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Investigation of Growth Medium Supplementation and Ethanol Tolerance of the Yeast *Saccharomyces cerevisiae*

Submitted by:

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CERTIFICATION OF DISSERTATION

I certify that the ideas, experimental work, analyses and conclusions reported in this thesis are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

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ABSTRACT

Ethanol tolerance is one of the most important properties of yeasts used for bioethanol production, and has been correlated with plasma membrane fluidity. This study investigates yeast membrane fluidity and ethanol tolerance, particularly in relation to proline and inositol supplementation. Three *Saccharomyces cerevisiae* strains (A12, PDM and K7) were selected, based on reported stress tolerance and ethanol productivity; an ethanol tolerant baker's yeast (A12), a wine yeast (PDM) and a sake yeast (K7), the latter produce up to 17 and 17.5 %(v/v) ethanol, respectively.

To determine the feasibility of these strains and supplementation for bioethanol production, a model system was devised using Yeast Nitrogen Base (YNB) with 18% (w/v) sucrose. YNB was chosen for its defined and consistent composition (limiting variation) and for its lower fluorescent background (enabling membrane fluidity assessment *in situ*). However growth of all strains was inconsistent and ferments stuck at high sugar levels. This was likely due to insufficient nitrogen or other essential nutrients, and could be ameliorated by a complex but undefined medium but with high and inexact levels of proline and inositol. In order to allow unequivocal discrimination of supplement effects, experiments were continued with media similar to previous laboratory studies; YNB with 2% (w/v) glucose.

When cultured in YNB with 2% (w/v) glucose, the three strains had similar growth rates and performance, although K7 maintained significantly higher viability. Comparison of generalized polarization (GP) of laurdan-labelled cells indicated that PDM had the highest membrane fluidity, followed in order by K7 and A12. Conversely A12 had the highest ethanol tolerance, followed in order by K7 and PDM, so unlike some published reports, higher ethanol tolerance related to lower membrane fluidity. Furthermore in

comparison to 6 h cultures, 24 h cultures of all strains had lower membrane fluidity and higher ethanol tolerance.

Two approaches were used to assess ethanol tolerance. The total plate count (TPC) is widely used to assess ethanol tolerance, while methylene violet staining has been proposed as a rapid alternative. Correlation analysis showed only weak correlations between viability assessment by methylene violet staining and viability by TPC, membrane fluidity by GP or culture age. In contrast there were strong correlations between membrane fluidity by GP, viability by TPC and culture age.

Despite showing promise in previously published studies as a stress tolerance enhancer, proline supplementation did not lead to any consistent significant change in membrane fluidity or ethanol tolerance. The only significant effect was the higher GP of the PDM strain with 0.5 g/L proline. However, no significant differences between levels of supplementation were detected in viability reduction in ethanol-stressed cultures (either by TPC or methylene violet staining). Therefore further study is needed to confirm this result. The present study failed to confirm reports that inositol supplementation increases ethanol tolerance. No significant changes of either GP or viability reduction upon ethanol stress were found when the medium was supplemented with various levels of inositol. Further investigation, including more variations in concentration, is needed to elucidate this possibility.

In summary, of the three *S. cerevisiae* strains tested, A12 seems to be the best for bioethanol production, followed by K7 and then PDM. Some relationships were found between culture age, ethanol stress tolerance and membrane fluidity, although supplementation of cultures with proline or inositol did not seem to enhance culture performance or ethanol tolerance.

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GLOSSARY OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
ATP	Adenosine tri phosphate
CDP-DAG	Cytidine diphospho-diacylglycerol
DLPC	dilauroyl-phosphatidylcholine
DNA	Deoxyribonucleic Acid
DPH	1,6-Diphenyl-1,3,5-hexatriene
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron Paramagnetic Resonance
FAD2	Δ12-fatty acid desaturase encoding gene
FTIR	Fourier Transform Infrared
G	Grating correction factor
g	Gravity, relative centrifugal force
GMO	Genetically modified organism
GP	Generalized polarization
GRAS	Generally recognized as safe
HPLC	High Performance Liquid Chromatography
INO1	*myo*-inositol-1-phosphate synthase encoding gene
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
Laurdan	6-lauroyl-2-dimethylamino naphthalene
LSD	Least Significant Difference
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
P5C	Δ^1-pyrroline-5-carboxylate
PA	Phospatidic acid
PC	Phosphatidylcholine
PC	Personal Computer
PDA	Photo Diode Array
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PMMA	polymethyl methacrylate
PRO1	γ-glutamyl kinase encoding gene

PS	Phosphatidylserine
PUT1	Proline oxidase encoding gene
PUT2	Δ^1-pyrroline-5-carboxylate dehydrogenase encoding gene
r	anisotropy
RID	Refractive Index Detector
ROS	Reactive oxygen species
SPS	Ssy1-Ptr3-Ssy5 complex
T_m	Melting Temperature of DNA
TPC	Total plate count
VR MVS	viability reduction by methylene violet staining
VR TPC	viability reduction by total plate count
YEP	Yeast extract peptone
YNB	Yeast nitrogen base
YNBNG	Yeast nitrogen base without glucose
YPD	Yeast extract peptone dextrose

