for yeast strain A15 grown in medium with inositol supplementation the proportion of C16:1 was significantly lower.

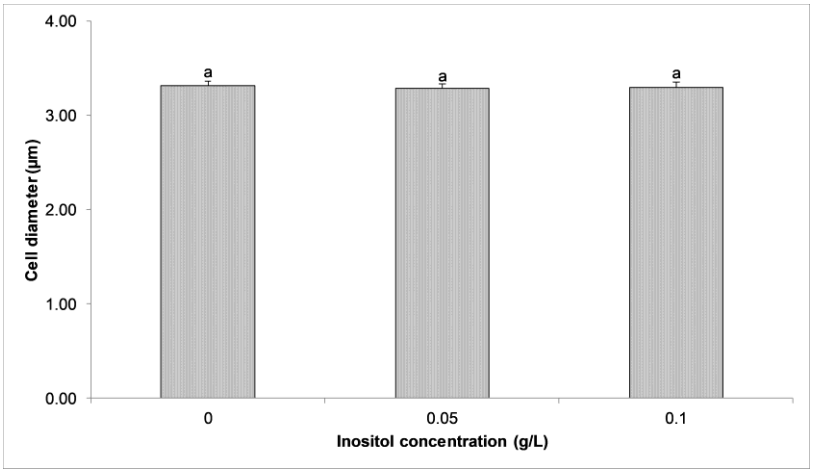
The unsaturation index (UI) values of the three yeast strains used in this study are presented in Table 5.4. While, in general, there appeared to be a higher proportion of unsaturated fatty acids in cells grown in medium with inositol supplementation (Figure 5.7), increases and decreases in the proportion of individual fatty acids balanced out and no significant overall differences were observed between cells grown in medium without or with inositol supplementation for yeast strain A12 or A15 . In contrast, yeast strain K7 grown in medium with inositol supplementation showed significantly higher unsaturation index values than the same strain grown in unsupplemented control medium, although the unsaturation index values of cells grown in media with the two different inositol supplementation levels were similar. It can be seen from Figure 5.8 that yeast strain K7 grown on medium with inositol supplementation had a markedly lower proportion of C16:0, although a slightly higher proportion of C18:0, with slightly lower proportion of C16:1, but a markedly higher proportion of C18:1, leading to a net increase in UI. These results indicate that supplementation of medium with inositol can affect fatty acid profiles and thereby UI, but is the effects are most likely strain-specific.

Table 5.4 Unsaturation index values of the yeast strains used in this study grown in media without or with inositol supplementation. The values are the means of data obtained from three independent experiments ± SD. The means in the same column with different superscript letters have differences that are statistically significant at α = 0.05 as assessed using Tukey's *post-hoc* test.

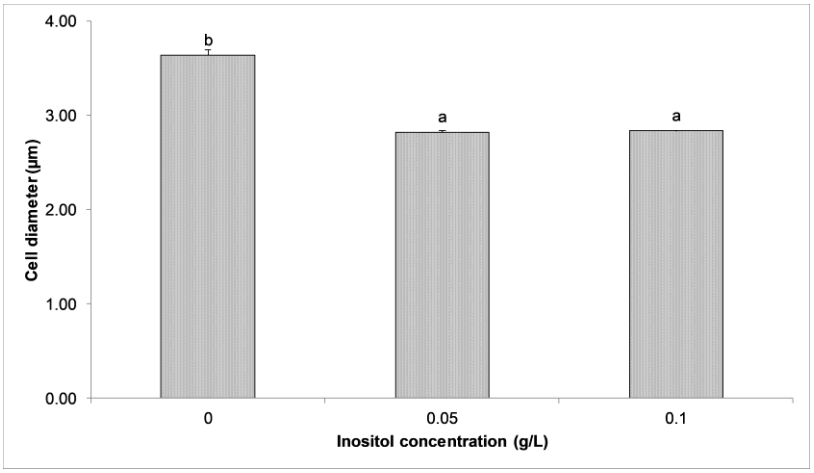
Level of Inositol Supplementation (g/L)	Unsaturation Index		
	A12	A15	K7
0.00	0.646±0.019[a]	0.639±0.010[a]	0.678±0.022[b]
0.05	0.703±0.032[a]	0.627±0.001[a]	0.721±0.008[a]
0.10	0.665±0.026[a]	0.630±0.006[a]	0.719±0.009[a]

5.2.6 Cell size

To investigate the effect of growth in medium supplemented with inositol on yeast cell size, the size of cells grown in media without or with inositol supplementation was determined using a MOXI Z cell counter and the results are presented in Figure 5.9. The cell diameter ranged from 3 to 4 μm. An effect of growth in media with inositol supplementation was only observed for yeast strain A15, where growth in media with inositol supplementation led to a significant decrease in cell size, although there was no significant difference in cell size between cells grown the two different levels of cells grown in media with inositol supplementation. In contrast, no effect of growth in medium with inositol supplementation was observed in the case of yeast strain A12 or K7. From the cell size measurement data, it was apparent that yeast strain K7 had a greater diversity of cell sizes compared to yeast strains A12 and A15, as indicated by the higher standard deviation values. This difference was only observed for the cells grown in inositol-supplemented media.



(A)



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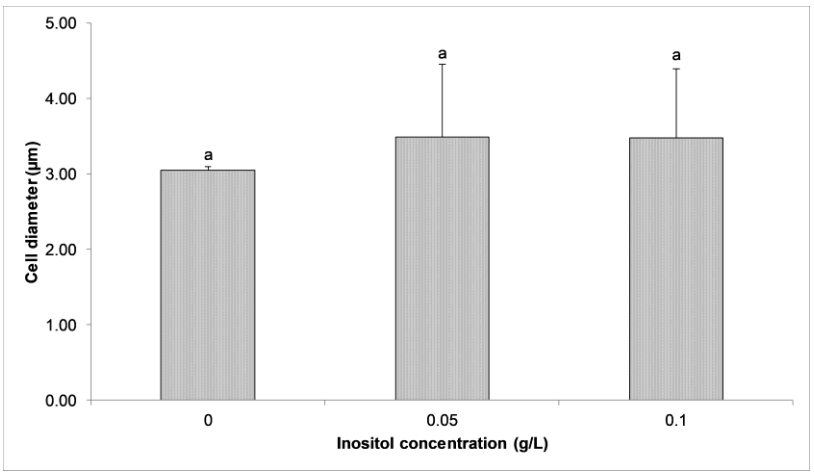

(C)

Figure 5.9 Cell size of yeast strain (A) A12 (B) A15 and (C) K7 grown in media without and with inositol supplementation. The values graphed are the means of data obtained from three independent experiments and the error bars represent SD. Different letters over bars indicate that the means have differences that are statistically significant at $\alpha = 0.05$ as assessed using Tukey's post-hoc test.

5.3 Discussion

Glucose consumption and ethanol production data confirmed the results of previous experiments (described in Chapter 4) which indicated that inositol supplementation of the medium improves glucose consumption and ethanol production. These effects of inositol supplementation of the medium on fermentation performance were also reported by other authors (Ji *et al.* 2008; Nikolić *et al.* 2009a).

The three yeast strains used in the present study showed different glucose consumption levels. Interestingly, at 24 hours of fermentation, yeast strain A15 had lower glucose consumption and ethanol production compared to yeast strains A12 and K7. However, at 96 hours A15 showed the highest glucose consumption and ethanol production. This phenomenon was also seen in the experiments reported in Chapter 3 (Figures 3.5 and 3.6) and Chapter 4 (Figures 4.9 and 4.10). Yeast strain A15 showed the lowest residual glucose in the fermentation media (about 2.7 and 1.0 mg/mL for growth media without and with inositol supplementation, respectively). For yeast cells grown without inositol supplementation, yeast strain A12 showed higher glucose consumption than (~8.7 vs ~27.4 mg/mL residual glucose). However, interestingly, when grown in inositol-supplemented fermentation media yeast strain K7 had higher glucose consumption and hence lower residual glucose (~1.9 and ~1.6 mg/mL for media with 0.05 and 0.1 g/L inositol supplementation, respectively) compared to A12 (~3.8 and ~3.8 mg/mL for media with 0.05 and 0.1 g/L inositol supplementation, respectively), which indicates that inositol supplementation of the medium has a greater effect on yeast strain K7 compared to yeast strain A12. These results suggest that inositol supplementation of the medium affects different yeast strains differently. It may improve fermentation performance for one strain more than for another strain. This is also supported by Qs and Qp values (Table 5.1, Table 5.2 and Table 5.3), which

are in agreement with the described results described earlier in the present study (see Table 4.1, Table 4.3, Table 4.4 and Table 4.5).

Total cell number data showed good agreement with OD_{600nm} data in that inositol supplementation of the medium increased cell numbers significantly (Figure 4.6 vs Figure 5.3). Furthermore, the total cell number data described in this chapter also showed similar trends to the $OD_{600\ nm}$ data. Yeast strain K7 had the highest cell number, followed by yeast strains A12 and A15. This result was also observed by Smit *et al*. (1992) where they found a positive correlation between cell dry weight and optical density, even though further exploration revealed that at a point when cell dry weight reached maximum value, the $OD_{600\ nm}$ values could still increase due to an increase in cell volume (Smit *et al.* 1992).

Three stress factors were tested in the present experiment, i.e. ethanol stress, hyperosmotic stress, and acetic acid stress. These stresses are commonly experienced by yeast during industrial fermentation (Basso, Rocha & Basso 2011). As presented in Figure 5.4, Figure 5.5 and Figure 5.6 there are indications that each strain has different susceptibility when exposed to the stresses tested in the present study. For ethanol stress, K7 is the most susceptible of the three yeast strains. When grown in fermentation medium with inositol supplementation, yeast strain A12 showed the highest improvement in ethanol stress tolerance, while yeast strain A15 showed the second-best improvement. An improvement in tolerance to ethanol stress when the fermentation medium is supplemented with inositol was also reported by Chi, Kohlwein & Paltauf (1999), Krause *et al*. (2007) and Furukawa *et al*. (2004). The present study, besides confirming previous reports that inositol supplementation of the medium increases ethanol stress tolerance, also found that each strain tested has a different response to inositol supplementation of the medium.

Hyperosmotic stress is very commonly experienced by yeast during fermentations for industrial bioethanol production as high gravity fermentation is attempted in order to increase ethanol production (Bafrncová *et al.* 1999; Chan-u-tit *et al.* 2013; Deesuth *et al.* 2012; Deesuth *et al.* 2015). In fuel ethanol production, the yeast cells are considered to be exposed to hyperosmotic stress when they are exposed to more than 27% (w/v) sugar. Fermentations that use such a high initial level of sugar are commonly known as very high gravity (VHG) fermentations (Thomas *et al.* 1993). The tolerance to hyperosmotic stress of the yeast strains used in the present study was very good. Again, for this stress yeast strain K7 showed the lowest tolerance, especially when grown in media without inositol supplementation (in which this strain exhibited ~60% relative growth), while the other strains exhibited ~80% relative growth. When grown in inositol-supplemented fermentation media, yeast strain K7 only achieved ~74% relative growth, while yeast strains A12 and A15 reached ~94% and 86% relative growth, respectively. As far as we are aware, investigation of the effects of inositol supplementation of the medium on hyperosmotic stress tolerance is rare. One of the possible mechanisms by which inositol may contribute to hyperosmotic stress tolerance may be by the provision of a substrate for the synthesis of phosphatidylinositol-3,5-bisphosphate, which acts as a second messenger in several stress response pathways (Dove *et al.* 1997). By increasing the level of phosphatidylinositol-3,5-bisphosphate, inositol may confer on the cells a greater stress tolerance.

The last stress examined in the present study was acetic acid stress. The concentration of acetic acid used in this study was 67 mM (4 g/L), as suggested by Zheng *et al*. (2011), which is, in fact, more than six times higher than the highest concentration of acetic acid (~10 mM; 0.6 g/L) considered normal in a fermentation (Sousa *et al.* 2012). Acetic acid is produced during both aerobic and anaerobic conditions and can accumulate as one of the byproducts of ethanolic fermentation

(Rodrigues, Ludovico & Leão 2006) or as a consequence of the presence of acetic acid bacteria in the fermentation culture (Vilela-Moura *et al.* 2011). The three yeast strains used in the present study showed different levels of tolerance to acetic acid stress. Yeast strain K7 was found to be the most susceptible strain as it exhibits the lowest relative growth when exposed to 67 mM acetic acid. Acetic acid is one of the factors that trigger programmed cell death (Sousa *et al*. 2012). Acetic acid also decreases the activity of some enzymes in the glycolytic and ethanol formation pathways (Sousa *et al.* 2012).

From the results of the present study, it is clear that inositol acts as a general protector against stress. The three yeast strains tested in the present study showed a statistically significant increase in tolerance to ethanol, hyperosmotic, and acetic acid stresses when grown in medium supplemented with inositol compared to when grown in unsupplemented medium. Most published studies have repported correlations between inositol supplementation of the medium and improved tolerance to ethanol stress (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Krause *et al.* 2007). Therefore, the present study extends the evidence that inositol supplementation of the medium protects yeast cells against ethanol stress to also include other stresses. The present study also found evidence that each yeast strain has a different response to inositol supplementation of the medium. This was clearly evident in the response to acetic acid stress, where yeast strain A15 showed the highest increase in relative growth when inositol was used to supplement the fermentation medium (about 9 times to the relative growth rate of the unsupplemented medium control), while in contrast, yeast strains A12 and K7 only showed about 2- and 5-fold increases in the relative growth when grown in inositol supplemented medium compared to unsupplemented medium control. The protective effect against the stress of inositol supplementation of the medium improves the ability of the yeast cells to grow in the presence of the stress. This

ability of inositol supplementation of the medium to protect yeast cell from stress may be an advantage that can be further exploited in the context of industrial fermentations.

The three yeast strains used in the present study showed different capabilities to synthesize glycerol in response to osmotic stress when grown in inositol supplemented medium. Yeast strain A15 exhibited a statistically significant increase in glycerol concentration when exposed to osmotic stress at both levels of inositol supplementation of the medium used in the present study compared to unsupplemented control. However, yeast strain A12 showed a statistically significant increase in glycerol concentration when exposed to osmotic stress when the fermentation medium was supplemented with 0.1 g/L inositol at 24 h of growth, while at 96 h of growth the statistically significant difference was observed when the fermentation medium was supplemented with both 0.05 and 0.1 g/L inositol compared to unsupplemented control. As for yeast strain K7, no statistically significant differences in glycerol concentration were observed for under osmotic stress. Glycerol is known to have an important role as a general protector against stress, particularly against hyperosmotic stress (Li *et al.* 2009). It should be noted, however, that glycerol is produced within the yeast cell to increase the internal osmotic pressure in order to counter the potential loss of water from the cell due to the higher osmotic pressure in the extracellular environment (Nevoigt & Stahl 1997). However, the plasma membrane of the cell is permeable to glycerol, resulting in the loss of glycerol to the extracellular environment. In this study we therefore measured the glycerol concentration in the medium. The concentration of glycerol in the medium is in equilibrium with the intracellular glycerol concentration. Under osmotic stress conditions, the glycerol concentration in the cell can be increased by increased glycerol synthesis, inhibiting the loss of glycerol

through the plasma membrane, or increased uptake of glycerol from the extracellular medium via a membrane transporter (Nevoigt & Stahl 1997).

As far as we are aware, no previous studyhas investigated possible correlation between the level of inositol supplementation of the medium and glycerol production. Possible mechanisms by which inositol supplementation of the growth medium may stimulate glycerol synthesis include effects on phosphoinositide signalling pathways or the conversion of excess inositol into glucose-6-phosphate which can then be converted into glycerol-3-phosphate. Glycerol-3-phosphate is a as precursor for the biosynthesis of glycerol. It has been reported that phosphoinositides play a role in high osmolarity glycerol (HOG) pathway signalling. The HOG pathway regulates the synthesis of glycerol in response to hyperosmotic stress (Adhikari & Cullen 2015; Dove *et al.* 1997). Thus, we can explain the (strain-specific) effects of inositol supplementation of the medium on glycerol accumulation through the link between inositol supplementation of the medium and stimulation of the production of phosphatidylinositol-based signalling molecules which may in turn activate of the HOG pathway which controls glycerol synthesis. Notwithstanding, the possible mechanisms by which inositol supplementation of the medium stimulates an increase in glycerol production needs to be further investigated.

The fatty acid composition and unsaturation index values indicate that the three yeast strains investigated in the present study have different responses to inositol supplementation of the medium. Generally, the level of most saturated fatty acids analyzed decreased when the fermentation medium was supplemented with inositol, with the exception of C18:0. The proportion of C16:0 tended to decrease when the medium was supplemented with inositol in all of the yeast strains assessed in the present study. This decrease seems to be compensated by an increase in the proportions of the other fatty acid, especially C18:0 and C18:1.

Changes in unsaturated fatty acid composition varied between the strains assessed. Yeast strains A12 and K7 only showed significant increase in C18:1, while yeast strain A15 had a significant increase in C18:1 and a significant decrease in C16:1.

Change in the fatty acid composition may lead to a changed unsaturation index value. However, the results of the present study indicated that only K7 had a statistically significant higher unsaturation index (UI) value when the fermentation medium was supplemented with inositol, largely due to significantly higher proportion of C18:1. The other two strains did not show any significant change. For yeast strains A12 and A15, the changes in unsaturation index value were not statistically significant. Yeast strain A12 showed the largest change in fatty acid composition for C18:1 when the fermentation medium was supplemented with inositol, but no statistically significant change in the UI value was observed. A slight decrease in C16:1 for yeast strain A12 when the medium was supplemented with 0.1 g/L inositol might compensate the increase of C18:1, even though this was not observed for 0.05 g/L inositol Supplementation. For yeast strain A15, even though there is a change in fatty acid composition, the disappearance of C16:1 is compensated by increasing C18:1, which led to insignificant change in UI. These results indicate that the membrane compositional response to inositol supplementation is strain-specific, whereby each strain will respond in a somewhat different manner.

An effect of inositol supplementation of the medium on cell size was only detected for yeast strain A15. The cell size decreased significantly when the fermentation medium were supplemented with inositol. Yeast strain A12 and K7 did not show any changes in cell size as relayed to inositol. The present result confirm the finding of Jiranek, Graves & Henry (1998) that a mutant strain which accumulated inositol evidenced higher cell growth and decreased cell size.

However, the present study further demonstrated that the effect of inositol again strain-specific, since only one (A15) of the three strains assessed had decreased cell size in response to inositol supplementation.

At this point it is useful to compare the levels of inositol supplementation reported in the various studies, and to discuss the minimal effective level of inositol as presented in Table 5.5.

Table 5.5 Comparison of fermentation medium inositol supplementation levels tested in previous published studies and the present study and the effective concentrations observed. The concentrations are all given in mg/L for ease of comparison.

Study	Organism	Level of inositol supplementation (mg/L)	Level of inositol stimulating improvement (mg/L)	Further improvement with higher levels of inositol?
This study	3 strains of *S. cerevisiae*	0, 50, 100, 200, 400, 800	50	No
Chi *et al.* (1999)	*Saccharomyces* sp.	0, 100	100	Not applicable
Furukawa *et al.* (2004)	*S. cerevisiae*	1.8, 16	No improvement reported	Not applicable
Ji et al. (2008)	*Pachysolen tanniphilus*	0, 10, 50, 100, 150, 200	10	Yes, up to 100 mg/L but not higher

As noted earlier in this thesis, Chi, Kohlwein & Paltauf (1999) and Ji *et al*. (2008) noted improvements in several parameters in cultures grown in inositol-supplemented media, while Furukawa *et al.* (2004) did not. The lack of response observed in the latter study may be explained by the level of inositol supplementation of the medium tested, for which the maximum was 16 mg/L. This level of inositol supplementation of the medium is much lower than the level used in the present study, i.e. 50 mg/L. Thus, the Furukawa et al. (2004) study may not have sufficient level of inositol supplementation of the medium to observe an effect.

Chi, Kohlwein & Paltauf (1999) noted an improvement in parameters with the use of a single inositol concentration in the medium of 100 mg/L, in agreement with the finding of the present study and Ji *et al.* (2008), although using a different yeast species, found an improvement in parameters with increasing level of inositol supplementation of the medium up to 100 mg/L of inositol supplementation. As described in Chapters 4 and 5, the present study found a positive response to inositol supplementation for most parameters measured at our minimal level of 50 mg/L of inositol supplementation, although no further significant improvement was seen at the higher inositol levels tested. In contrast to the findings of Ji *et al.* (2008), we did not observe a decrease in performance with increasing levels of inositol supplementation of the medium, even at up to 800 mg/L inositol. Thus, it can be concluded that supplementation of the medium with 50 mg/L of inositol is sufficient to elicit the positive effects, and that adding more inositol does not significantly improve the response. It is generally considered that inositol is a necessary "vitamin" for the growth of yeast and defined media such as YNB contain 2 mg/L inositol, a concentration deemed necessary for growth. However, here we can differentiate between the proposed role of inositol as an essential growth factor and its capacity to improve fermentation performance and stress tolerance. To improve fermentation performance and stress tolerance seems to require a concentration of inositol in the medium that is an order of magnitude higher than that needed for growth.

5.4 Conclusions

Inositol supplementation of the medium seems to have a different effect on each yeast strain tested in the present study. Inositol supplementation of the medium greatly affected the fermentation performance of yeast strains A12 and K7 throughout the fermentation, while for yeast strain A15 it only seemed to have an

effect early in the respiro-fermentative phase of growth. Inositol supplementation of the medium significantly improved the glucose consumption and ethanol production of yeast strain A12 and K7. Even though yeast strain A15 produced the highest amount of ethanol, the presence or absence of inositol supplementation of the medium appeared to have no effect on these cells inositol supplementation had an effect on 24 h just not at 96 h (for glucose concentration and ethanol concentration). This could also be seen in the fermentation kinetic data, where only yeast strains A12 and K7 exhibited a significant increase in glucose consumption and ethanol production when the fermentation medium was supplemented with inositol.

Inositol supplementation of the medium also influenced glycerol production by yeast strains A12 and A15, for which an increase in extracellular glycerol concentration was noted, while in the case of yeast strain K7 no change in glycerol production in response to inositol supplementation of the medium was observed. These strain-specific effect of inositol supplementation of the medium are likely to be caused by the ability of inositol to stimulate the production of phosphoinositide based signalling molecules which may in turn stimulate the HOG pathway to up-regulate glycerol synthesis. K7 was the only yeast strain tested in this study to show a significant increase in the unsaturation index value when grown in fermentation medium supplemented with inositol. With respect to cell size, yeast strains A12 and K7 seemed to not be affected by growth in medium with inositol supplementation, while yeast strain A15 showed a significant decrease in cell diameter when grown in medium with inositol supplementation.

Based on the results of the present study, it can be concluded that the effect of inositol supplementation of the medium is strain-specific, i.e. each yeast strain responds differently to inositol supplementation of the medium in terms of the various parameters assessed. Inositol supplementation of the medium may

improve fermentation performance (yeast strains A12 and K7), increase glycerol production (yeast strains A12 and A15), increase the unsaturation index (yeast strain K7), and decrease cell size (yeast strain A15). However, inositol supplementation of the medium seems to have a general effect of improving the tolerance to several stresses (e.g. ethanol, acetic acid and hyperosmotic stresses), even though each yest strain had a different basal level of tolerance to the stress and magnitude of response to inositol supplementation of the medium in terms of tolerance to stresses. Finally, this study has demonstrated that an inositol supplementation level of 50 mg/L of inositol is sufficient to elicit these positive effects and that higher levels of supplementation up to 800 mg/L of inositol do not significantly enhance these positive effects.

CHAPTER SIX: GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Discussion

The present study investigates the role of inositol in yeast adaptation to stress. To be able to detect any differential effects of inositol on the cell physiology, stress tolerance and/or fermentation performance in the three *S. cerevisiae* strains investigated under the constraints of the chosen fermentation conditions an optimum initial concentration of glucose is required. An initial concentration of glucose is chosen such that in the absence of inositol supplementation of the medium each strain has a fermentation profile that can be distinguished from that of the other strains in terms of the time during the fermentation when the complete utilization of glucose occurs and/or the level of some residual glucose at the end of the fermentation. If the initial sugar concentration is too low ferments by each yeast strain may finish at close to the same time. On the other hand, if too high an initial sugar concentration is used then none of the ferments by the different yeast strain may complete in the time available. The optimal fermentation conditions should enable discrimination between the yeast strains based on their fermentation performance and the detection of improvements in response to inositol supplementation of the medium. Therefore, the first experiment was directed towards the determination of the optimal initial glucose concentration for this purpose.

The three yeast (*S. cerevisiae*) strains used in the present study were initially selected on the basis of different patterns of stress tolerance and ethanol productivity. The medium used was yeast nitrogen base (YNB), which was selected to enable the assessment of cellular membrane fluidity of the yeasts under the prevailing fermentation conditions in the fermentation medium at the time of

the fermentation sample was collected. The yeast strains were grown with three initial glucose concentrations, i.e. 5, 10 or 15% initial glucose concentration. YNB is a minimal medium and is considered a relatively nutritionally-poor medium compared to more complex media that contain components such as yeast extract and/or peptone (Ishmayana, Learmonth & Kennedy 2011). Therefore, the effects of hyperosmotic stress were observed in fermentations with 15% (w/v) initial glucose concentration, as indicated by stuck fermentation, whereas in rich media this phenomenon is usually observed only under what is termed very high gravity (VHG) condition, in which there is greater than a 27% (w/v) initial glucose concentration (Ishmayana, Learmonth & Kennedy 2011; Thomas, Hynes & Ingledew 1994). Thus, we have observed that the perceived intensity of a stress relates to the richness of the culture medium, as has also been observed in many previous studies (Northcott 1994). This consideration led us to standardize the media used for the assessment of stress tolerance.

The present study showed that compared to the growth, as indicated by final $OD_{600\ nm}$ value, of yeast strains A12 and A15, the growth of yeast strain K7 was the highest and the growth significantly decreased when the initial glucose concentration increased further from 5 to 10 and 15% (w/v) initial glucose concentration, i.e. there was a marked decrease in cell density at each time point as the initial glucose concentration increased (Figure 3.1).In addition, yeast strain K7 left the highest concentration of residual glucose in the fermentation medium when the medium contained 10 or 15% (w/v) initial glucose concentration (Figure 3.5). This result indicates that yeast strain K7 is the most susceptible strain to hyperosmotic stress. This was confirmed in a subsequent experiment that assessed the sensitivity of the yeast strains to hyperosmotic stress as yeast strain K7 exhibited the lowest relative growth of the three yeast strains when exposed to 27% (w/v) sorbitol (Figure 5.5). K7 only achieved about 60% relative growth when

the growth medium was not supplemented with inositol, although this increased to about 75% relative growth when inositol was used to supplement the fermentation medium. The other two yeast strains exhibited higher relative growth when exposed to 27% (w/v) sorbitol, which also increased when the fermentation medium was supplemented with inositol.

The present finding confirms that in nutritionally-poor media, yeast cells are more vulnerable to stresses. This is most evident when the yeast cells are grown under hyperosmotic conditions (i.e. when a relatively high initial concentration of glucose is used). Findings similar to those of the present study were reported by Krause *et al*. (2007). They presented a growth curve of a yeast strain grown in synthetic medium with two different initial glucose concentrations (v.i.z. 2 and 12% w/v) and it was observed that yeast cells grown in 12% (w/v) glucose exhibited a significantly lower growth curve, only achieving about half of the cell growth observed for the same strain when grown in medium with 2% (w/v) initial glucose concentration. However, in rich medium it was found that the cells could still maintain their high growth in media with up to a 30% (w/v) initial glucose concentration (Deesuth *et al.* 2015; Thomas & Ingledew 1990). Therefore, for industrial bioethanol production, in which a very high initial glucose concentration is used, rich media need to be used so that the yeast cells can survive under the hyperosmotic conditions. However, for studies that require a simple medium in order to investigate the effect of addition and omission of individual medium components, lower initial glucose concentrations should be used to avoid the induction of hyperosmotic stress.

The data presented in this study reveal that a standardized medium is required for investigation of the effect of medium supplementation so that the effect of supplementation can be easily determined. The present study proved that when the medium is not optimised it is hard to determine the effect of supplementation.

When the fermentation rate is too fast (i.e. the initial glucose concentration is too low), no difference may be observed with or without supplementation, and the same issue will be encountered if the fermentation rate is too slow (i.e. the initial glucose concentration is too high) as shown in Figure 3.5. Therefore, the present study provides a standardized condition for supplementation studies, i.e. the use of synthetic medium with 10% (w/v) initial sugar concentration.

Notwithstanding the differing impacts on cell final density, the maximum growth rate of each strain was not strongly affected by the initial glucose concentration no significant difference in the μ_{max} or most $OD_{600\ nm}$ values (Table 3.1). Similarly, the $OD_{600\ nm}$ values at 12 h were not markedly affected by the initial glucose concentration, although at later stages of the cultures some differences in $OD_{600\ nm}$ values can be observed, especially in the case of yeast strain K7 for which the $OD_{600\ nm}$ was only slightly decreased when grown in medium with a 10% (w/v) initial glucose concentration but significantly decreased when grown in medium with a 15% (w/v) initial glucose concentration (Figure 3.1, Table 3.1). Notwithstanding, it is clear that the three yeast strains used in the present study can achieve different maximum cell densities, such that yeast strain K7 can reach the highest cell density, followed by yeast strains A15 and A12. The $OD_{600\ nm}$ essentially measures the loss of transmission of incident light due to scatter, the magnitude of which is determined by: a) the cell density and b) the average cell size and shape. Thus, comparisons of $OD_{600\ nm}$ between different yeast strains need to take into account both of these factors. To check, if $OD_{600\ nm}$ is an accurate measure of cell density, the data in Figures 3.1, 3.3 and 3.4 as well as in Table 5.1 was used to calculate the ratios of $OD_{600\ nm}$ and the total cell count multiplied by the average cell size of the various strains relative to the reference strain at the 36 h timepoint. Relative to yeast strain A12, the ratios of $OD_{600\ nm}$ values were 1.40 for yeast strain K7 and 1.12 for yeast strain A15, while the ratios of cell density times

average cell size were 1.33 for yeast strain K7 and 1.26 for yeast strain A15. While these ratios are not identical, it can be concluded that the measurement of cell density using $OD_{600\ nm}$ is valid. It is interesting to note that at the 12 hour time point of the fermentation, yeast strain K7 showed relatively low $OD_{600\ nm}$ compared to the other two yeast strains, but reached the highest $OD_{600\ nm}$ of any of the three yeast strains at time point 36 hour (Table 3.1), which indicates that yeast strain K7 has a relatively higher growth rate at the late stages of the fermentation than the other two yeast strains.

This conclusion that yeast strain K7 has better growth later in the fermentation is supported by the cell viability and total viable cell count data (Figure 3.3 and Figure 3.4). Both cell viability and total viable cell values were higher for yeast strain K7 compared to yeast strains A12 and A15 during the later stages of the fermentation. Therefore, even though cell growth started to decline for all strains late in the fermentation, yeast strain K7 performed better in this respect than the other two yeast strains due to its higher cell viability. Yeast strain A15 showed an interesting phenomenon in that its cell viability decreased at an early stage of the fermentation, but was then maintained and relatively stable for a long period of time, while the cell viability of the other strains decreased gradually. This can explain the relatively high $OD_{600\ nm}$ value of yeast strain A15 at the later stage of the fermentation (Figure 3.1).

The parameters that were the most affected when the yeast strains were grown in media with different initial glucose concentrations were the glucose consumption and ethanol production. Higher initial glucose concentrations led to lower glucose consumption and consequently lower ethanol production. For all three of the yeast strains the ethanol production was most notably decreased when the initial glucose concentration was increased from 10 to 15% (w/v) (Table 3.3). This is most likely due to the hyperosmotic stress experienced by the cells which

led to a decreased ability to ferment glucose to ethanol (da Cruz, Batistote & Ernandes 2003; Ishmayana, Learmonth & Kennedy 2011). Since the objective of the present study was to differentiate between the fermentation performances of the three yeast strains and to assess the potential for enhancement of fermentation performance by inositol supplementation of the medium, it seemed that a 5% (w/v) initial glucose was too low with all ferments effectively concluded within 36 h and with little apparent difference between yeast strain, while a 15% (w/v) initial glucose concentration was too high with all ferments stuck and with 34 to 51% of the initial glucose remaining and with 0 to 4.4% cell viability (Table 3.2, Figure 3.3, and Figure 4.3). Therefore, we decided that a 10% (w/v) initial glucose concentration was an appropriate concentration for further study, with yeast strain A15 completing the fermentation in about 84 h, yeast strain A12 completing the fermentation at more or less the end of the experimental period (168 h) and yeast strain K7 not completing the fermentation with about 26% of the initial sugar remaining. At this initial concentration of glucose, the fermentation performance of each yeast strain could easily be differentiated and there was potential for improvement of the fermentation performance of each yeast strain upon inositol supplementation of the medium.