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CHAPTER ONE: LITERATURE REVIEW AND BACKGROUND

1.1 Introduction

Production of ethanol as a fossil fuel replacement has become more important because of the decrease in the availability of fossil fuels, their increasing price and also environmental issues (Bai, Anderson & Moo-Young 2008; Thomsen, Medina & Ahring 2003). Ethanol derived from plant sources is known as bioethanol, and is mainly produced from sugar rich feedstocks such as molasses, cellulose or starch (Thomsen, Medina & Ahring 2003). These feedstocks are converted to bioethanol through fermentation by microorganisms, especially baker's yeast (*Saccharomyces cerevisiae*). Recently the bacterium *Zymomonas mobilis* has also been exploited for the fermentation process (Cazetta *et al.* 2007; Dien, Cotta & Jeffries 2003). However, *S. cerevisiae* is still preferred over *Z. mobilis* (Bai, Anderson & Moo-Young 2008; Dien, Cotta & Jeffries 2003). Bai, Anderson & Moo-Young (2008) summarized reasons why *Z. mobilis* is not suitable for industrial application. Firstly, *Z. mobilis* has narrow substrate preference, utilizing D-glucose, D-fructose and sucrose. When sucrose is used as a substrate, formation of by products lowers ethanol productivity. Therefore, *Z. mobilis* is not suitable for industrial feedstocks with complex mixture of sugars. Secondly, even though *Z. mobilis* is recognized as a generally recognized as safe (GRAS) microorganism, unlike *S. cerevisiae*, *Z. mobilis* is not generally accepted for animal feed, and therefore it will generate problems for utilization of biomass waste if *S. cerevisiae* is replaced by *Z. mobilis* in industrial ethanol production. Lastly, *Z. mobilis* is reported to be oscillatory (cycles of increase and decrease of metabolites during fermentation) when applied in continuous fermentation, indicated by cycling of concentrations of either substrate

or product under a particular condition. This property eventually inhibits sugar utilization and ethanol production. Therefore *S. cerevisiae* is still extensively used for industrial bioethanol production (Bai, Anderson & Moo-Young 2008).

The requirement for highly efficient processes of bioethanol production has led to the need for yeasts with capability for high levels of ethanol production. However, high ethanol concentrations cause a problem, in that the fermentative microorganisms have limited ethanol tolerance, which therefore limits the ethanol produced during the fermentation process (Ingram 1986). Many efforts have been conducted to increase the ethanol tolerance and fermentative efficiency of yeasts, to improve their capability to produce high levels of ethanol (Chi, Kohlwein & Paltauf 1999; Takagi *et al.* 2005; Xue *et al.* 2008).

Genetically modified yeasts have been introduced by several investigators. Metabolic pathways of these yeasts were improved so that they can tolerate various stresses during the fermentation process and produce higher concentrations of ethanol. Genetic modifications of the yeast include changes to yeast protein expression leading to increasing unsaturation index of membrane fatty acids (Kajiwara *et al.* 2000; You, Rosenfield & Knipple 2003) or yeast accumulation of specific metabolites that increase stress tolerance (Takagi *et al.* 2005; Terao, Nakamori & Takagi 2003).

Other investigators have modified the growth medium by adding components that have been shown to be protective agents against various stresses. These components include minerals (Xue *et al.* 2008), trehalose (Hottiger *et al.* 1994), inositol (Chi, Kohlwein & Paltauf 1999; Ji *et al.* 2008) and proline (Takagi 2008; Takagi *et al.* 2005).

A combination of both genetic modification and modification of growth medium has also been performed (Krause *et al.* 2007; Takagi *et al.* 2000; Takagi *et al.* 2005). These experiments indicated improved performance of ethanol

production by increasing tolerance of the yeast against various stresses, such as heat, freezing, high osmotic pressure and high ethanol concentration. By increasing the yeast tolerance against various stress factors, the yeast can continue to produce ethanol in the presence of these stresses during the fermentation. This can lead to continued conversion and to higher concentrations of ethanol produced in the fermentation process, further decreasing the cost of the distillation process, a very important consideration in industrial process.

The current study emphasizes the effect of growth media on the ethanol tolerance of yeast. The two supplements investigated in this study are inositol and L-proline. Inositol has been shown to have protective properties against ethanol stress (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004). Proline supplementation has been investigated in *S. cerevisiae* that have disrupted proline synthesis, but at only one concentration (Takagi 2008; Takagi *et al.* 2005). Inositol supplementation has been investigated in *Saccharomyces* yeasts, but at only one concentration; either 75 µM (Krause *et al.* 2007) or 100 µg/mL (Chi, Kohlwein & Paltauf 1999). Inositol supplementation has been investigated at several concentrations in a non-*Saccharomyces* yeast (Ji *et al.* 2008). Thus none of the previous studies have investigated the influence of different concentrations of the supplements proline or inositol on *S. cerevisiae* health.

L-proline has also been shown to lead to protection against various stresses, including freezing, desiccation, oxidation, and ethanol (Takagi 2008). Nevertheless, previous studies mainly used genetic engineering to modify the metabolic pathway of the yeast so that it can accumulate high concentrations of L-proline inside the cell (Takagi 2008; Takagi *et al.* 2000; Takagi *et al.* 2005). It has been shown that *S. cerevisiae* has the capacity to assimilate extracellular L-proline under aerobic conditions with limited assimilation under anaerobic conditions (Horak & Kotyk 1986; Ingledew, Magnus & Solsuski 1987). Focussing

on the role of L-proline as an osmoprotectant, Thomas, Hynes & Ingledew (1994) showed that L-proline supplementation stimulated yeast growth and improved viability and ethanol productivity; however, they did not report effects on ethanol tolerance *per se*. Thus there are no published reports which specifically address the influence of extracellular L-proline on ethanol tolerance.