To further characterize the experimental system, a preliminary experiment was conducted using a 15 % (w/v) initial glucose concentration with only yeast strain A15. In this experiment it was seen that inositol supplementation of the medium increased membrane fluidity (as indicated by a lower generalized polarization value for yeast cell with laurdan-labelled cellular membranes) (Figure 4.4). The preliminary experiment was only performed in duplicate, therefore statistical analysis of the data could not be performed. For the main experiment, three biological replicates were performed, thereby allowing statistical analysis of the data. The results of the main inositol supplementation experiment confirmed

the results of the preliminary experiment in that as measured at 24-hour time point a significantly lower generalized polarization value was observed for the cellular membranes of laurdan-labelled cells of yeast strain A15 when the cells were grown in medium supplemented with inositol (Figure 4.11B). Besides yeast strain A15, the other two yeast strains (A12 and K7) were assessed as well (Figure 4.11 A and C). It seems that each yeast strain responds differently to inositol supplementation of the medium. For yeast strain A12 the only statistically significant change was an increase in the generalized polarization of the membranes of laurdan-labelled cells, indicating a decrease in membrane fluidity, at the highest level of inositol supplementation (0.80 g/L). Yeast strain K7 showed a significantly higher generalized polarization, indicating a decrease in membrane fluidity, at all levels of inositol supplementation of the medium. Thus, for another stress the responses to inositol supplementation of the medium were different, with two yeast strains (K7 and A12) exhibiting a decrease in membrane fluidity and the other yeast strain (A15) exhibiting an increase in membrane fluidity. When we compare this finding to the fatty acid composition (Figure 5.8) and unsaturation index values (Table 5.4), some anomalies appear.

It was noted earlier that the fatty acid composition may correlate with the fluidity of the cellular membranes, although other factors, such as protein and sterol composition, may also affect the membranes fluidity (Alexandre, Rousseaux & Charpentier 1994; Learmonth 2012). Inositol supplementation of the medium seemed, in general, to result in a decrease in the proportion of saturated fatty acids, with the exception of C18:0, which increased (Figure 5.8). When unsaturated fatty acids were considered, although yeast strains K7 and A15 exhibited decreased proportions of C16:1, all yeast strains exhibited higher proportions of C18:1.

The changes in the proportions of individual fatty acids seemed to largely balance out with no significant change in UI for yeast strains A12 and A15. The

data for yeast strain A12 are consistent, in that the cellular membrane fluidity of laurdan-labelled cells was not significantly changed at the levels of inositol supplementation of the medium (0.05 and 0.10 g/L) at which the fatty acid proportions and UI were assessed. However, when grown in medium supplemented with inositol yeast strain K7 had substantially and significantly higher UI values (Table 5.4), which is usually assumed to indicate an increase in membrane fluidity, while in contrast, the generalized polarization data for laurdan-labelled cells indicated significant decrease in cellular membrane fluidity when the cells were grown in media with inositol supplementation (Figure 4.11(C)). For yeast strain A15, the unsaturation index value showed no statistically significant change when the yeast strain was grown in media with inositol supplementation (Table 5.4), while generalized polarization data showed a significant decrease indicating more fluid cellular membranes of laurdan-labelled cells when grown in media with inositol supplementation (Figure 4.11(B)). Like for yeast strain K7, the data obtained for yeast strain A15 also inconsistent between unsaturation index and generalized polarization data. Thus, we observed a conflict between the membrane fluidity inferred from fatty acid composition data and the fluidity assessed by generalized polarization of laurdan-labelled cells. The present study confirms that, while it may be considered a useful indicator, unsaturation index alone cannot be used as a measure of membrane fluidity. Membrane fluidity may be affected by numerous other membrane compositional, regulatory and/or environmental factors.

It is well-known that when yeast cells are exposed to ethanol, the yeast cellular membranes become more fluid, due to the fluidizing effect of ethanol (Alexandre, Rousseaux & Charpentier 1994; Ding *et al.* 2009). In the present study we examined the baseline membrane fluidity, the magnitude of the ethanol-initiated decrease in cellular membrane fluidity and whether inositol may have a protective

effect against the ethanol fluidization. The preliminary experiment indicated that in the case of yeast strain A15 inositol-supplementation of the medium led to a higher baseline membrane fluidity (i.e. a lower generalized polarization value) and a more substantial decrease in generalized polarization (i.e. increase in membrane fluidity) upon ethanol addition (Figures 4.4 and 4.5 and Table 4.2). This pattern was also seen for yeast strain A15 in the main experiment, which had more biological replicates and a more robust statistical analysis (Figures 4.11 and 4.12 and Table 4.6). In contrast, yeast strain A12, as noted above, tended to have higher generalized polarization values, indicating a lower level of membrane fluidity, when grown in inositol-supplemented medium but this difference was only statistically significant at 0.80 g/L of inositol supplementation. Despite the slightly lower baseline cellular membrane fluidity, yeast strain A12 also had a more pronounced decrease in generalized polarization (increase in fluidity) upon ethanol addition. Notably, yeast strain K7 had a significantly higher generalized polarization (lower fluidity) at all levels of inositol supplementation of the medium and furthermore had a smaller decrease in generalized polarization (increase in fluidity) upon addition of ethanol, thus its lower cellular membrane fluidity helped to limit the fluidizing effect of ethanol. After the initial shock of the added ethanol, the three yeast strains had somewhat different capacities to recover the level of membrane fluidity that they had exhibited prior to the ethanol exposure. Interestingly, for yeast strain A12, the generalized polarization value after ethanol exposure increased gradually to reach a value equal to the baseline value of this strain even without inositol supplementation of the medium (Figure 4.12A). The results of the cellular membrane fluidity measurements indicate that even though inositol-supplementation of the medium affects the membrane fluidity, it does not inhibit the dynamic systems of the yeast cells which give them the ability to actively modulate their level of membrane fluidity.

Based on the results of the present study, in general, inositol-supplementation of the medium does not affect the ethanol yield (mg ethanol per mg glucose), however it does affect glucose consumption and ethanol productivity (the rate of ethanol production), except for yeast strain A15 for which the differences in the kinetic parameters between cells grown with and without inositol-supplementation of the medium were not statistically significant (Table 5.1, Table 5.2 and Table 5.3). Even though the ethanol yield parameter of none of the strains was affected by inositol-supplementation of the medium, the final ethanol concentration achieved was significantly increased when the fermentation medium was supplemented with inositol for yeast strains A12 and K7 (Figure 5.2 and Table 5.1 and 5.3). In agreement with the fermentation kinetic data, yeast strain A15 did not show any statistically significant difference in ethanol concentration with or without inositol-supplementation of the medium. The results of the present study also indicate that the effect of inositol supplementation of the medium on fermentation performance are strain-specific, with some yeast strains significantly affected by inositol-supplementation of the medium (yeast strains A12 and K7) and others not affected (yeast strain A15).

The effect of inositol-supplementation of the medium on yeast that was most anticipated was its effect on stress tolerance. Several published studies have highlighted the effect of inositol-supplementation of the medium on ethanol tolerance, but have not investigated the effect on the tolerance to other stresses (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Krause *et al.* 2007). Therefore, in the present study, in addition to ethanol stress, we also examined the effects of inositol-supplementation of fermentation medium on hyperosmotic and acetic acid stresses. The results obtained in the present study indicate that all three yeast strains assessed had a significantly increased tolerance to each of the stresses tested when grown in medium with inositol supplementation. The

enhancement of tolerance by growth in medium with inositol was greater for exposure to ethanol and acetic acid stresses than for exposure to hyperosmotic stress. Yeast strain K7 only had about a 2% relative growth when exposed to acetic acid stress but this increased to about 10% relative growth when grown in medium supplemented with inositol, which was still much lower, however, than that of the other yeast strain grown in medium supplemented with inositol. This result indicates that yeast strain K7 is the most susceptible strain to acetic acid, although growth in medium supplemented with inositol had a positive effect. Yeast strain K7 was also the most susceptible strain to exposure to ethanol and hyperosmotic stresses. Thus, it can be seen again that the effects of inositol supplementation of the medium are strain-dependent. Furthermore, this report confirmed the previous reports that inositol-supplementation of the medium enhances ethanol tolerance and, in addition, extends the previous research to reveal that inositol-supplementation of the medium increases the tolerance to a range of other stresses.

It is well known that glycerol synthesis increases in response to high osmotic pressure. This was also confirmed in the present study, where use of a higher initial glucose concentration in the fermentation medium led to in increase in glycerol production and higher extracellular glycerol concentration for the three yeast strains studied (Figure 3.7 and Figure 5.7). The increase in glycerol production could have potentially affected the ability of the yeast to produce ethanol, although the data shown in Table 3.3 tend to indicate that, in general, glycerol production did not greatly affect ethanol production. Notwithstanding, there were some exceptions to this generalization, for example less ethanol was observed at the end of the fermentation when the ferments contained a 15% (w/v initial glucose concentration (Figure 3.6 and Figure 4.3).

The effect of inositol-supplementation of the medium on glycerol synthesis was also examined in the present study. Inositol-supplementation of the medium seemed to result in an increase in glycerol synthesis by yeast strains A12 and A15, as indicated by a significantly higher extracellular glycerol concentration when the cells were grown in medium with inositol supplementation (Figure 5.7). In contrast, yeast strain K7 did not show any difference in extracellular glycerol concentration when cultured in media without or with inositol supplementation. This is consistent with the low tolerance to hyperosmotic stress of yeast strain K7 (Figure 5.5).

The present study also found some evidence that inositol supplementation of the medium may affect cell size. However, only one yeast strain, yeast strain A15, exhibited a change in cell size, which was decreased in cell diameter, in response to growth in medium with inositol supplementation. This result is consistent with the previously published results of Jiranek, Graves & Henry (1998). Furthermore, the present study demonstrated that the effect of inositol-supplementation of the medium is not the same for all yeast strains on cell size.

In terms of all the parameters examined in the present study, yeast strain A15 seems to be the most promising for further development for use in ethanol production. High ethanol production by the yeast strain is considered the most important commercial consideration in ethanol production. Even though in terms of stress tolerance yeast strain A12 performed better than yeast strain A15, yeast strain A15 still had reasonable stress tolerance and, importantly, produced the highest concentration of ethanol of any of the three yeast strains assessed. Therefore, we recommend that further studies on the optimization of bioethanol production should use this yeast strain.

6.2 Conclusions and Contribution of This Study

The data collected in the present study led us to some important conclusions. For growth medium supplementation studies, a 10% (w/v) initial glucose concentration in a chemically-defined fermentation medium is the optimum medium for investigation of the effect of inositol supplementation of the growth medium on the ethanolic fermentation. By using this initial glucose concentration, we can clearly observe the effect of supplementation of the medium with a particular compound, since use of a lower initial glucose concentration results in all the glucose being consumed before we can observe any effect of supplementation of the growth medium, while the use of a higher glucose concentration in the fermentation results in a stuck fermentation and no effect of supplementation of the growth medium can be observed.

Inositol is already known to have important roles in yeast cell growth and fermentation performance. However, the exact concentration at which inositol elicits these positive effects has not yet been elucidated. As summarized in Table 5.5, some studies used very low concentrations of inositol and a limited range of inositol concentration (Krause et al 2007; Chi et al. 1999; Ji et al. 2008). One study which reported that when too high a concentration of inositol is present in the fermentation medium, it can have negative effects, such as inhibit cell growth (Ji et al 2008). The present study used a wider range of inositol concentrations, from 0.05 – 0.80 g/L. However, no negative effects were observed even at the highest level of inositol supplementation of the fermentation media, which was a higher concentration of inositol than that used in the previous studies (Ji et al 2008; Krause et al 2007; Chi et al 199). In the range of inositol concentration used in the present study, no statistically significant differences in term of cell growth or fermentation performance were observed between cells grown in media with low or high levels of inositol supplementation. Therefore, we conclude that a relatively

low level of inositol supplementation of the medium, 0.05 g/L, is sufficient to elicit the positive effect in terms of enhancing the fermentation performance of yeast cells.

Based on all the parameters investigated in the present study, the effects of growth in medium supplemented with inositol seem to be strain-specific. This was observed for the membrane fluidity parameter (generalized polarization of laurdan-labelled yeast cells, membrane fatty acid unsaturation index), extracellular glycerol concentration, cell growth and, most importantly, fermentation performance. However, while all of these effects may be of different magnitudes in the individual yeast strains studied, there was a generalized effect of growth in medium supplemented with inositol in promoting increased stress tolerance in the yeast strains used in the present study. Growth in medium supplemented with inositol increased the tolerance to ethanol, hyperosmotic and acetic acid stresses for all yeast strains. The present study, besides confirming the previous reports that growth in medium supplemented with inositol protects yeast cells against ethanol stress, also found that growth in medium supplemented with inositol can protect yeast cells against other stresses. This led us to conclude that inositol, or the biomolecules it is converted into in cells, plays an important role as a general stress protector.

6.3 Future Directions

Yeast stress tolerance is one of the factors that can have important effects on ethanol fermentation performance. Growth in medium supplemented with Inositol, which is known to elicit protection against stress, is recommended with the inositol to be added as a supplement to the fermentation media at a level of 0.05 g/L . Considering the possible mechanisms for the positive effects of inositol we note that it is also required for the synthesis of phosphatidyl inositol, one of phospholipid

components of the yeast cellular membranes and a precursor for the synthesis of important signalling molecules. Therefore, further investigation following up on the present study could be directed towards:

1. *Investigate the effects of supplementation of the medium with inositol on the phospholipid class composition of cellular membranes*

The presence of free inositol in the fermentation medium may affect the phospholipid composition of the cellular membranes. This possible effect would be due to the availability of inositol for phospatidylinositol synthesis. However, in the present study, the impact of inositol on the phospholipid composition of the cellular membranes was not assessed, due to time constraints. Many parameters examined in the present study indicated that responses to inositol-supplementation of the medium are strain specific. Therefore, it is recommended that further investigations include testing effect on multiple yeast strains.

2. *Investigate the effects of supplementation of the medium with inositol on the activity of the plasma membrane H^+-ATPase*

Furukawa *et al*. (2004) suggested that inositol may affect the activity of the plasma membrane H^+-ATPase. The plasma membrane H^+-ATPase is a key transporter regulating ionic balance and essential for creating a proton gradient across the plasma membrane that is utilized for secondary transport of ions and nutrients into the cell. This enzyme is also a key determinant of stress tolerance and its activity known be affected by the phospholipid composition of the plasma membrane (Furukawa et al 2004). Thus, a change in the phospholipid composition of the plasma membrane may also change the activity of the plasma membrane H^+-ATPase, since most of the enzyme structure is embedded in the phospholipid bilayer of the plasma membrane phospholipid and therefore a change in the

phospholipid bilayer environment may also affect the structure and therefore the activity of the enzyme. Therefore, investigations to reveal how changes in the phospholipid composition of the plasma membrane can affect the activity of the plasma membrane H^+-ATPase will lead to a better understanding of the role of this crucial enzyme in cell physiology and in adaptation to environmental stresses.

3. *Investigate the effects of supplementation of the medium with inositol on fermentation performance in rich fermentation medium under VHG conditions*

The present study used a chemically defined fermentation medium, so that the effect of growth in medium supplemented with inositol could be easily differentiated from the effects of other nutritional components, as well as to enable assessment of membrane fluidity under the prevailing conditions in the culture. However, such chemically-defined fermentation media are unsuitable for practical application in the bioethanol industry since they are considered poor media which cannot support high fermentation performance, especially when VHG fermentation is required. Therefore, it will be interesting to investigate whether inositol-supplementation of rich media can also increase ethanol production.

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APPENDIX 1
RECIPIES FOR MEDIA USED IN THIS STUDY

Appendix 1.1 Recipes for media

Yeast Extract Peptone Medium (YEP)

- 0.5% Yeast extract (w/v) from Difco
- 0.5% Bacteriological peptone (w/v) from Difco
- 0.3% $(NH_4)_2SO_4$ (w/v)
- 0.3% KH_2PO_4 (w/v)
- 1% Glucose (w/v)
- Made up with Milli-Q water

Note: For YEP agar, 1.5% bacteriological agar was added

10×Yeast Nitrogen Base (YNB) with 2% glucose

- 6.7 g Bacto yeast nitrogen base from Difco
- 100 mL Milli-Q water

Filter sterilized using a 0.22 μm membrane filter into a sterile Schott bottle. Stored at 4°C until required. D-glucose stock solution was made at a concentration of 50% (w/v), filter sterilized and added to the media to achieve the required concentration.

10× Yeast Nitrogen Base (YNB) without inositol

- 6.9 g yeast nitrogen base without inositol and amino acid (ForMedium)
- 0.05 g amino acid mixture (Sunrise Science)
- 100 mL Milli-Q water

Filter sterilized using a 0.22 μm membrane filter into a sterile Schott bottle. Stored at 4°C until required. D-glucose stock solution was made at a concentration of 50% (w/v), filter sterilized and added to the media to achieve the required concentration. Inositol stock solution was made at a concentration of 10 g/L, filter sterilized and added to the media to achieve the required concentration.

Appendix 1.2 Formula for media with varying initial glucose concentrations

Initial glucose concentration (g/L)	10× YNB (mL)	Glucose stock solution* (mL)	MilliQ water (mL)	Final Volume (mL)
5	5	5	40	50
10	5	10	35	50
15	5	15	30	50

Note : * = Glucose stock solution concentration is 50% (w/v)

Appendix 1.3 Formula for media with inositol supplementation (for 10% initial glucose concentration)

Inositol Concentration (g/L)	10× YNB without inositol (mL)	Glucose stock solution# (mL)	Inositol stock solution## (mL)	MilliQ water (mL)	Final Volume (mL)
0	5	10	0	35	50
0.05	5	10	0.25	34.75	50
0.1	5	10	0.5	34.5	50
0.2	5	10	1	34	50
0.4	5	10	2	33	50
0.8	5	10	4	31	50

Note : # = Glucose stock solution concentration is 50% (w/v)
= Inositol stock solution concentration is 10 g/L

APPENDIX 2
RAW DATA USED TO CREATE GRAPHS AND FIGURES IN THIS THESIS

Appendix 2.1 Raw data from experiment monitoring optical density at 600 nm. Cultures were grown under aerobic conditions in YNB medium with the initial glucose concentration indicated in the table. Fermentation was conducted at 30°C and 180 opm. The data represent the means of three independent experiments. (Figure 3.1)

Mean value of $OD_{600\ nm}$

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	0.122	0.124	0.128	0.092	0.088	0.094	0.188	0.175	0.149
6	1.184	1.116	1.127	0.573	0.566	0.684	0.989	0.927	1.022
12	3.036	2.967	2.918	2.687	2.743	2.785	3.075	3.013	3.052
18	3.633	3.582	3.482	4.469	4.049	4.028	3.922	3.877	3.833
24	3.863	3.824	3.799	5.255	5.030	4.778	4.326	4.295	4.298
30	3.995	3.949	3.918	5.695	5.423	5.034	4.487	4.412	4.458
36	4.081	4.093	4.002	5.977	5.744	5.327	4.623	4.591	4.566
48	4.121	4.176	4.072	6.014	5.791	5.424	4.624	4.876	4.715
60	4.074	4.144	4.014	6.148	5.757	5.444	4.600	4.897	4.737
72	3.971	4.138	3.949	6.094	5.620	5.478	4.716	4.967	4.928
84	4.070	4.246	3.951	6.348	5.909	5.568	4.781	5.177	4.951
96	3.830	3.966	3.976	6.210	5.963	5.572	4.747	5.237	5.000
108	4.099	4.281	4.060	6.265	6.055	5.588	4.781	5.145	5.118
120	4.071	4.359	4.089	6.296	6.089	5.594	4.765	5.120	5.149
132	4.102	4.357	4.143	6.347	6.116	5.674	4.763	5.167	5.191
144	4.094	4.431	4.199	6.381	6.202	5.711	4.809	5.202	5.210
156	4.201	4.514	4.279	6.478	6.268	5.797	4.918	5.276	5.277
168	4.236	4.575	4.332	6.600	6.380	5.862	4.971	5.403	5.326

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v) unit

Standard Deviation

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	0.008	0.013	0.007	0.016	0.012	0.021	0.048	0.028	0.019
6	0.324	0.295	0.398	0.152	0.202	0.183	0.287	0.254	0.205
12	0.217	0.202	0.161	0.548	0.501	0.217	0.192	0.213	0.114
18	0.145	0.095	0.075	0.454	0.230	0.084	0.183	0.203	0.064
24	0.135	0.124	0.007	0.436	0.275	0.106	0.278	0.297	0.047
30	0.101	0.030	0.015	0.354	0.241	0.136	0.256	0.300	0.064
36	0.094	0.100	0.056	0.235	0.187	0.101	0.152	0.187	0.054
48	0.043	0.072	0.022	0.143	0.202	0.198	0.131	0.084	0.045
60	0.109	0.066	0.036	0.183	0.107	0.248	0.079	0.210	0.181
72	0.199	0.142	0.112	0.375	0.260	0.151	0.130	0.137	0.059
84	0.150	0.120	0.116	0.113	0.164	0.196	0.150	0.056	0.066
96	0.420	0.337	0.066	0.106	0.173	0.136	0.182	0.182	0.048
108	0.132	0.074	0.066	0.135	0.191	0.209	0.118	0.183	0.054
120	0.141	0.138	0.069	0.171	0.185	0.154	0.160	0.182	0.066
132	0.137	0.141	0.087	0.200	0.183	0.234	0.204	0.198	0.085
144	0.154	0.112	0.165	0.276	0.252	0.322	0.126	0.257	0.165
156	0.134	0.096	0.240	0.212	0.283	0.264	0.183	0.227	0.048
168	0.197	0.116	0.277	0.188	0.301	0.212	0.234	0.219	0.143

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Appendix 2.2 Raw data from experiment monitoring viability. Cultures were grown under aerobic conditions in YNB medium with the initial glucose concentration indicated in the table. Fermentation was conducted at 30°C and 180 opm. The data represent the means of three independent experiments. (Figure 3.3)

Mean value of viability (%)

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	78.7	81.2	81.1	95.2	95.4	95.6	50.7	49.2	51.8
6	96.0	96.3	96.0	96.9	94.1	96.0	89.1	88.0	86.1
12	79.8	80.0	69.2	94.3	96.5	94.3	60.8	56.8	49.5
18	72.3	70.5	71.3	91.9	86.1	80.5	57.4	53.6	46.9
24	60.7	58.2	60.2	84.1	79.8	74.6	56.0	53.7	45.7
30	55.8	51.7	53.0	80.8	75.7	74.4	54.1	48.9	45.8
36	50.7	48.2	51.2	76.7	75.8	71.8	51.2	48.6	46.1
48	41.4	40.2	40.9	72.5	66.4	65.1	49.9	48.8	45.2
60	35.0	33.0	26.9	64.4	51.7	53.7	47.2	48.1	46.1
72	28.6	27.3	18.9	59.0	34.6	45.2	39.4	45.5	41.7
84	25.6	25.1	15.7	43.2	23.6	37.2	34.3	43.2	38.2
96	18.5	21.6	11.5	5.5	19.7	28.9	23.4	27.8	33.9
108	13.6	18.3	11.0	0.2	14.8	25.0	11.9	3.5	29.3
120	7.7	12.0	8.4	0.0	10.0	17.7	4.8	0.1	16.9
132	3.4	9.4	12.3	0.0	2.0	3.8	2.5	0.0	9.9
144	1.3	6.1	7.8	0.0	0.0	0.0	1.7	0.1	4.3
156	0.8	4.6	7.6	0.0	0.0	0.0	1.0	0.1	2.0
168	0.1	2.0	4.4	0.0	0.0	0.0	0.2	0.0	0.8

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Standard deviation

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	3.5	6.3	5.3	0.7	2.3	2.1	6.2	6.2	5.4
6	1.0	1.3	0.6	0.7	2.3	3.2	2.9	1.8	1.1
12	2.3	3.9	3.8	4.6	3.4	1.3	5.4	5.4	1.3
18	1.7	3.2	1.9	5.1	9.5	4.8	0.7	4.0	1.6
24	1.1	1.3	3.0	7.7	3.0	3.4	2.1	1.8	3.4
30	4.3	6.0	1.7	7.5	1.9	5.9	2.5	2.1	3.6
36	5.3	4.6	2.3	8.9	3.7	2.8	1.3	2.7	0.9
48	5.1	2.9	4.4	12.0	4.7	4.0	1.1	1.2	0.6
60	4.7	1.1	2.8	11.8	5.7	1.5	0.9	1.1	3.8
72	0.9	2.7	1.7	16.1	10.1	6.3	2.3	2.3	1.3
84	0.9	2.2	2.2	22.9	2.8	7.5	4.0	1.4	0.3
96	3.5	0.6	1.2	4.7	1.6	4.5	6.4	3.4	1.2
108	3.8	1.0	2.5	0.2	1.7	2.8	4.2	1.8	0.8
120	2.1	4.3	0.7	0.1	3.9	5.2	3.1	0.2	4.3
132	1.0	4.0	4.7	0.0	2.1	1.3	1.8	0.0	3.0
144	1.7	5.7	1.6	0.0	0.0	0.0	1.5	0.1	1.8
156	0.5	4.9	2.7	0.0	0.0	0.0	0.9	0.1	0.4
168	0.2	3.3	4.5	0.0	0.0	0.0	0.4	0.0	0.8

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Appendix 2.3 Raw data from experiment monitoring viable cell count. Cultures were grown under aerobic conditions in YNB medium with the initial glucose concentration indicated in the table. Fermentation was conducted at 30°C and 180 opm. The data represent the means of three independent experiments. (Figure 3.4)

Mean of viable cell count ($\times 10^6$)

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	0.47	0.47	0.51	0.60	0.55	0.55	0.54	0.54	0.52
6	9.11	8.85	7.35	4.88	4.65	5.74	6.07	5.40	7.08
12	34.13	33.03	32.50	32.77	34.43	38.53	26.70	24.70	23.93
18	35.03	32.70	36.80	54.10	43.23	42.87	32.27	31.97	27.27
24	31.87	27.77	29.57	43.90	48.73	35.03	34.87	33.07	29.07
30	28.13	29.10	27.70	54.73	45.60	40.57	30.93	28.40	29.20
36	22.33	24.37	28.07	57.67	55.17	53.23	28.87	28.20	28.73
48	20.73	25.00	23.17	65.03	52.17	46.13	31.53	29.30	31.43
60	18.70	18.33	13.93	52.53	40.30	39.03	29.00	35.30	34.97
72	16.17	15.27	10.67	50.17	27.13	40.63	26.90	32.23	29.57
84	13.93	14.17	9.07	33.33	17.83	34.23	22.80	31.67	27.43
96	9.93	12.37	6.13	4.30	14.40	23.37	14.10	17.87	24.27
108	6.70	11.43	5.77	0.13	11.60	20.07	7.67	2.13	22.23
120	4.10	6.30	4.20	0.03	7.77	13.30	2.73	0.07	11.50
132	1.77	4.87	7.43	0.00	1.30	2.83	1.43	0.00	6.53
144	0.73	3.40	4.20	0.00	0.00	0.00	0.90	0.03	3.03
156	0.40	2.17	4.53	0.00	0.00	0.00	0.60	0.03	1.43
168	0.07	0.93	2.73	0.00	0.00	0.00	0.13	0.00	0.50

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Standard deviation

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	0.04	0.06	0.05	0.21	0.21	0.11	0.07	0.02	0.03
6	3.87	3.33	2.52	1.05	1.39	2.81	1.95	1.22	2.53
12	4.02	7.52	3.89	10.14	7.31	8.71	2.63	3.14	6.95
18	4.45	5.86	10.00	25.15	14.11	20.05	2.80	4.28	5.55
24	2.18	7.58	2.11	5.63	7.47	7.31	7.95	7.17	3.93
30	4.44	7.69	3.28	9.74	7.77	1.95	5.65	2.88	6.36
36	1.02	0.59	8.78	15.44	9.11	12.46	2.28	4.16	6.21
48	2.94	1.71	3.38	17.44	5.66	6.99	2.18	4.73	1.75
60	1.70	1.67	3.05	4.95	5.71	8.95	1.45	2.60	7.39
72	3.05	3.62	3.14	15.87	13.58	12.83	5.83	4.97	4.12
84	1.84	3.66	2.95	20.15	2.69	10.46	4.20	4.45	3.98
96	3.55	1.63	0.85	3.83	2.23	5.99	4.73	3.12	3.76
108	1.95	1.12	1.94	0.12	2.96	3.06	2.82	1.06	3.05
120	1.57	2.00	0.69	0.06	3.45	3.93	1.77	0.12	1.82
132	0.61	1.42	3.71	0.00	1.35	1.00	1.07	0.00	1.72
144	1.01	2.98	1.40	0.00	0.00	0.00	0.82	0.06	1.05
156	0.30	1.87	2.58	0.00	0.00	0.00	0.56	0.06	0.25
168	0.12	1.53	3.04	0.00	0.00	0.00	0.23	0.00	0.46

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Appendix 2.4 Raw data from experiment monitoring glucose concentration. Cultures were grown under aerobic conditions in YNB medium with the initial glucose concentration indicated in the table. Fermentation was conducted at 30°C and 180 opm. The data represent the means of three independent experiments. (Figure 3.5)

Mean of glucose concentration (%w/v)

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	4.443	9.248	14.153	4.308	8.882	14.093	3.704	9.595	14.634
6	4.046	8.104	12.798	3.688	8.336	12.301	3.457	8.754	12.403
12	2.924	7.745	12.352	2.907	8.190	11.652	2.619	7.993	11.616
18	1.832	6.395	11.204	1.787	7.033	10.989	1.667	6.881	10.908
24	1.044	5.342	10.795	1.256	6.046	10.526	1.193	6.266	10.421
30	0.589	4.730	9.464	0.711	5.036	9.648	0.644	5.848	10.117
36	0.214	4.301	8.777	0.288	4.336	9.603	0.111	4.449	9.733
48	0.118	3.432	8.705	0.086	3.796	8.432	0.054	3.288	9.129
60	0.109	2.996	8.228	0.080	3.392	8.769	0.057	2.305	8.772
72	0.108	2.294	7.853	0.072	2.960	8.616	0.054	1.026	7.116
84	0.104	1.829	7.714	0.064	2.662	8.401	0.054	0.071	6.441
96	0.085	1.247	7.633	0.056	2.548	8.118	0.053	0.045	5.578
108	0.077	0.977	7.380	0.055	2.506	8.342	0.054	0.043	5.352
120	0.085	0.642	7.353	0.054	2.439	8.286	0.053	0.044	5.311
132	0.086	0.524	7.142	0.054	2.414	7.892	0.052	0.044	5.106
144	0.087	0.329	6.856	0.056	2.382	7.902	0.053	0.045	4.944
156	0.081	0.269	6.878	0.054	2.274	7.843	0.047	0.044	4.701
168	0.073	0.245	6.716	0.053	2.284	7.630	0.053	0.043	4.854

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Standard deviation

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	0.361	1.005	1.489	1.219	1.239	1.643	1.130	0.559	0.880
6	0.735	0.981	1.364	1.686	1.479	1.067	1.231	0.780	1.140
12	0.399	0.453	1.258	1.231	0.742	0.898	0.897	1.104	1.122
18	0.233	0.531	1.267	0.944	1.035	0.590	0.667	0.886	1.029
24	0.121	0.272	2.082	0.580	1.382	1.172	0.445	1.067	1.200
30	0.096	0.311	1.017	0.307	1.237	1.303	0.244	0.848	1.096
36	0.058	0.416	1.180	0.182	1.494	0.989	0.078	0.400	0.941
48	0.012	0.226	0.700	0.010	1.192	1.599	0.008	0.552	0.467
60	0.007	0.346	0.557	0.011	0.860	1.293	0.003	0.297	0.951
72	0.010	0.515	0.585	0.006	1.179	1.270	0.010	0.407	1.009
84	0.005	0.520	0.617	0.005	1.359	1.510	0.009	0.033	0.771
96	0.002	0.496	0.636	0.002	1.452	1.558	0.008	0.008	0.652
108	0.014	0.492	0.715	0.002	1.411	0.916	0.009	0.007	0.673
120	0.005	0.502	0.681	0.003	1.327	0.930	0.008	0.005	0.602
132	0.004	0.380	0.677	0.002	1.247	1.445	0.007	0.005	0.739
144	0.003	0.278	0.713	0.005	1.194	1.169	0.006	0.004	0.836
156	0.012	0.186	0.690	0.004	1.081	0.989	0.006	0.003	0.986
168	0.012	0.138	0.803	0.006	0.931	0.795	0.008	0.000	0.807

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Appendix 2.5 Raw data from experiments monitoring ethanol concentration. Cultures were grown under aerobic conditions in YNB medium with the initial glucose indicated in the table. Fermentation was conducted at 30°C and 180 opm. The data represent the means of three independent experiments. (Figure 3.6)