The major objective of this study was the identification of appropriate yeast strains and culture conditions to improve the efficiency of bioethanol production, leading to increased amounts of ethanol produced by the fermentation process. In addition to increasing the efficiency of this phase of manufacture, increased ethanol production will decrease the cost and energy expenditure of the distillation phase. The ultimate goal of this study is to improve the efficiency of bioethanol production, thereby lowering the cost, inputs and waste.

1.2 The Yeast Plasma Membrane

The plasma membrane of eukaryotic cells, including yeast, is one of the most important constituents of the cell (Nipper 2007). The membranes are thin, flexible, and relatively stable sheet-like structures that covers all living cells and organisms (McKee & McKee 2003). The yeast plasma membrane is about 7.5 nm in width (van der Rest *et al.* 1995). It is the first barrier that separates the cell from the environment and therefore becomes the first component to be damaged when the cell is exposed to various environmental stresses (Learmonth 2011; Learmonth & Gratton 2002; Rodrıguez-Vargas *et al.* 2007). The plasma membrane also has functions for transporting substances in to and out of the cells, signal transduction, maintaining the shape of the cells, interaction among cells and overall metabolism of the cells (Elliot & Elliot 1997; Nipper 2007).

Like other biological membranes, the yeast plasma and intracellular membranes are also composed of various types of membrane lipid (Nipper

2007). Basically, they are lipid bilayers composed of phospholipids, sterols and other lipid molecules, in which various proteins are inserted. Phospholipid structures are composed of two distinctive parts that make them suitable for their structural roles; a hydrophilic “head” group and hydrophobic part composed of two fatty acid chains (McKee & McKee 2003). The membrane proteins give special abilities to membrane such as molecule and ion transport, energy generation and signal transduction. The amount and type of proteins in specific cell membranes are different, depending on the environment of the cell (McKee & McKee 2003).

1.2.1 Phospholipids

Phospholipids are the main component of the yeast plasma membrane. Most phospholipids are phosphoglycerides, with the sphingolipid sphingosine also present in some membranes. A phosphoglyceride is composed of a glycerol with sn1 and sn2 positions esterified to fatty acids, and the sn3 position attached to a phosphate group which may then be attached to a hydrophilic molecule (Beltran 2005; Daum *et al.* 1998). The fatty acids may vary in length and unsaturation. A saturated fatty acid is commonly present on the sn1 position, with the sn2 position commonly occupied by an unsaturated fatty acid (Beltran 2005). Palmitic (16:0), stearic (18:0), palmitoleic (16:1) and oleic (18:1) acids are the most abundant fatty acids found in the phospholipids (Beltran 2005). Minor amounts of myristic (14:0) and C-26 fatty acid have also been found in *S. cerevisiae* membranes (Daum *et al.* 1998).

One hydroxyl group of the phosphate can be linked to a polar molecule and make up the hydrophilic part of the phospholipids which is responsible for certain physical properties of the phospholipids. The head group component can be used as a basis of classification (Daum *et al.* 1998). The main polar molecules

attached to the phosphate group of phospholipids in the yeast plasma membrane are ethanolamine, choline, serine and inositol (Beltran 2005). The leaflet of the lipid bilayer facing the interior of the cell is rich in phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), while the external leaflet is rich in phosphatidylcholine (PC) and sphingolipids (van der Rest *et al.* 1995). Figure 1.1 shows the main classes of phospholipids of *S. cerevisiae*.

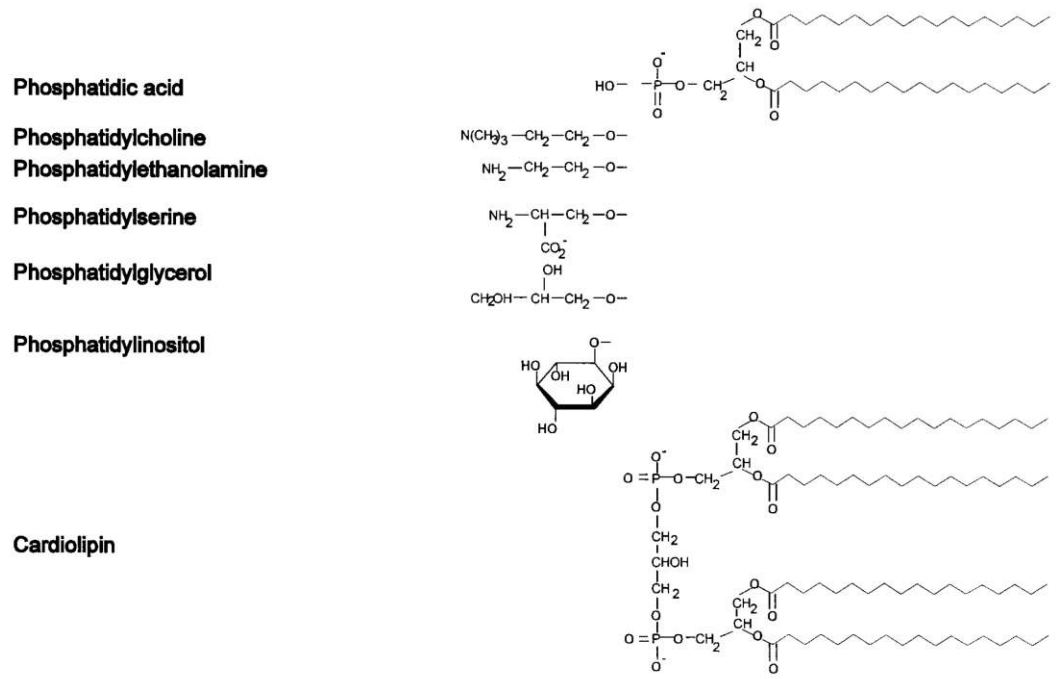


Figure 1.1 Phospholipids found in the plasma membrane of *S. cerevisiae* (from van der Rest *et al.* 1995)

Several authors have reported different compositions of *S. cerevisiae* plasma membrane phospholipids. The composition of phospholipids 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 composition of plasma membrane phospholipids from several reports are presented in Table 1.1.

Yeast plasma membrane phospholipids are mainly synthesised via the CDP-DAG pathway (Carman 2005; Gaspar *et al.* 2006). In this pathway PS, PE and

PC are synthesised from CDP-DAG via reactions catalysed by *CHO1*-encoded PS synthase, *PSD1*/*PSD2*-encoded PS decarboxylase, *CHO2*-encoded PE methyltransferase and *OPI3*-encoded phospholipid methyltransferase (Carman 2005). Figure 1.2 shows the synthetic pathways of phospholipids in *S. cerevisiae*.

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)	Blagovic *et al.* (2005)	Butcher (2008)
Phosphatidyl choline	17.0	16.8	11.3	18.7	27.2
Phosphatidyl ethanolamine	14.0	20.3	24.6	16.6	19.8
Phosphatidyl inositol	27.7	17.7	27.2	36.6	24.7
Phosphatidyl serine	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

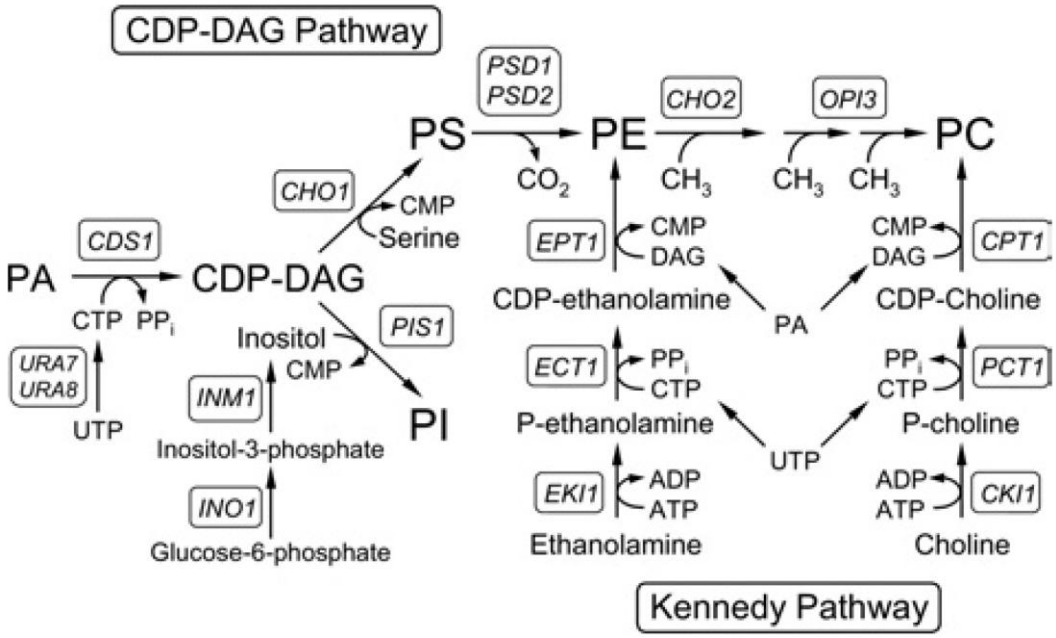


Figure 1.2 Pathways for synthesis of phospholipids in *S. cerevisiae* (from Carman 2005)

The present study investigates the influence of inositol supplementation on ethanol tolerance of the yeast. The presence of inositol in the growth medium has been shown to influence the levels of PC. According to Gaspar *et al.* (2006), inositol addition increases PC turnover. When the medium was supplemented by inositol, the PI proportion increased and the PC proportion decreased, while no significant changes were observed for other phospholipid classes. Inositol can act as a noncompetitive inhibitor at the major pathway branch point by inhibiting PS synthase. 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 (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 composition of the yeast membrane phospholipids. Other changes in phospholipid composition have been reported following inositol supplementation (Chi, Kohlwein & Paltauf 1999; Gaspar *et al.* 2006). In the presence of inositol, the PI content increased while PC and PE levels decreased (Chi, Kohlwein & Paltauf 1999). However, Carman (2005) suggested that it is possible to compensate for the lowered availability of PS as a substrate by activating the Kennedy pathway. Further studies are still needed to confirm this hypothesis (Carman 2005). Increased levels of membrane PI could contribute to increased signalling activity by PI – based signalling pathways, thereby contributing to enhanced stress responses.

1.2.2 Sphingolipids

Sphingolipids are another important constituent of the yeast plasma membrane. Sphingolipids are composed of a long chain amino alcohol, to which is attached a fatty acid and a hydrophilic head group (McKee & McKee 2003). In yeast, the long chain amino alcohol is primarily phytospingosine (D-erythro-2-amino-octadecane-1,3,4-triol) which is also a major component of plant plasma membranes but a minor component of the plasma membrane of animal cells (Daum *et al.* 1998). The phytospingosine is N-acylated with a fatty acid and the acylated molecule is known as a ceramide. In the *S. cerevisiae* plasma membrane the main fatty acids are characteristically C18, C20 and C26 (Patton & Lester 1991; van der Rest *et al.* 1995). The polar alcohol group of the phytospingosine is joined by glycosidic or phosphodiester linkages (Nelson & Cox 2008).