Mean of ethanol concentration (%w/v)

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	0.026	0.020	0.018	0.016	0.019	0.013	0.030	0.023	0.019
6	0.165	0.153	0.177	0.110	0.113	0.099	0.137	0.116	0.098
12	0.613	0.583	0.468	0.419	0.520	0.417	0.544	0.445	0.418
18	0.760	0.738	0.536	0.692	0.667	0.514	0.485	0.596	0.481
24	0.950	0.950	0.812	0.835	0.818	0.725	0.848	0.711	0.621
30	0.993	0.989	0.918	0.906	1.196	0.769	1.056	1.001	0.703
36	0.944	1.245	1.060	0.963	1.106	0.938	1.167	1.017	0.858
48	0.990	1.359	0.767	1.070	1.092	1.107	0.973	1.170	0.950
60	0.893	1.366	0.828	0.993	1.468	1.321	0.916	1.655	1.077
72	0.785	1.438	0.761	1.075	1.604	1.343	0.869	2.009	1.540
84	0.733	1.664	0.864	0.929	1.678	1.214	0.792	2.072	1.675
96	0.688	1.640	0.758	0.924	1.626	1.140	0.756	2.186	1.667
108	0.663	1.635	0.723	0.820	1.464	1.056	0.559	2.121	1.622
120	0.560	1.539	0.715	0.741	1.266	0.955	0.529	2.023	1.421
132	0.559	1.356	0.596	0.578	1.073	0.952	0.486	1.766	1.333
144	0.459	1.318	0.491	0.485	1.092	0.675	0.407	1.633	1.130
156	0.342	1.097	0.400	0.398	0.937	0.661	0.305	1.378	1.047
168	0.264	0.947	0.298	0.318	0.699	0.533	0.249	1.296	0.859

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Standard deviation

Time (hours)	A12			K7			A15		
	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)	(5%)	(10%)	(15%)
0	0.004	0.001	0.004	0.006	0.013	0.005	0.011	0.012	0.010
6	0.052	0.033	0.127	0.017	0.060	0.037	0.027	0.036	0.062
12	0.194	0.159	0.118	0.101	0.090	0.166	0.103	0.010	0.176
18	0.311	0.164	0.125	0.131	0.069	0.256	0.011	0.184	0.350
24	0.275	0.199	0.293	0.194	0.057	0.271	0.283	0.396	0.386
30	0.432	0.368	0.320	0.192	0.176	0.439	0.408	0.367	0.424
36	0.464	0.350	0.471	0.293	0.193	0.475	0.475	0.513	0.529
48	0.521	0.186	0.194	0.432	0.215	0.448	0.407	0.811	0.598
60	0.428	0.333	0.286	0.465	0.689	0.739	0.460	0.994	0.775
72	0.482	0.288	0.313	0.569	1.103	0.819	0.404	1.074	0.709
84	0.351	0.299	0.366	0.552	0.868	0.734	0.301	1.128	0.977
96	0.358	0.221	0.320	0.600	1.075	0.797	0.375	1.087	0.933
108	0.281	0.294	0.391	0.424	0.867	0.729	0.131	1.006	1.076
120	0.310	0.261	0.348	0.393	0.717	0.754	0.292	0.974	0.769
132	0.306	0.193	0.247	0.249	0.495	0.732	0.251	0.785	0.757
144	0.234	0.111	0.310	0.177	0.600	0.495	0.229	0.679	0.505
156	0.162	0.118	0.329	0.170	0.484	0.421	0.095	0.440	0.495
168	0.157	0.038	0.227	0.135	0.486	0.502	0.111	0.413	0.319

Note: Percentages in parentheses are the initial glucose concentrations in units of % (w/v)

Appendix 2.6 Raw data for experiments monitoring glycerol concentration at 96 hours of fermentation. Glycerol was measured using HPLC. Cultures were grown under aerobic conditions in YNB medium with the initial glucose indicated in the table. Fermentation was conducted at 30°C and 180 opm. The data represent the means of three independent experiments. (Figure 3.7)

Strain	**Glucose Conc. (% w/v)**	**Glycerol Concentration (mg/mL)**	
		Mean	**Standard deviation**
A12	5	0.357	0.064
	10	0.675	0.036
	15	0.772	0.091
K7	5	0.159	0.014
	10	0.254	0.027
	15	0.384	0.056
A15	5	0.314	0.047
	10	0.416	0.070
	15	0.606	0.007

Appendix 2.7 Raw data from experiments monitoring generalized polarization of yeast cell strains at different initial glucose concentrations. Cultures were grown under aerobic conditions in YNB medium with the initial glucose concentration indicated in the table. Fermentation was conducted at 30°C and 180 opm. Measurements were conducted on samples collected at the indicated time. The data represent the means of three independent experiments. (Figure 3.8)

Glucose concentration (% w/v)	Strain	Generalized Polarization			
		Mean		Standard deviation	
		6 hours	24 hours	6 hours	24 hours
5	A12	0.4655	0.6056	0.0091	0.0176
	K7	0.3982	0.6262	0.0107	0.0184
	A15	0.4387	0.5768	0.0064	0.0151
10	A12	0.4421	0.5256	0.0707	0.0433
	K7	0.2191	0.5088	0.0189	0.0660
	A15	0.3220	0.5584	0.0180	0.0063
15	A12	0.4534	0.4702	0.0605	0.0136
	K7	0.2322	0.4274	0.0166	0.0191
	A15	0.2936	0.5259	0.0293	0.0036

Appendix 2.8 Raw data from experiment monitoring generalized polarization of laurdan labelled yeast cell strains grown in different initial glucose concentrations and exposed to 18% (v/v) ethanol at the 10th minute. Cultures were grown under aerobic conditions in YNB medium with the initial glucose concentration indicated in the table. Fermentation was conducted at 30°C and 180 opm. Measurements were conducted on samples collected at the indicated times. The data represent the means of three independent experiments. (Figure 3.11)

5% w/v initial glucose

Time (minutes)	Mean			Standard deviation		
	A12	K7	A15	A12	K7	A15
0	0.6150	0.5997	0.5899	0.0221	0.0320	0.0406
5	0.6106	0.6013	0.5944	0.0099	0.0341	0.0328
10	0.5416	0.5405	0.5191	0.0259	0.0338	0.0402
15	0.5448	0.5380	0.5257	0.0264	0.0395	0.0387
20	0.5493	0.5281	0.5325	0.0281	0.0260	0.0382
25	0.5619	0.5295	0.5265	0.0299	0.0228	0.0300
30	0.5560	0.5362	0.5289	0.0202	0.0221	0.0271

10% w/v initial glucose

Time (minutes)	Mean			Standard deviation		
	A12	K7	A15	A12	K7	A15
0	0.5196	0.5142	0.5592	0.0463	0.0692	0.0073
5	0.5301	0.5114	0.5559	0.0426	0.0663	0.0087
10	0.5062	0.4876	0.5041	0.0217	0.0517	0.0089
15	0.5254	0.5120	0.5162	0.0216	0.0432	0.0106
20	0.5328	0.5213	0.5121	0.0150	0.0338	0.0079
25	0.5328	0.5232	0.5021	0.0108	0.0247	0.0019
30	0.5401	0.5138	0.5053	0.0111	0.0215	0.0017

15% w/v initial glucose

Time (minutes)	Mean			Standard deviation		
	A12	K7	A15	A12	K7	A15
0	0.4667	0.4231	0.5269	0.0241	0.0182	0.0016
5	0.4729	0.4344	0.5267	0.0082	0.0208	0.0074
10	0.4587	0.4538	0.4736	0.0178	0.0206	0.0015
15	0.4928	0.4916	0.4826	0.0197	0.0226	0.0023
20	0.5083	0.5123	0.4786	0.0176	0.0143	0.0012
25	0.5097	0.5154	0.4806	0.0160	0.0107	0.0027
30	0.5117	0.5178	0.4793	0.0201	0.0021	0.0047

Appendix 2.9 Raw data from experiments monitoring survival rates of the three yeast strains after exposure to 18% (v/v) ethanol for 1 hour followed by growing on an agar plate. Cultures were grown under aerobic conditions in YNB medium with 5% (w/v) initial glucose. Fermentation was conducted at 30°C and 180 opm for 24 hours. The data represent the means of three independent experiments. (Figure 3.12)

Strain	1st exp.	2nd exp.	3rd exp.	Means	SD
A12	68.8%	54.2%	56.1%	59.7%	8.0%
K7	88.8%	45.3%	79.3%	71.2%	22.9%
A15	33.9%	19.8%	25.6%	26.4%	7.1%

Appendix 2.10 Raw data used to calculate the Pearson correlation and the results of the correlation analysis performed using Minitab 15 from experiments monitoring generalized polarization and survival rates after exposure to 18% (v/v) ethanol. Cultures were grown under aerobic conditions in YNB medium with 5% (w/v) initial glucose. Fermentation was conducted at 30°C and 180 opm for 24 hours. The survival rates were determined using the total plate count method.

Strain	Generalized Polarization	Survival rate (%)
A12	0.6219	68.8
	0.6078	54.2
	0.587	56.1
K7	0.6452	88.8
	0.6251	45.3
	0.6084	79.3
A15	0.5763	33.9
	0.5921	19.8
	0.5619	25.6

Correlations: GP24, Survival rate

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Pearson correlation of GP24 and Survival rate = 0.753
P-Value = 0.019
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Appendix 2.11 Raw data from experiments monitoring the optical density at 600 nm. Cultures of A15 yeast strain were grown under aerobic conditions in YNB medium with 15% (w/v) initial glucose concentration. The fermentation was conducted at 30°C and 180 opm. The data represent the means of two independent experiments. (Figure 4.1)

Time (hours)	Mean			Standard deviation		
	Inositol Concentration (g/L)					
	0.0	0.1	0.4	0.0	0.1	0.4
0	0.027	0.030	0.024	0.012	0.005	0.005
6	0.101	0.158	0.147	0.062	0.076	0.076
12	0.584	1.907	1.862	0.324	0.633	0.642
18	1.660	3.509	3.400	0.451	0.473	0.483
24	2.463	3.914	3.818	0.351	0.532	0.496
30	2.930	4.186	4.106	0.362	0.574	0.583
36	3.201	4.312	4.225	0.339	0.599	0.716
48	3.649	4.458	4.432	0.289	0.663	0.677
60	3.910	4.689	4.627	0.319	0.677	0.691
72	4.004	4.782	4.713	0.297	0.689	0.658
84	4.123	4.900	4.751	0.374	0.627	0.661
96	4.277	5.105	4.995	0.258	0.646	0.594

Appendix 2.12 Raw data from experiment monitoring cell viability. Cultures of A15 yeast strain were grown under aerobic conditions in YNB medium with 15% (w/v) initial glucose concentration. The fermentation was conducted at 30°C and 180 opm. The data represent the means of two independent experiments. (Figure 4.2)

Time (hours)	Mean			Standard deviation		
	Inositol Concentration (g/L)					
	0.0	0.1	0.4	0.0	0.1	0.4
0	83.6	88.7	83.7	16.0	0.1	7.5
6	94.9	97.5	95.3	3.0	0.4	3.1
12	95.7	82.7	81.7	1.8	7.4	10.7
18	95.8	37.0	38.6	2.5	11.0	10.9
24	89.7	30.4	33.1	0.4	10.9	14.2
30	86.8	31.4	29.3	1.2	9.9	13.5
36	83.2	31.2	32.1	1.9	12.2	8.7
48	82.3	26.9	30.9	3.6	7.1	12.2
60	81.8	30.7	31.2	1.6	13.5	11.0
72	80.6	29.5	31.8	4.1	8.1	9.8
84	77.4	30.0	30.2	3.2	7.1	11.2
96	78.1	30.3	29.4	4.4	8.0	6.3

Appendix 2.13 Raw data from experiments monitoring glucose consumption and ethanol production. Cultures of A15 yeast strain were grown under aerobic conditions in YNB medium with 15% (w/v) initial glucose concentration. The fermentation was conducted at 30°C and 180 opm. The data represent the means of two independent experiments. (Figure 4.3)

Mean value of glucose and ethanol concentration

Time (hours)	Glucose (% w/v)			Ethanol (% w/v)		
	Inositol Concentration (g/L)					
	0.0	0.1	0.4	0.0	0.1	0.4
0	16.05	17.50	17.82	0.00	0.00	0.00
6	15.49	16.25	16.40	0.01	0.02	0.03
12	15.19	15.98	15.43	0.10	0.31	0.32
18	14.53	15.70	14.90	0.30	0.59	0.77
24	14.00	14.41	14.10	0.50	0.86	0.84
30	12.72	14.06	13.25	0.81	0.83	0.90
36	12.32	13.30	12.47	1.10	0.93	1.10
48	11.97	11.90	12.14	1.28	1.14	1.31
60	11.51	11.79	11.60	1.48	1.21	1.42
72	11.23	11.59	11.13	1.63	1.29	1.51
84	9.72	11.31	10.29	1.78	1.33	1.64
96	8.49	11.19	10.21	2.02	1.24	1.64

Standard deviation

Time (hours)	Glucose (% w/v)			Ethanol (% w/v)		
	Inositol Concentration (g/L)					
	0.0	0.1	0.4	0.0	0.1	0.4
0	1.78	1.82	1.49	0.00	0.00	0.00
6	1.48	1.23	1.53	0.01	0.01	0.02
12	1.41	1.24	1.37	0.03	0.02	0.10
18	1.31	1.55	1.09	0.08	0.14	0.21
24	0.71	0.14	0.42	0.03	0.31	0.20
30	0.26	0.55	0.00	0.23	0.47	0.51
36	0.16	1.24	1.00	0.40	0.54	0.76
48	0.29	2.95	1.15	0.29	0.77	0.93
60	0.61	2.85	1.62	0.51	0.68	1.01
72	1.28	3.03	2.20	0.74	1.19	1.24
84	0.75	3.17	2.58	0.56	1.00	1.32
96	2.14	3.31	2.62	0.83	0.84	1.45

Appendix 2.14 Raw data from experiments monitoring the generalized polarization of laurdan-labelled yeast cells. Cultures of A15 yeast strain were grown under aerobic conditions in YNB medium with 15% (w/v) initial glucose concentration. The fermentation was conducted at 30°C and 180 opm. The data represent the means of two independent experiments. (Figure 4.4)

Inositol Concentration (g/L)	Generalized Polarization	
	Mean	Standard deviation
0.0	0.5849	0.0089
0.1	0.5196	0.0405
0.4	0.5087	0.0191

Appendix 2.15 Raw data from experiments monitoring generalized polarization change of laurdan-labelled yeast cells after exposure to 18% (v/v) ethanol. The cells were exposed to 18% (v/v) ethanol at the 10th minute. Cultures of A15 yeast strain were grown under aerobic conditions in YNB medium with 15% (w/v) initial glucose concentration. The fermentation was conducted at 30°C and 180 opm. The data represent the means of two independent experiments. (Figure 4.5)

Time (minutes)	Mean value			Standard deviation		
	Inositol concentration (g/L)					
	0.0	0.1	0.4	0.0	0.1	0.4
0	0.5862	0.5200	0.5035	0.0101	0.0465	0.0184
1	0.5857	0.5235	0.5056	0.0147	0.0405	0.0150
2	0.5838	0.5228	0.5077	0.0110	0.0443	0.0184
3	0.5826	0.5203	0.5053	0.0119	0.0382	0.0199
4	0.5862	0.5134	0.5080	0.0084	0.0322	0.0188
5	0.5886	0.5163	0.5059	0.0090	0.0427	0.0099
6	0.5763	0.5187	0.5139	0.0004	0.0367	0.0219
7	0.5833	0.5157	0.5132	0.0041	0.0341	0.0236
8	0.5821	0.5226	0.5125	0.0071	0.0451	0.0218
9	0.5895	0.5252	0.5102	0.0097	0.0401	0.0147
10	0.5895	0.5181	0.5137	0.0115	0.0439	0.0230
11	0.5058	0.4039	0.3962	0.0174	0.0435	0.0248
12	0.5170	0.4111	0.4103	0.0101	0.0462	0.0364
13	0.5199	0.4158	0.4105	0.0176	0.0503	0.0263
14	0.5222	0.4180	0.4085	0.0231	0.0500	0.0323
15	0.5192	0.4185	0.4102	0.0205	0.0408	0.0332
16	0.5147	0.4247	0.4178	0.0174	0.0503	0.0322
17	0.5133	0.4284	0.4177	0.0125	0.0483	0.0347
18	0.5034	0.4327	0.4225	0.0146	0.0489	0.0351
19	0.5016	0.4386	0.4280	0.0019	0.0530	0.0263
20	0.4998	0.4364	0.4326	0.0046	0.0478	0.0260