There are three main sphingolipids found in the yeast plasma membrane: inositolphospho-ceramide; mannosyl-inositolphospho-ceramide; and mannosyl-diinositolphospho-ceramide (Beltran 2005; van der Rest *et al.* 1995). The structures of the sphingolipids found in the yeast plasma membrane are shown in Figure 1.3.

Patton & Lester (1991) suggest that 90% or more of the yeast sphingolipids are located in the plasma membrane. They also found that sphingolipids comprise more than 30% of total phospholipids in the yeast plasma membrane.

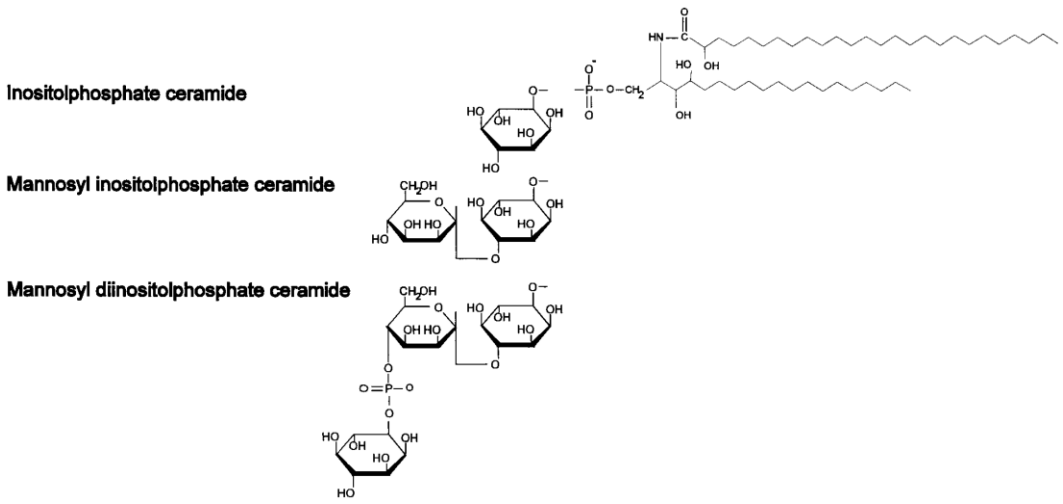


Figure 1.3 Major sphingolipids found in the *S. cerevisiae* plasma membrane (from van der Rest *et al.* 1995)

1.2.3 Fatty acyl chains

Although other yeast genera can produce polyunsaturated fatty acids, *S. cerevisiae* does not have the capability to synthesis polyunsaturated fatty acids, so the fatty acid composition of the *S. cerevisiae* plasma membrane is rather simple (Schneiter *et al.* 1999). The main fatty acids found in *S. cerevisiae* plasma membranes are palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1) and oleic (C18:1) acids, with minor amounts of other fatty acids (Daum *et al.* 1998; van der Rest *et al.* 1995). It appears that some strains of *S. cerevisiae* may form polyunsaturated fatty acids under some circumstances, although they also readily take up fatty acids from extracellular sources (Kajiwara *et al.* 1996). The composition of fatty acids in the *S. cerevisiae* plasma membrane is presented in Table 1.2.

Table 1.2 Composition of fatty acids in *S. cerevisiae* plasma membranes (from van der Rest *et al.* 1995)

Chain length and saturation	% of total fatty acid
10:0-14:0	7.0
16:0	12.8
16:1	32.8
18:0	8.0
18:1	28.0
18:3	1.4
20-24	8.0

The packing of the fatty acyl chains is a major determinant of plasma membrane fluidity. The packing increases with increasing fatty acyl chain length and decreases with increasing degree of unsaturation. Thus, increasing fatty acyl chain length and lowering degree of unsaturation could be expected to lead to a more ordered membrane structure and lower fluidity (van der Rest *et al.* 1995). Increasing hydrostatic pressure, lowering temperature and addition of sterols to phospholipids can result in lowering fluidity, since these treatments affect the bilayer by decreasing the relative area of lipid molecules (van der Rest *et al.* 1995). Other factors which may also influence membrane fluidity include phospholipid class distribution, ionic strength, pH, protectant molecules, membrane proteins, and cell metabolic status. The balance of these factors will determine the membrane fluidity (Learmonth 2011). Membrane fluidity has been suggested as mechanism for adaptation to various environmental stresses (Learmonth & Gratton 2002; Rodrıguez-Vargas *et al.* 2007), as will be further discussed in section 1.4.

1.2.4 Sterols

Sterols comprise another important lipid component of yeast plasma membranes. This component is essential for eukaryotic membrane stability and affects important characteristics of plasma membranes such as fluidity, flexibility and permeability (Daum *et al.* 1998). Sterols are rigid polycyclic hydrophobic molecules which may or may not contain a flexible aliphatic moiety and with a hydrophilic hydroxyl group that makes them weakly amphipathic (Beltran 2005). Ergosterol (Figure 1.4) is the major sterol component of yeast plasma membranes with zymosterol being the minor component (Zinser, Paltauf & Daum 1993).

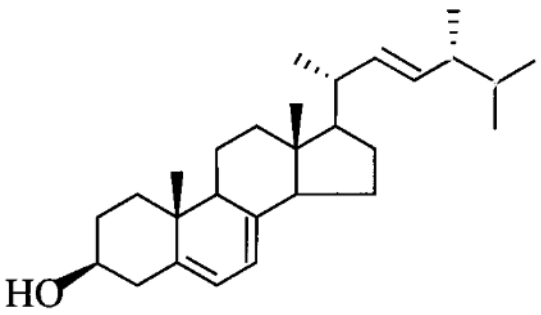


Figure 1.4 Chemical structure of ergosterol, the main sterol of yeast plasma membranes (from Daum *et al.* 1998)

Biochemical and biophysical studies have concluded that sterols are important regulators of membrane fluidity and permeability (Beltran 2005; Daum *et al.* 1998) and may also affect the lateral movement and the activity of membrane proteins (van der Rest *et al.* 1995).

1.3 Ethanol Stress and Yeast Tolerance

During ethanolic fermentation, yeast cells are exposed to various stress conditions such as temperature upshifts, high osmolarity, free radicals, nutrient starvation, organic acids (Siderius & Mager 2003) and also high ethanol concentrations (Dinh *et al.* 2008; Learmonth 2011; Learmonth & Gratton 2002; Taylor *et al.* 2008). These stress conditions will be sensed by the yeast cells and

will induce signal transduction pathways that lead to changes in gene expression and metabolism. The aim of the responses can be repair of the damage caused by the stress, protection of cell components and/or induction of stress tolerance. After these mechanisms become effective, the growth and functional activity of the cells can generally be resumed (Siderius & Mager 2003). A generalized scheme of yeast responses to stress conditions is presented in Figure 1.5.

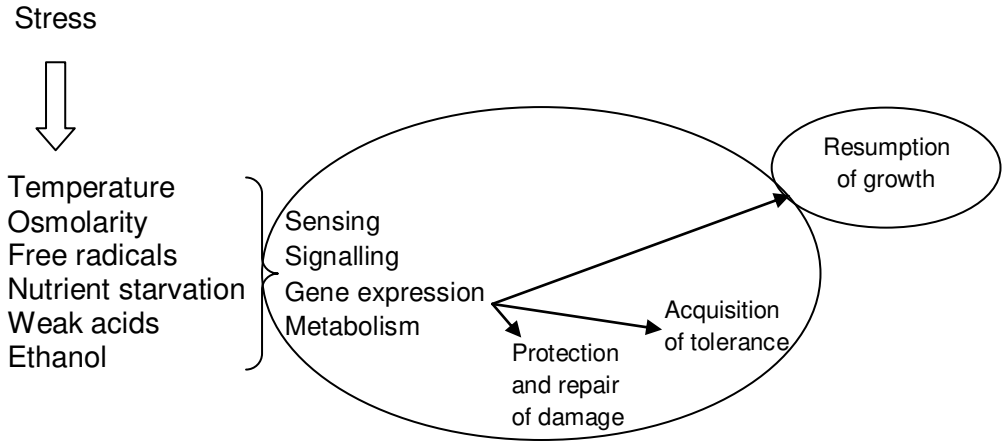


Figure 1.5 General scheme illustrating the main principles of yeast response to stress (from Siderius & Mager 2003; with addition from Dinh *et al.* 2008).

One condition considered as a stress for the yeast cells is high ethanol concentration. Although ethanol is the main product of the fermentation process, it is well known as a toxic substance for yeast cells. It has been reported to damage mitochondrial DNA and can act as an inactivator of several enzymes such as hexokinase (You, Rosenfield & Knipple 2003) and alcohol dehydrogenase (Nagodawithana & Steinkraus 1976). Ethanol inhibits amino acid and glucose transport systems which leads to inhibition of growth and a decrease in cell viability (Lei *et al.* 2007). Ethanol also causes fluidisation of cell membranes (Learmonth & Gratton 2002).

One of the most studied adaptations of yeast to high ethanol concentration is increasing the unsaturation index of the yeast plasma membrane (Dinh *et al.* 2008; Rodrıguez-Vargas *et al.* 2007; You, Rosenfield & Knipple 2003), i.e. by

increasing the proportion of unsaturated fatty acid in the yeast plasma membrane. Since the fatty acid composition of the yeast plasma membrane is rather simple (mainly composed of palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1) and oleic (C18:1) acids), the changes of these fatty acids in the presence of high ethanol concentration can be simply monitored.

Increasing ethanol concentration leads to increasing the amounts of monounsaturated fatty acids in the phospholipid component of the plasma membrane with corresponding decreasing amounts of saturated fatty acids, which in turn leads to a higher unsaturation index. Alexandre, Rousseaux & Charpentier (1994) found that in the presence of high ethanol concentration, the amount of C18:1 was increased dramatically while C16:1 did not change significantly. This change was associated with a decrease of C16:0 and C18:0, with the former showing the most dramatic changes. Their work showed that the unsaturation index was increased by ~30% in the presence of ethanol. Interestingly, while the unsaturation index went up, the average length of the fatty acyl chains increased, with a decrease in C16:0 and increase in C18:1.

Changes in ergosterol composition of the plasma membrane have also been observed when yeasts are grown in the presence or absence of ethanol. The proportion of ergosterol in yeast cells increases at the expense of other sterols when grown in the presence of ethanol. This suggests that ethanol tolerance is highly correlated with high proportions of ergosterol. Determination of sterol:protein and sterol:phospholipid ratios revealed that both ratios were decreased relative to control when the yeast were grown in the presence of ethanol. These changes combined to increase the measured fluidity of the plasma membrane (Alexandre, Rousseaux & Charpentier 1994).