Appendix 2.16 Raw data from experiments monitoring the optical density at 600 nm. Cultures of the A12 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.6(A))

Mean value of optical density of A12 strain

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.099	0.112	0.092	0.075	0.067	0.064
6	0.829	1.154	1.108	0.999	0.992	1.010
12	2.248	3.165	3.200	3.116	3.142	3.107
18	3.024	3.658	3.768	3.641	3.666	3.634
24	3.416	4.042	4.114	3.967	4.064	3.984
30	3.531	4.266	4.279	4.147	4.189	4.206
36	3.785	4.375	4.421	4.338	4.407	4.281
48	3.982	4.489	4.567	4.446	4.605	4.538
60	4.243	4.615	4.692	4.646	4.653	4.588
72	4.360	4.667	4.738	4.634	4.711	4.554
84	4.386	4.649	4.730	4.644	4.673	4.659
96	4.464	4.716	4.776	4.668	4.704	4.662

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.008	0.018	0.029	0.009	0.004	0.004
6	0.099	0.220	0.231	0.181	0.182	0.178
12	0.156	0.020	0.065	0.048	0.063	0.034
18	0.048	0.121	0.189	0.097	0.029	0.036
24	0.087	0.127	0.134	0.105	0.084	0.125
30	0.163	0.181	0.136	0.004	0.137	0.143
36	0.101	0.142	0.221	0.146	0.091	0.102
48	0.149	0.214	0.208	0.096	0.122	0.072
60	0.110	0.183	0.061	0.138	0.114	0.081
72	0.188	0.198	0.063	0.137	0.104	0.186
84	0.188	0.189	0.135	0.117	0.176	0.057
96	0.124	0.188	0.081	0.107	0.091	0.058

Appendix 2.17 Raw data from experiments monitoring the optical density at 600 nm. Cultures of the A15 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.6(B))

Mean value of optical density of A15 strain

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.081	0.074	0.073	0.072	0.084	0.075
6	0.472	0.807	0.838	0.719	0.768	0.747
12	1.601	3.351	3.438	3.320	3.354	3.320
18	2.723	4.135	4.243	4.217	4.257	4.198
24	3.348	4.545	4.581	4.514	4.604	4.541
30	3.707	4.737	4.792	4.796	4.829	4.730
36	3.892	4.822	4.865	4.811	4.915	4.888
48	4.159	5.001	5.044	5.057	5.129	5.021
60	4.273	5.118	5.191	5.264	5.226	5.173
72	4.407	5.294	5.330	5.368	5.375	5.258
84	4.488	5.367	5.447	5.426	5.460	5.383
96	4.602	5.473	5.587	5.602	5.557	5.439

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.012	0.022	0.019	0.018	0.023	0.025
6	0.133	0.157	0.173	0.107	0.149	0.101
12	0.449	0.085	0.090	0.069	0.173	0.137
18	0.401	0.176	0.043	0.095	0.102	0.242
24	0.349	0.038	0.046	0.043	0.087	0.083
30	0.313	0.125	0.060	0.084	0.128	0.097
36	0.354	0.152	0.043	0.156	0.203	0.034
48	0.346	0.086	0.080	0.094	0.060	0.104
60	0.307	0.076	0.070	0.012	0.113	0.104
72	0.393	0.026	0.089	0.045	0.104	0.117
84	0.300	0.047	0.139	0.125	0.117	0.119
96	0.336	0.089	0.042	0.029	0.093	0.146

Appendix 2.18 Raw data from experiments monitoring the optical density at 600 nm. Cultures of the K7 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.6(C))

Mean value of optical density of K7 strain

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.060	0.048	0.051	0.050	0.062	0.062
6	0.393	0.484	0.503	0.482	0.495	0.490
12	1.975	2.852	2.893	2.748	2.740	2.716
18	3.747	4.447	4.537	4.441	4.462	4.463
24	4.706	5.422	5.404	5.421	5.436	5.533
30	5.313	6.025	6.065	6.025	6.005	5.997
36	5.717	6.105	6.206	6.193	6.253	6.222
48	6.224	6.139	6.297	6.235	6.217	6.179
60	6.479	6.250	6.399	6.240	6.259	6.343
72	6.663	6.263	6.432	6.340	6.369	6.344
84	6.769	6.286	6.443	6.360	6.275	6.442
96	6.651	6.342	6.392	6.378	6.357	6.363

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.008	0.009	0.006	0.005	0.008	0.011
6	0.073	0.146	0.107	0.167	0.179	0.141
12	0.215	0.275	0.209	0.211	0.335	0.153
18	0.201	0.297	0.280	0.342	0.419	0.268
24	0.130	0.257	0.307	0.325	0.398	0.316
30	0.019	0.381	0.267	0.469	0.457	0.317
36	0.235	0.328	0.345	0.466	0.500	0.314
48	0.392	0.376	0.448	0.498	0.508	0.357
60	0.525	0.484	0.433	0.494	0.539	0.409
72	0.574	0.455	0.453	0.512	0.527	0.408
84	0.561	0.386	0.427	0.534	0.498	0.445
96	0.516	0.435	0.416	0.530	0.566	0.343

Appendix 2.19 Raw data from experiments monitoring cell viability. Cultures of the A12 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.7(A))

Mean value of cell viability (%)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	96.97	96.54	95.60	96.06	96.18	97.95
6	97.61	97.95	97.47	98.74	97.74	98.29
12	96.84	77.67	75.24	75.99	73.49	73.20
18	93.38	72.93	71.60	71.16	74.40	71.90
24	89.56	69.39	72.04	72.17	69.50	70.94
30	87.28	67.10	67.35	69.53	68.49	67.33
36	85.72	61.65	65.84	63.14	63.55	65.25
48	83.40	48.17	50.34	49.52	48.20	46.84
60	79.30	38.63	36.59	36.80	37.31	36.34
72	75.17	27.02	27.45	29.35	27.90	26.70
84	70.73	23.69	23.31	22.03	22.73	21.79
96	65.07	16.67	16.41	17.14	16.36	17.83

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	1.32	1.54	2.11	2.34	0.74	1.16
6	0.12	0.92	0.66	0.37	1.36	0.67
12	0.40	3.30	2.10	2.33	1.60	0.55
18	2.96	4.21	2.39	2.14	1.66	2.08
24	0.86	3.79	3.59	0.57	2.33	2.89
30	1.39	5.17	3.91	0.66	4.33	1.27
36	2.70	6.23	5.10	3.25	3.92	0.99
48	2.27	4.09	2.50	3.39	3.52	1.71
60	5.39	2.19	4.00	5.21	3.51	1.95
72	2.46	2.73	2.63	0.68	1.76	0.52
84	3.38	1.07	1.91	2.42	3.85	1.23
96	4.58	1.47	2.40	0.37	1.91	1.03

Appendix 2.20 Raw data from the experiment monitoring the cell viability. Cultures of the A15 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.7(B))

Mean value of cell viability (%)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	92.53	92.99	91.28	93.83	93.87	94.19
6	94.46	97.41	98.31	98.52	97.87	97.58
12	94.71	50.92	49.10	50.16	50.85	49.84
18	91.26	38.50	34.13	38.93	40.59	39.41
24	87.18	39.54	38.26	40.09	41.28	39.50
30	87.23	37.69	37.44	39.59	40.87	37.96
36	86.12	36.84	38.98	38.65	38.85	37.98
48	82.22	38.73	36.96	38.86	39.46	36.71
60	80.98	36.95	38.96	38.59	40.06	39.26
72	74.16	35.83	34.41	36.40	35.06	35.28
84	73.05	34.66	33.78	34.16	36.03	35.40
96	64.70	32.38	31.56	32.97	33.93	32.75

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	4.91	6.56	2.91	3.90	5.90	1.35
6	1.13	0.78	0.64	0.62	1.05	1.82
12	1.82	1.28	1.40	4.44	3.44	1.01
18	1.62	0.99	1.04	1.99	1.75	3.04
24	0.95	1.48	3.52	3.59	2.51	1.85
30	3.58	3.92	2.88	1.17	1.46	2.57
36	2.57	1.91	0.77	0.99	3.28	1.29
48	0.88	2.46	2.01	0.94	3.72	3.84
60	1.61	2.11	2.27	1.47	1.85	1.10
72	1.13	1.98	3.35	4.43	3.90	3.15
84	2.82	1.89	2.97	2.15	2.95	1.45
96	6.77	2.90	3.88	3.08	2.36	1.28

Appendix 2.21 Raw data from experiment monitoring the cell viability. Cultures of the K7 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.7(C))

Mean value of cell viability (%)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	98.59	88.96	98.93	98.15	97.21	98.78
6	98.64	98.13	98.72	99.15	99.00	99.12
12	99.24	97.64	98.02	96.99	97.72	97.62
18	93.07	88.61	89.21	89.69	87.34	89.81
24	82.76	86.18	85.69	85.96	83.11	86.25
30	80.33	85.96	82.64	86.71	84.42	85.77
36	79.98	85.51	84.45	86.38	82.82	84.97
48	78.16	81.63	81.09	84.33	80.53	81.59
60	77.34	69.35	70.83	72.10	67.03	69.58
72	75.49	45.17	51.80	50.84	43.21	45.28
84	72.20	30.55	34.10	33.90	24.45	32.10
96	68.27	20.30	17.76	16.44	16.09	19.58

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	1.11	16.84	0.73	1.01	4.42	1.31
6	0.14	1.34	0.85	0.47	0.55	0.69
12	0.42	0.68	0.53	1.20	0.56	1.11
18	3.66	4.13	3.26	2.87	4.25	0.93
24	4.54	4.58	6.41	5.98	6.27	3.76
30	6.11	5.43	8.14	4.03	5.48	5.12
36	6.60	6.19	6.54	5.22	7.20	4.73
48	6.60	8.13	7.83	6.30	8.81	6.79
60	8.03	8.43	9.40	8.34	9.50	5.66
72	6.12	10.53	5.02	8.73	10.29	5.39
84	5.81	10.63	5.35	3.92	2.19	7.14
96	6.97	11.96	15.15	8.88	3.20	7.10

Appendix 2.22 Raw data from experiments monitoring viable cell count. Cultures of the A12 yeast strains were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.8(A))

Mean value of viable cell count (×10^6 cell/mL)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	1.08	1.13	1.21	1.08	1.15	1.02
6	10.06	20.97	20.04	15.64	15.95	14.72
12	33.97	44.37	39.57	38.47	38.70	40.30
18	34.90	40.10	38.40	40.53	40.07	35.77
24	34.97	40.77	39.07	42.93	37.80	37.07
30	32.97	37.83	36.47	38.83	38.70	40.50
36	36.10	35.60	37.47	38.03	36.33	36.00
48	35.80	27.23	30.63	28.77	27.53	27.20
60	36.83	24.30	22.20	20.90	21.97	20.10
72	35.77	15.97	16.47	17.37	15.80	15.47
84	36.10	13.80	14.30	13.43	12.73	12.30
96	31.33	9.53	9.87	9.97	9.37	9.90

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.14	0.18	0.17	0.24	0.06	0.02
6	0.93	3.93	3.46	5.08	6.00	4.88
12	2.55	2.29	2.72	1.27	1.37	0.44
18	1.71	3.26	6.52	3.59	3.65	3.99
24	1.56	0.90	5.33	1.21	3.20	4.58
30	3.44	3.10	2.48	0.47	2.43	1.71
36	4.07	2.46	6.12	4.12	4.40	2.95
48	2.15	2.73	4.22	3.29	3.50	2.65
60	0.50	2.35	4.80	4.25	3.06	2.55
72	1.00	2.98	3.32	2.28	1.01	0.51
84	1.01	1.57	2.88	2.57	2.42	1.08
96	2.83	1.75	2.66	0.59	1.35	1.14

Appendix 2.23 Raw data from experiment monitoring the viable cell count. Cultures of the A15 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.8(B))

Mean value of viable cell count (×10^6 cell/mL)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.88	0.99	0.92	0.90	0.92	1.06
6	6.22	14.43	14.48	11.59	13.49	12.82
12	20.50	34.73	31.43	30.40	34.77	32.60
18	31.73	29.07	24.13	28.63	29.17	28.67
24	37.63	30.03	29.17	30.63	30.90	28.63
30	35.33	28.70	29.63	30.87	31.63	30.43
36	38.77	28.07	30.67	29.83	29.17	29.53
48	38.30	31.03	30.17	31.87	31.77	28.57
60	40.40	29.53	32.60	32.27	33.77	33.57
72	35.80	30.13	29.90	30.37	29.40	28.70
84	35.87	27.70	27.30	28.23	29.37	27.70
96	34.73	26.77	28.73	27.77	29.30	28.03

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.12	0.17	0.08	0.27	0.18	0.01
6	1.87	4.77	3.82	2.54	2.50	2.42
12	6.92	1.63	2.04	0.89	7.03	3.50
18	2.99	2.32	3.96	3.01	3.46	6.81
24	2.14	2.64	3.19	5.12	6.35	3.39
30	3.63	3.84	6.09	0.95	6.20	2.55
36	6.70	2.42	1.92	0.51	4.22	0.35
48	7.44	3.23	4.52	2.78	5.91	4.33
60	2.19	3.21	3.72	1.44	2.66	1.50
72	9.15	2.67	4.92	3.85	6.50	5.92
84	3.70	2.89	4.69	4.10	5.77	2.29
96	1.57	4.39	6.01	4.56	1.87	2.55

Appendix 2.24 Raw data from experiment monitoring the viable cell count. Cultures of the K7 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.8(C))

Mean value of viable cell count (×10^6 cell/mL)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.93	0.90	1.03	1.11	1.05	1.20
6	6.59	10.62	10.50	11.86	9.45	9.84
12	41.90	60.50	64.33	58.33	61.43	55.37
18	56.70	72.13	69.77	66.43	68.40	65.30
24	61.90	78.20	77.67	73.10	74.97	78.63
30	65.43	84.83	79.53	80.43	81.33	80.90
36	58.73	77.37	80.07	80.03	78.53	78.43
48	60.67	80.87	83.83	83.93	83.40	80.27
60	67.33	79.03	77.87	75.50	66.10	71.33
72	68.53	49.27	61.13	55.17	47.80	48.97
84	64.13	30.80	36.13	34.50	24.13	33.07
96	65.27	21.10	18.17	16.13	16.00	19.47

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.05	0.08	0.08	0.13	0.17	0.15
6	1.54	3.77	2.63	7.47	3.79	2.51
12	5.78	3.30	1.27	3.81	4.86	2.27
18	4.42	3.00	3.54	4.16	4.75	4.29
24	8.72	7.30	6.30	10.84	5.32	9.16
30	7.54	9.19	13.72	5.36	7.82	9.25
36	6.39	5.27	1.10	5.80	5.23	0.38
48	2.24	5.58	9.43	6.95	7.89	5.41
60	13.66	15.87	15.15	10.47	10.46	7.50
72	6.83	10.63	8.11	12.69	14.38	5.35
84	2.20	10.27	5.16	5.05	0.85	7.02
96	9.14	14.32	15.94	9.39	3.76	8.26

Appendix 2.25 Raw data from experiment monitoring glucose consumption. Cultures of the A12 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.9(A))

Mean value of glucose concentration (% w/v)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	10.7	10.6	10.7	10.8	10.7	10.9
6	10.3	9.8	10.3	10.4	10.0	10.2
12	9.2	8.4	8.4	8.0	8.6	8.6
18	6.8	6.2	7.1	6.5	7.3	6.8
24	6.2	5.4	5.7	6.1	5.5	5.4
30	5.5	4.6	4.6	5.0	4.8	5.0
36	5.2	4.0	4.3	4.0	4.2	3.9
48	4.4	2.7	3.1	2.9	2.9	2.6
60	3.9	2.1	2.4	2.0	2.1	2.0
72	3.0	1.5	1.6	1.5	1.6	1.5
84	2.5	1.1	1.3	1.2	1.2	1.2
96	1.8	0.7	0.6	0.7	0.6	0.6

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.1	0.2	0.7	0.3	0.4	0.4
6	0.3	0.7	0.6	0.5	0.9	0.4
12	0.3	0.5	0.7	0.6	0.6	0.2
18	0.5	0.4	0.9	1.0	0.7	1.3
24	0.3	0.2	0.4	1.1	0.7	1.2
30	0.7	0.3	0.5	0.3	0.6	1.0
36	0.6	0.1	0.5	0.1	0.7	0.3
48	0.3	0.1	0.3	0.2	0.5	0.2
60	0.2	0.1	0.6	0.1	0.2	0.4
72	0.2	0.2	0.4	0.2	0.2	0.5
84	0.2	0.1	0.6	0.3	0.2	0.5
96	0.2	0.0	0.3	0.2	0.1	0.2

Appendix 2.26 Raw data from experiment monitoring glucose consumption. Cultures of the A15 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.9(B))

Mean value of glucose concentration (% w/v)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	10.7	10.7	10.9	10.7	11.0	10.8
6	10.3	10.4	10.6	10.5	10.5	10.4
12	9.4	8.6	8.9	9.0	8.9	8.9
18	6.9	6.8	6.7	6.8	6.6	5.9
24	6.3	6.2	5.9	6.3	6.0	5.2
30	5.5	5.7	5.3	5.8	5.5	5.0
36	4.7	5.1	4.8	4.9	5.1	4.8
48	3.6	4.1	4.0	3.8	3.9	3.4
60	2.6	3.1	3.2	2.7	2.8	2.7
72	1.3	2.2	2.0	1.8	1.8	2.0
84	0.7	1.6	1.5	1.3	1.1	1.4
96	0.1	1.0	0.8	0.6	0.5	0.7

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.2	0.3	0.3	0.0	0.3	0.3
6	0.5	0.2	0.4	0.1	0.3	0.2
12	0.1	0.4	0.7	0.0	0.3	0.1
18	0.8	0.4	1.1	0.8	0.5	0.9
24	0.6	0.4	0.6	0.8	0.6	0.9
30	0.9	0.8	0.4	0.7	0.4	0.7
36	0.7	0.6	0.4	0.4	0.2	0.6
48	1.0	0.5	0.6	0.6	0.5	0.4
60	0.9	0.5	0.6	0.4	0.8	0.2
72	0.9	0.4	0.2	0.4	0.4	0.1
84	0.6	0.3	0.1	0.4	0.4	0.2
96	0.1	0.2	0.2	0.3	0.4	0.3

Appendix 2.27 Raw data from experiment monitoring glucose consumption. Cultures of the K7 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.9(C))

Mean value of glucose concentration (% w/v)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	10.9	10.7	11.0	10.6	10.9	10.7
6	10.2	10.0	9.9	10.3	10.1	10.2
12	9.1	8.9	9.0	9.3	9.1	9.0
18	6.5	5.9	6.1	6.3	6.4	6.6
24	5.7	4.9	5.1	5.5	5.7	5.1
30	5.0	4.2	4.4	4.5	4.7	4.7
36	4.7	3.6	3.9	3.7	4.0	3.8
48	3.8	2.2	2.2	2.4	2.1	2.2
60	3.4	1.3	1.3	1.5	1.4	1.4
72	2.9	0.6	0.6	0.6	0.7	0.6
84	2.6	0.4	0.3	0.3	0.5	0.3
96	2.0	0.2	0.3	0.2	0.3	0.2

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	1.0	0.7	0.9	0.6	0.7	0.7
6	1.2	1.0	1.1	0.9	1.2	1.0
12	1.3	1.4	1.0	0.6	1.1	1.3
18	0.7	0.4	0.4	1.1	0.4	1.1
24	0.7	0.5	0.7	0.5	0.6	0.5
30	0.6	0.5	0.3	0.4	0.9	0.8
36	0.6	0.4	0.0	0.3	0.3	0.5
48	0.5	0.5	0.4	0.4	0.3	0.6
60	0.7	0.6	0.5	0.6	0.5	0.6
72	0.7	0.2	0.1	0.2	0.3	0.1
84	0.6	0.3	0.2	0.2	0.4	0.1
96	0.9	0.1	0.1	0.1	0.1	0.0

Appendix 2.28 Raw data from experiment monitoring ethanol production. Cultures of the A12 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.10(A))

Mean value of ethanol concentration (% w/v)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.02	0.02	0.02	0.02	0.02	0.02
6	0.16	0.20	0.20	0.15	0.15	0.15
12	0.43	0.63	0.62	0.59	0.63	0.61
18	0.74	1.11	0.98	1.13	1.12	0.97
24	0.91	1.37	1.36	1.35	1.37	1.29
30	0.94	1.61	1.67	1.55	1.63	1.40
36	1.04	1.80	1.56	1.76	1.61	1.68
48	1.20	1.92	1.93	2.01	1.94	2.01
60	1.23	2.05	2.11	2.15	2.00	2.21
72	1.42	2.14	2.23	2.23	2.24	2.31
84	1.49	2.36	2.31	2.36	2.44	2.37
96	1.66	2.48	2.43	2.59	2.52	2.46

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.00	0.00	0.00	0.00	0.00	0.00
6	0.04	0.05	0.05	0.01	0.01	0.01
12	0.21	0.29	0.19	0.27	0.33	0.28
18	0.18	0.06	0.21	0.13	0.14	0.25
24	0.21	0.10	0.12	0.21	0.14	0.28
30	0.23	0.05	0.19	0.24	0.14	0.31
36	0.17	0.36	0.33	0.26	0.30	0.45
48	0.19	0.45	0.39	0.49	0.45	0.54
60	0.26	0.29	0.27	0.32	0.48	0.46
72	0.30	0.37	0.20	0.36	0.31	0.37
84	0.30	0.26	0.27	0.31	0.21	0.43
96	0.16	0.28	0.29	0.21	0.22	0.39

Appendix 2.29 Raw data from experiment monitoring ethanol production. Cultures of the A15 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.10(B))

Mean value of ethanol concentration (% w/v)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.02	0.02	0.02	0.02	0.02	0.02
6	0.11	0.16	0.17	0.15	0.16	0.16
12	0.39	0.72	0.73	0.77	0.68	0.71
18	0.70	1.12	1.06	1.07	1.04	1.05
24	0.96	1.39	1.37	1.34	1.30	1.41
30	1.22	1.57	1.56	1.51	1.52	1.63
36	1.43	1.66	1.66	1.74	1.75	1.73
48	1.97	2.06	2.09	2.02	2.13	1.93
60	2.26	2.24	2.41	2.32	2.42	2.24
72	2.67	2.54	2.66	2.59	2.68	2.35
84	3.04	2.82	2.85	2.73	2.89	2.61
96	3.30	2.88	2.96	2.93	3.07	2.89

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.00	0.00	0.00	0.00	0.00	0.00
6	0.03	0.03	0.03	0.02	0.03	0.02
12	0.10	0.08	0.07	0.09	0.08	0.10
18	0.21	0.21	0.23	0.22	0.20	0.19
24	0.20	0.43	0.33	0.25	0.22	0.33
30	0.36	0.35	0.26	0.22	0.30	0.34
36	0.22	0.35	0.19	0.20	0.21	0.28
48	0.30	0.32	0.29	0.24	0.52	0.31
60	0.36	0.37	0.41	0.24	0.43	0.45
72	0.54	0.33	0.48	0.38	0.47	0.44
84	0.64	0.24	0.50	0.44	0.48	0.56
96	0.68	0.16	0.43	0.44	0.52	0.58

Appendix 2.30 Raw data from experiment monitoring ethanol production. Cultures of the K7 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.10(C))

Mean value of ethanol concentration (% w/v)

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.01	0.01	0.01	0.01	0.01	0.01
6	0.07	0.10	0.10	0.09	0.09	0.09
12	0.34	0.57	0.62	0.56	0.53	0.56
18	0.64	0.98	1.00	1.11	1.01	0.98
24	0.91	1.34	1.41	1.48	1.50	1.45
30	1.13	1.63	1.65	1.65	1.78	1.66
36	1.21	1.85	1.82	2.05	1.98	1.87
48	1.52	2.22	2.18	2.40	1.93	2.25
60	1.61	2.39	2.27	2.52	2.23	2.35
72	1.97	2.48	2.41	2.66	2.38	2.49
84	2.10	2.63	2.49	2.79	2.45	2.72
96	2.44	2.79	2.75	2.93	2.66	3.09

Standard deviation

Time (hours)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.00	0.00	0.00	0.00	0.00	0.00
6	0.01	0.03	0.03	0.04	0.04	0.04
12	0.06	0.06	0.05	0.13	0.09	0.07
18	0.04	0.12	0.32	0.30	0.17	0.26
24	0.04	0.06	0.36	0.48	0.25	0.18
30	0.05	0.22	0.31	0.42	0.42	0.22
36	0.10	0.31	0.26	0.48	0.28	0.22
48	0.10	0.12	0.28	0.39	0.13	0.33
60	0.14	0.14	0.24	0.46	0.16	0.30
72	0.37	0.18	0.22	0.46	0.15	0.26
84	0.44	0.15	0.26	0.51	0.16	0.27
96	0.85	0.17	0.18	0.42	0.15	0.42

Appendix 2.31 Raw data from experiment monitoring generalized polarization of laurdan-labelled yeast cells. Cultures of the yeast strains were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.11)

Inositol Concentration (g/L)	Generalized polarization					
	A12		A15		K7	
	Mean value	Standard deviation	Mean value	Standard deviation	Mean value	Standard deviation
0.00	0.5105	0.0101	0.5678	0.0075	0.5638	0.0136
0.05	0.5437	0.0239	0.5046	0.0095	0.6019	0.0122
0.10	0.5394	0.0132	0.5263	0.0104	0.6150	0.0026
0.20	0.5405	0.0106	0.5335	0.0129	0.6069	0.0131
0.40	0.5383	0.0165	0.5262	0.0123	0.6069	0.0061
0.80	0.5591	0.0208	0.5196	0.0061	0.6099	0.0133

Appendix 2.32 Raw data from experiment monitoring the change in generalized polarization of laurdan-labelled yeast cells after exposure to 18% v/v ethanol at the 10th minutes. Cultures of the A12 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.12(A))

Mean value of generalized polarization

Time (min)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.5087	0.5524	0.5410	0.5409	0.5404	0.5596
1	0.5047	0.5485	0.5407	0.5448	0.5388	0.5559
2	0.4985	0.5439	0.5378	0.5376	0.5343	0.5508
3	0.5079	0.5377	0.5387	0.5358	0.5403	0.5625
4	0.5105	0.5452	0.5331	0.5397	0.5342	0.5608
5	0.5139	0.5413	0.5361	0.5390	0.5356	0.5560
6	0.5104	0.5486	0.5406	0.5371	0.5394	0.5613
7	0.5150	0.5473	0.5405	0.5428	0.5352	0.5611
8	0.5156	0.5354	0.5418	0.5420	0.5390	0.5611
9	0.5155	0.5381	0.5428	0.5421	0.5422	0.5574
10	0.5148	0.5425	0.5403	0.5435	0.5415	0.5641
11	0.4624	0.4556	0.4566	0.4607	0.4564	0.4690
12	0.4708	0.4646	0.4819	0.4768	0.4647	0.4807
13	0.4847	0.4778	0.4812	0.4883	0.4759	0.4951
14	0.4894	0.4730	0.4935	0.4942	0.4868	0.4951
15	0.4964	0.4925	0.4984	0.5026	0.4950	0.5023
16	0.5025	0.5000	0.5031	0.5098	0.5009	0.5112
17	0.5085	0.5034	0.5122	0.5149	0.5090	0.5137
18	0.5120	0.5151	0.5185	0.5193	0.5206	0.5197
19	0.5172	0.5176	0.5265	0.5244	0.5255	0.5253
20	0.5169	0.5176	0.5253	0.5302	0.5294	0.5280

Standard deviation

Time (min)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.0027	0.0278	0.0115	0.0131	0.0133	0.0229
1	0.0191	0.0263	0.0182	0.0090	0.0135	0.0268
2	0.0250	0.0240	0.0201	0.0086	0.0175	0.0221
3	0.0157	0.0208	0.0133	0.0120	0.0204	0.0223
4	0.0122	0.0312	0.0207	0.0098	0.0183	0.0230
5	0.0106	0.0238	0.0060	0.0106	0.0205	0.0212
6	0.0118	0.0307	0.0156	0.0116	0.0219	0.0232
7	0.0087	0.0206	0.0130	0.0089	0.0153	0.0175
8	0.0050	0.0165	0.0099	0.0104	0.0160	0.0151
9	0.0067	0.0167	0.0156	0.0132	0.0116	0.0218
10	0.0092	0.0269	0.0113	0.0143	0.0174	0.0174
11	0.0131	0.0318	0.0162	0.0122	0.0282	0.0176
12	0.0056	0.0238	0.0070	0.0228	0.0230	0.0180
13	0.0045	0.0226	0.0049	0.0095	0.0211	0.0129
14	0.0102	0.0202	0.0075	0.0119	0.0183	0.0115
15	0.0149	0.0128	0.0009	0.0110	0.0108	0.0102
16	0.0082	0.0051	0.0062	0.0057	0.0080	0.0104
17	0.0100	0.0116	0.0042	0.0019	0.0073	0.0085
18	0.0104	0.0055	0.0059	0.0047	0.0062	0.0046
19	0.0124	0.0062	0.0044	0.0080	0.0036	0.0005
20	0.0091	0.0046	0.0090	0.0061	0.0009	0.0010

Appendix 2.33 Raw data from experiment monitoring the change in generalized polarization of laurdan-labelled yeast cells after exposure to 18% (v/v) ethanol at the 10th minutes. Cultures of the A15 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.12(B))

Mean value of generalized polarization

Time (min)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.5693	0.5029	0.5200	0.5284	0.5189	0.5121
1	0.5630	0.4970	0.5215	0.5293	0.5217	0.5165
2	0.5648	0.5026	0.5237	0.5325	0.5231	0.5061
3	0.5648	0.5022	0.5242	0.5305	0.5280	0.5200
4	0.5676	0.5078	0.5245	0.5352	0.5257	0.5194
5	0.5630	0.5034	0.5253	0.5345	0.5224	0.5224
6	0.5696	0.5037	0.5258	0.5354	0.5302	0.5227
7	0.5691	0.5050	0.5324	0.5322	0.5299	0.5254
8	0.5711	0.5075	0.5329	0.5381	0.5305	0.5245
9	0.5703	0.5106	0.5292	0.5377	0.5308	0.5254
10	0.5728	0.5084	0.5295	0.5350	0.5273	0.5210
11	0.4861	0.4062	0.4423	0.4422	0.4370	0.4294
12	0.5012	0.4197	0.4485	0.4565	0.4482	0.4425
13	0.5033	0.4248	0.4485	0.4518	0.4544	0.4409
14	0.5083	0.4319	0.4560	0.4575	0.4590	0.4531
15	0.5037	0.4381	0.4611	0.4590	0.4660	0.4542
16	0.5113	0.4440	0.4649	0.4644	0.4667	0.4618
17	0.5160	0.4458	0.4658	0.4656	0.4696	0.4611
18	0.5153	0.4485	0.4648	0.4671	0.4680	0.4610
19	0.5187	0.4500	0.4740	0.4666	0.4756	0.4663
20	0.5156	0.4529	0.4728	0.4695	0.4782	0.4725

Standard deviation

Time (min)	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.20	0.40	0.80
0	0.0054	0.0084	0.0126	0.0156	0.0152	0.0130
1	0.0072	0.0099	0.0078	0.0134	0.0174	0.0093
2	0.0126	0.0128	0.0076	0.0206	0.0084	0.0179
3	0.0083	0.0079	0.0153	0.0069	0.0118	0.0083
4	0.0114	0.0103	0.0090	0.0133	0.0129	0.0083
5	0.0112	0.0103	0.0154	0.0093	0.0123	0.0084
6	0.0041	0.0122	0.0105	0.0096	0.0155	0.0089
7	0.0054	0.0085	0.0074	0.0124	0.0105	0.0061
8	0.0081	0.0102	0.0100	0.0134	0.0086	0.0048
9	0.0087	0.0115	0.0105	0.0156	0.0106	0.0070
10	0.0078	0.0090	0.0094	0.0158	0.0131	0.0060
11	0.0136	0.0139	0.0145	0.0249	0.0297	0.0167
12	0.0161	0.0027	0.0047	0.0145	0.0195	0.0076
13	0.0105	0.0039	0.0059	0.0154	0.0152	0.0202
14	0.0120	0.0065	0.0036	0.0174	0.0143	0.0103
15	0.0160	0.0068	0.0082	0.0116	0.0189	0.0133
16	0.0107	0.0041	0.0033	0.0183	0.0136	0.0096
17	0.0072	0.0023	0.0063	0.0133	0.0093	0.0115
18	0.0083	0.0031	0.0102	0.0118	0.0114	0.0143
19	0.0135	0.0089	0.0026	0.0117	0.0094	0.0079
20	0.0069	0.0041	0.0039	0.0154	0.0032	0.0073