Some researchers also reported morphological differences in yeast cells related to ethanol tolerance (Canetta, Adya & Walker 2006; Dinh *et al.* 2008; Lei

et al. 2007). These studies showed that upon exposure to stepwise increasing ethanol concentrations, yeast adapt by increasing their size. Ethanol-adapted strains were observed to have larger cell size than non-adapted strains. While the shortest diameter of adapted and non-adapted cells were similar, clear differences were seen in the longest diameter (Dinh *et al.* 2008). In contrast, Canetta, Adya & Walker (2006) found that upon acute exposure to 30% ethanol yeast cells exhibited shrinkage and cell surface roughness as detected by atomic force microscopy.

Manipulation of floculence by adjusting mechanical stirring rates in a bioreactor resulted in different floc populations containing yeast of different size and different ethanol tolerance. Lei *et al*. (2007) compared four different yeast floc sizes (100, 200, 300 and 400 µm) and found that the floc population with the smallest cell size showed the lowest ethanol tolerance. The ethanol tolerance was found to increase with increasing floc cell size up to 300 µm, while further increases in floc cell size resulted in decreasing ethanol tolerance (Lei *et al.* 2007). Figure 1.6 presents the influence of different yeast floc cell size on ethanol tolerance of the yeast.

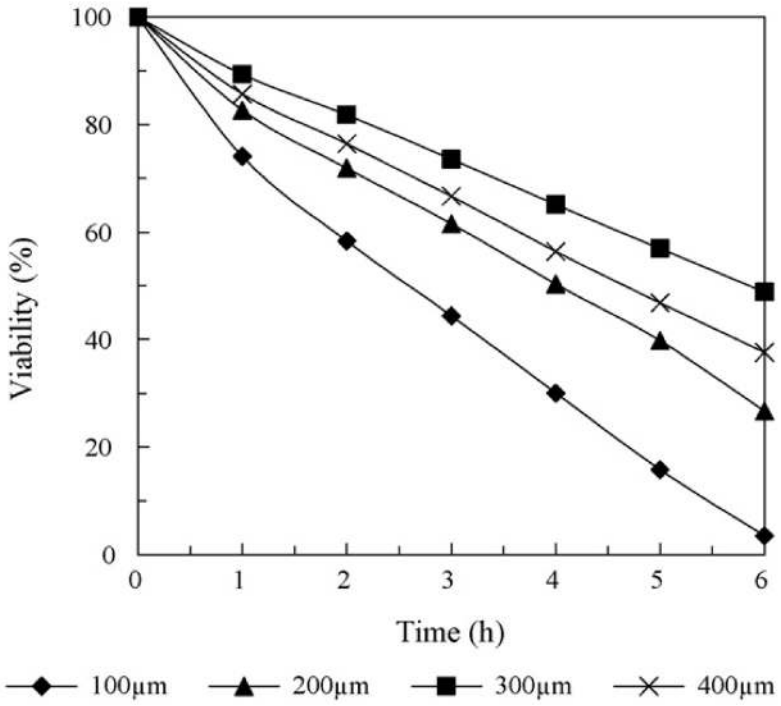


Figure 1.6 Viability of the yeast floc populations after exposure to 20% (v/v) ethanol shock at 30°C. Different floc cell sizes are indicated by different symbols in the graph (from Lei *et al.* 2007).

The same study also confirmed a positive effect of ergosterol on ethanol tolerance. Cell ergosterol content followed the same pattern as ethanol tolerance, increasing in content up to the 300 µm cell size floc population, and then decreasing in the 400 µm cell size floc population, thus increasing ethanol tolerance was related to increasing ergosterol content (Lei *et al.* 2007).

Phospholipids are one of the most important components of the yeast plasma membrane. Phospholipid composition is also affected when yeast cells are exposed to high ethanol concentrations. By monitoring the plasma membrane phospholipid composition during the ethanol fermentation process, Chi, Kohlwein & Paltauf (1999) found that during ethanol production, PI increased while other components (phospatidic acid (PA), PC, PE and PS) were decreased, especially PC and PE which decreased rapidly, as will be detailed in section 1.4.2.

Relationships between phospholipid composition and yeast floc cell size were also observed by Lei *et al*. (2007) in yeast cells that had adapted to high ethanol concentration. The relationship between plasma membrane PI and PE levels and floc cell size was similar to that between ergosterol level, floc cell size, and ethanol tolerance. In contrast, PC showed a different relationship in that the highest PC level was observed in the 200 µm floc cell size and PC level decreased dramatically in the 300 µm floc cell size (where the concentration of other phospholipids were maximal). This result is in agreement with that of previous study by Chi, Kohlwein & Paltauf (1999) in that a more ethanol tolerant yeast had a higher proportion of PI in the plasma membrane.

1.4 General Stress Protectants

Many researchers have found that the lethal effect of a stress condition can be reduced by the addition of supplement(s) to the growth media (Redón *et al.* 2009; Xue *et al.* 2008), metabolic engineering of the yeast to accumulate specific compounds that can act as protectors (Rodrıguez-Vargas *et al.* 2007; Takagi *et al.* 2005) or a combination of both treatments (Krause *et al.* 2007).