Appendix 2.34 Raw data from experiment monitoring the change in generalized polarization of laurdan-labelled yeast cells after exposure to 18% (v/v) ethanol at the 10th minutes. Cultures of the K7 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 4.12(C))

Mean value of generalized polarization

	Inositol concentration (g/L)					
Time (min)	0.00	0.05	0.10	0.20	0.40	0.80
0	0.5564	0.6042	0.6154	0.6083	0.6065	0.6173
1	0.5600	0.6021	0.6129	0.6025	0.6086	0.6100
2	0.5613	0.6029	0.6156	0.6060	0.6066	0.6120
3	0.5625	0.6020	0.6174	0.6069	0.6065	0.6101
4	0.5618	0.6024	0.6127	0.6068	0.6067	0.6083
5	0.5644	0.6019	0.6156	0.6054	0.6043	0.6113
6	0.5636	0.6017	0.6128	0.6069	0.6067	0.6069
7	0.5665	0.5996	0.6145	0.6089	0.6056	0.6089
8	0.5688	0.6038	0.6167	0.6061	0.6071	0.6094
9	0.5672	0.5989	0.6150	0.6089	0.6087	0.6060
10	0.5690	0.6014	0.6161	0.6088	0.6091	0.6090
11	0.4888	0.5393	0.5528	0.5475	0.5462	0.5457
12	0.5066	0.5420	0.5609	0.5530	0.5585	0.5510
13	0.5004	0.5373	0.5577	0.5496	0.5503	0.5433
14	0.5002	0.5385	0.5589	0.5496	0.5512	0.5409
15	0.5028	0.5353	0.5550	0.5502	0.5547	0.5455
16	0.5074	0.5386	0.5536	0.5517	0.5527	0.5444
17	0.5034	0.5399	0.5550	0.5528	0.5536	0.5424
18	0.5009	0.5387	0.5538	0.5545	0.5530	0.5420
19	0.5021	0.5361	0.5551	0.5478	0.5513	0.5389
20	0.5072	0.5386	0.5512	0.5494	0.5516	0.5413

Standard deviation

	Inositol concentration (g/L)					
Time (min)	0.00	0.05	0.10	0.20	0.40	0.80
0	0.0124	0.0155	0.0059	0.0154	0.0141	0.0108
1	0.0129	0.0148	0.0025	0.0158	0.0112	0.0119
2	0.0146	0.0113	0.0027	0.0180	0.0082	0.0117
3	0.0166	0.0125	0.0062	0.0163	0.0032	0.0119
4	0.0145	0.0113	0.0058	0.0163	0.0081	0.0122
5	0.0130	0.0133	0.0010	0.0135	0.0076	0.0119
6	0.0122	0.0116	0.0049	0.0116	0.0054	0.0137
7	0.0131	0.0142	0.0046	0.0132	0.0054	0.0171
8	0.0129	0.0117	0.0015	0.0123	0.0007	0.0129
9	0.0157	0.0109	0.0033	0.0104	0.0029	0.0166
10	0.0136	0.0120	0.0027	0.0072	0.0076	0.0170
11	0.0134	0.0203	0.0214	0.0229	0.0225	0.0173
12	0.0051	0.0132	0.0067	0.0195	0.0105	0.0134
13	0.0056	0.0092	0.0045	0.0112	0.0088	0.0167
14	0.0083	0.0169	0.0079	0.0063	0.0034	0.0182
15	0.0188	0.0181	0.0110	0.0088	0.0028	0.0222
16	0.0246	0.0198	0.0146	0.0143	0.0025	0.0211
17	0.0273	0.0237	0.0187	0.0198	0.0074	0.0246
18	0.0273	0.0221	0.0198	0.0177	0.0052	0.0281
19	0.0314	0.0256	0.0208	0.0229	0.0090	0.0241
20	0.0375	0.0254	0.0169	0.0233	0.0110	0.0325

Appendix 2.35 Raw data from experiment monitoring glucose concentration (mg/mL). Cultures of the yeast cells were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 5.1)

Strain	Inositol Concentration (g/L)	Mean			Standard deviation		
		Time (hours)					
		0	24	96	0	24	96
A12	0.00	105.614	58.474	8.721	1.061	1.119	1.936
	0.05	106.097	51.578	3.859	1.610	3.584	0.597
	0.10	106.527	49.803	3.815	1.514	2.134	0.171
A15	0.00	113.877	69.714	2.670	1.854	3.888	1.015
	0.05	114.722	62.523	1.025	2.540	4.597	0.026
	0.10	114.760	62.777	1.045	0.799	1.692	0.018
K7	0.00	108.819	66.513	27.401	6.501	2.793	3.504
	0.05	110.890	57.367	1.922	4.520	2.446	0.219
	0.10	109.720	52.496	1.603	5.013	3.212	0.236

Appendix 2.36 Raw data from experiment monitoring ethanol concentration (mg/mL). Cultures of the yeast cells were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 5.2)

Strain	Inositol Concentration (g/L)	Mean			Standard deviation		
		Time (hours)					
		0	24	96	0	24	96
A12	0.00	0.167	10.287	28.692	0.026	0.201	1.170
	0.05	0.171	16.265	32.334	0.027	0.285	0.731
	0.10	0.168	15.971	31.428	0.027	0.416	0.695
A15	0.00	0.235	10.278	38.028	0.039	0.660	1.681
	0.05	0.244	15.621	39.802	0.040	0.432	0.517
	0.10	0.240	15.714	39.312	0.042	0.585	0.755
K7	0.00	0.099	10.513	25.693	0.016	0.560	2.576
	0.05	0.099	16.716	36.882	0.017	1.165	1.061
	0.10	0.099	17.143	37.356	0.017	0.992	1.433

Appendix 2.37 Raw data from experiment monitoring total cell count ($\times 10^6$ cell/mL). Cultures of the yeast cells were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 5.3)

Strain	Inositol concentration (g/L)	Cell number ($\times 10^6$ cell/mL)	
		Average	SD
A12	0.00	59.4	1.4
	0.05	93.2	1.6
	0.10	92.7	5.2
A15	0.00	25.2	1.8
	0.05	61.7	6.3
	0.10	62.7	1.6
K7	0.00	79.3	8.2
	0.05	109.7	12.4
	0.10	111.3	15.3

Appendix 2.38 Raw data from experiment monitoring ethanol stress tolerance. Cultures of the yeast cells were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. Yeast cells were then taken from the culture at the 24 hour time point and exposed to 18% v/v ethanol. Relative growth was measured based on optical density at 600 nm of yeast cells exposed to the stress condition relative to the control after 24 hours of growth. The data presented are the means of four independent experiments. (Figure 5.4)

Strain	Inositol Concentration (g/L)	Survival rate (%)	
		Mean value	Standard deviation
A12	0.00	31.25	3.87
	0.05	64.74	10.73
	0.10	55.68	13.97
A15	0.00	17.03	3.00
	0.05	44.86	6.82
	0.10	48.37	4.29
K7	0.00	11.18	0.99
	0.05	37.69	1.27
	0.10	38.89	2.39

Appendix 2.39 Raw data from experiment monitoring hyperosmotic stress tolerance. Cultures of the yeast cells were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. Yeast cells were then taken from thw culture at the 24 hour time point and exposed to 27% w/v sorbitol. Relative growth was measured based on optical density at 600 nm of yeast cells exposed to the stress condition relative to the control after 24 hours of growth. The data presented are the means of four independent experiments. (Figure 5.5)

Strain	Inositol Concentration (g/L)	Survival rate (%)	
		Mean value	Standard deviation
A12	0.00	83.46	3.11
	0.05	94.50	1.32
	0.10	94.12	2.83
A15	0.00	76.81	1.48
	0.05	86.17	1.85
	0.10	85.80	1.16
K7	0.00	58.91	0.78
	0.05	76.65	5.60
	0.10	73.53	6.10

Appendix 2.40 Raw data from experiment monitoring acetic acid stress tolerance. Cultures of the yeast cells were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. Yeast cells were then taken from the culture at the 24 hour time point and exposed to 67 mM acetic acid. Relative growth was measured based on optical density at 600 nm of yeast cells exposed to the stress condition relative to the control after 24 hours growth. The data presented are the means of four independent experiments. (Figure 5.6)

Strain	Inositol Concentration (g/L)	Survival rate (%)	
		Mean value	Standard deviation
A12	0.00	37.64	2.12
	0.05	70.75	3.92
	0.10	69.47	3.29
A15	0.00	7.33	0.34
	0.05	64.80	3.19
	0.10	63.08	3.21
K7	0.00	2.07	0.09
	0.05	10.19	1.92
	0.10	10.80	1.60

Appendix 2.41 Raw data from experiment monitoring external glycerol concentration (mg/mL). Cultures of the yeast cells were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 5.7)

Strain	Inositol Concentration (g/L)	Mean			Standard deviation		
		Time (hours)					
		0	24	96	0	24	96
A12	0.00	0.032	3.046	5.360	0.009	0.223	0.278
	0.05	0.033	3.839	6.396	0.010	0.260	0.527
	0.10	0.033	4.313	6.344	0.011	0.586	0.191
A15	0.00	0.050	2.588	7.215	0.010	0.523	0.131
	0.05	0.049	3.363	7.967	0.009	0.115	0.278
	0.10	0.049	3.658	8.308	0.011	0.151	0.241
K7	0.00	0.016	2.743	4.378	0.003	0.475	0.439
	0.05	0.016	2.822	4.486	0.003	0.455	0.208
	0.10	0.017	2.772	4.559	0.004	0.549	0.292

Appendix 2.42 Raw data from experiment monitoring fatty acid composition (%). Cultures of the A12 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 5.8(A))

Fatty acid	Mean			Standard deviation		
	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.00	0.05	0.10
C6:0	0.02	0.01	0.04	0.02	0.02	0.01
C8:0	0.05	0.04	0.17	0.04	0.07	0.06
C10:0	0.27	0.35	0.63	0.24	0.31	0.17
C12:0	1.24	0.34	0.75	0.49	0.34	0.13
C14:0	2.64	1.31	1.49	0.04	0.27	0.27
C14:1	0.57	0.14	0.46	0.50	0.25	0.07
C16:0	20.93	18.68	18.90	0.56	0.91	1.31
C16:1	23.08	24.41	20.58	2.45	3.36	1.85
C18:0	10.31	9.00	11.50	2.06	2.53	1.19
C18:1	40.63	45.63	45.21	3.52	1.56	2.15
C18:2	0.18	0.04	0.12	0.23	0.08	0.12
C18:3	0.00	0.00	0.00	0.00	0.00	0.00
C20:0	0.09	0.04	0.15	0.08	0.08	0.09

Appendix 2.43 Raw data from experiment monitoring fatty acid composition (%). Cultures of the A15 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 5.8(B))

Fatty acid	Mean			Standard deviation		
	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.00	0.05	0.10
C6:0	0.02	0.02	0.01	0.01	0.00	0.00
C8:0	0.06	0.11	0.11	0.01	0.03	0.03
C10:0	0.86	1.24	1.30	0.08	0.25	0.25
C12:0	1.56	1.13	1.13	0.06	0.09	0.08
C14:0	3.58	1.69	1.66	0.09	0.13	0.09
C14:1	0.68	0.34	0.35	0.04	0.03	0.03
C16:0	19.89	18.16	17.89	0.48	0.39	0.48
C16:1	20.84	17.27	17.67	0.74	0.44	0.24
C18:0	9.74	14.83	14.67	0.52	0.60	0.39
C18:1	42.39	44.88	44.70	0.41	0.63	0.88
C18:2	0.00	0.10	0.16	0.00	0.13	0.15
C18:3	0.00	0.00	0.00	0.00	0.00	0.00
C20:0	0.38	0.23	0.36	0.02	0.10	0.05

Appendix 2.44 Raw data from experiment monitoring fatty acid composition (%). Cultures of the K7 yeast strain were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 5.8(C))

Fatty acid	Mean			Standard deviation		
	Inositol concentration (g/L)					
	0.00	0.05	0.10	0.00	0.05	0.10
C6:0	0.01	0.03	0.04	0.01	0.02	0.01
C8:0	0.06	0.10	0.16	0.03	0.03	0.03
C10:0	0.79	0.75	1.02	0.29	0.24	0.17
C12:0	1.25	0.92	1.03	0.56	0.15	0.23
C14:0	1.97	1.05	0.92	0.85	0.13	0.06
C14:1	0.37	0.21	0.20	0.20	0.01	0.04
C16:0	18.33	13.56	13.89	1.00	0.44	0.52
C16:1	20.37	19.55	20.16	1.63	1.36	0.73
C18:0	9.82	11.96	11.32	1.54	1.08	0.05
C18:1	46.74	51.29	50.87	1.12	1.33	1.64
C18:2	0.14	0.51	0.35	0.19	0.41	0.13

C18:3	0.00	0.00	0.00	0.00	0.00	0.00
C20:0	0.15	0.06	0.05	0.09	0.02	0.00

Appendix 2.45 Raw data from experiment monitoring cell diameter (µm). Cultures of the yeast cells were grown under aerobic conditions in YNB medium with 10% (w/v) initial glucose concentration. Inositol concentration in the media was varied as indicated in the table. The fermentation was conducted at 30°C and 180 opm. The data presented are the means of three independent experiments. (Figure 5.9)

Strain	Inositol concentration (g/L)	Cell Size (µm)	
		Average	SD
A12	0.00	3.307	0.049
	0.05	3.280	0.054
	0.10	3.297	0.051
A15	0.00	3.632	0.064
	0.05	2.819	0.021
	0.10	2.832	0.006
K7	0.00	3.051	0.042
	0.05	3.480	0.966
	0.10	3.478	0.912

APPENDIX 3
EXAMPLE OF STATISTICAL ANALYSIS

Raw data was initially compiled into Minitab 15® for Windows®. This software package was then used to perform analysis of variance, which compared the variance of each parameter (i.e. μ_{max}, Q_S, Q_P, GP, OD_{600nm}, total cell number, viable cell number, cell viability, glucose concentration, ethanol concentration and supplement concentration) between three strains with the variability within each replicate experiment of each strain. Significant differences between the data were determined from p-value. When $p < 0.05$, the null hypothesis was rejected, which means there is a significant difference between the data.

When significant differences detected, the test was followed by Tukey's HSD (honestly significant difference) posthoc test to determine which variable did and did not differ significantly ($p<0.05$) from each other. Pairwise comparison was used to determine which value different significantly.

This appendix provides examples of output from the statistical analysis.

Appendix 3.1 Analysis of variance between groups for determination of significant variance between maximum growth rate of strain (A12, A15 and K7) and glucose concentration (5, 10 and 15% w/v), followed by Tukey's HSD test.

General Linear Model: umax versus Strain, Glucose Conc.

```
Method

Factor coding  (-1, 0, +1)


Factor Information

Factor         Type   Levels  Values
Strain         Fixed       3  A12, A15, K7
Glucose Conc.  Fixed       3  5, 10, 15


Analysis of Variance

Source                  DF    Adj SS    Adj MS  F-Value  P-Value
  Strain                 2  0.007876  0.003938    16.79    0.000
  Glucose Conc.          2  0.000079  0.000040     0.17    0.846
  Strain*Glucose Conc.   4  0.000583  0.000146     0.62    0.653
Error                   18  0.004221  0.000235
Total                   26  0.012760


Model Summary

        S    R-sq  R-sq(adj)  R-sq(pred)
0.0153139  66.92%     52.21%      25.56%
```

Comparisons for umax

Tukey Pairwise Comparisons: Response = umax, Term = Strain

```
Grouping Information Using the Tukey Method and 95% Confidence

Strain  N      Mean  Grouping
K7      9  0.283409  A
A12     9  0.264512      B
A15     9  0.241637         C

Means that do not share a letter are significantly different.
```

Tukey Pairwise Comparisons: Response = umax, Term = Glucose Conc.

```
Grouping Information Using the Tukey Method and 95% Confidence

Glucose
Conc.    N      Mean  Grouping
15       9  0.265333  A
10       9  0.263090  A
5        9  0.261137  A

Means that do not share a letter are significantly different.
```

Tukey Pairwise Comparisons: Response = umax, Term = Strain*Glucose Conc.

```
Grouping Information Using the Tukey Method and 95% Confidence

Strain*Glucose
Conc.          N      Mean  Grouping
K7 10          3  0.286471  A
K7 15          3  0.283292  A
K7 5           3  0.280465  A   B
A12 5          3  0.268072  A   B  C
A12 10         3  0.265017  A   B  C
A12 15         3  0.260447  A   B  C
A15 15         3  0.252258  A   B  C
A15 10         3  0.237780      B  C
A15 5          3  0.234873         C

Means that do not share a letter are significantly different.
```