Some well known supplements that can act as stress protectors include unsaturated fatty acids (Redón *et al.* 2009), trehalose (Hirasawa *et al.* 2001), metal ions (Birch & Walker 2000; Walker 2004; Xue *et al.* 2008), ergosterol (Redón *et al.* 2009; Swan & Watson 1998) and inositol (Ji *et al.* 2008).

Addition of different types of fatty acids to growth media for anaerobic culture of yeasts can result in modification of plasma membrane fatty acid composition (Steels, Learmonth & Watson 1994). Yeast grown anaerobically in unsaturated fatty acids supplemented medium incorporate the supplemented unsaturated fatty acids, while yeasts grown aerobically typically modulate their unsaturated fatty acids composition and do not incorporate polyunsaturated lipids like C18:2

and C18:3 into their membranes (Steels, Learmonth & Watson 1994). Redón *et al.* (2009) studied fatty acids supplementation in wine yeast and found a slight increase in ethanol tolerance in yeasts which incorporated a palmitoleic (C16:1) acid supplement, but not of those which incorporated a linolenic (C18:3) acid supplementation. Furthermore, other studies have found that oleic (C18:1) acid gives even more significant effects on ethanol tolerance than palmitoleic (C16:1) acid (Alexandre, Rousseaux & Charpentier 1994; You, Rosenfield & Knipple 2003).

Trehalose is another compound that can act as a stress protectant. Previous studies suggest that trehalose can protect yeast cell against heat, ethanol, oxidative stress, freezing, high osmotic pressure and weak acid (Lewis *et al.* 1997). One of the possible mechanisms of trehalose protection against stress has been proposed to be both protection of proteins against damage (Hottiger *et al.* 1994) and membrane stabilisation (Wiemken 1990).

Ethanol tolerance can be affected by supplementation of the growth media with metal ions (Birch & Walker 2000; Walker 2004; Walker *et al.* 2006). The ethanol tolerance of yeast was found to be strongly affected by the addition of zinc, magnesium and calcium (Birch & Walker 2000; Walker 2004; Xue *et al.* 2008) (see Figure 1.7). However, a further study suggested that excess zinc can be toxic to the yeast cell and lead to lower viability (Xue *et al.* 2008). Therefore, addition of metal ions to the growth medium needs to be optimized in order to achieve the best results.

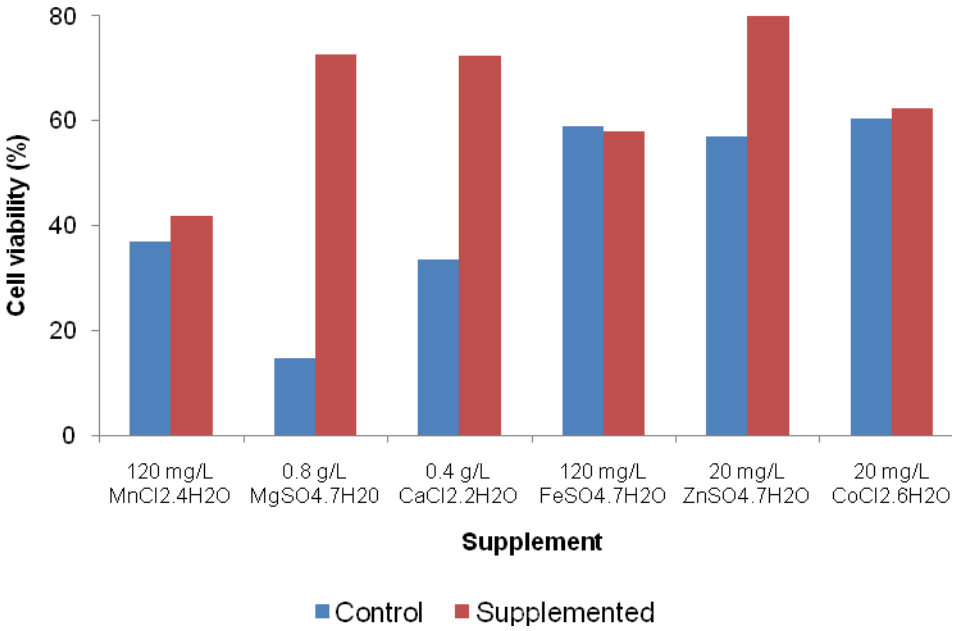


Figure 1.7 Effect of metal ion supplementation on cell viability after ethanol stress (18% v/v, 1 h) of a self flocculating yeast (adapted from Xue *et al.* 2008).

1.4.1 L-Proline

Beside its main role as amino acid component of protein molecules, L-proline is also considered to function as osmoprotectant, enhancing the stability of proteins and/or membranes during freezing, dehydration, and increasing temperature. It has also been reported to lower the melting temperature of DNA due to destabilization of the double helix during salt stress, and also to increase the solubility of protein thus inhibiting protein aggregation during folding/refolding and acting as an antioxidant by scavenging reactive oxygen species (ROS) (Takagi 2008).

Previous studies of *S. cerevisiae* showed that L-proline has protective effects against freezing (Morita, Nakamori & Takagi 2003; Sekine *et al.* 2007; Terao, Nakamori & Takagi 2003), desiccation (Takagi *et al.* 2000) and high ethanol concentration (Takagi *et al.* 2005). These studies mainly focused on developing yeast strains which accumulate L-proline inside the cell by means of genetic

manipulations. The targets of these studies were mainly the enzymes involved in L-proline biosynthesis and metabolism as shown in Figure 1.8 (Morita, Nakamori & Takagi 2003; Takagi *et al.* 2005; Terao, Nakamori & Takagi 2003).

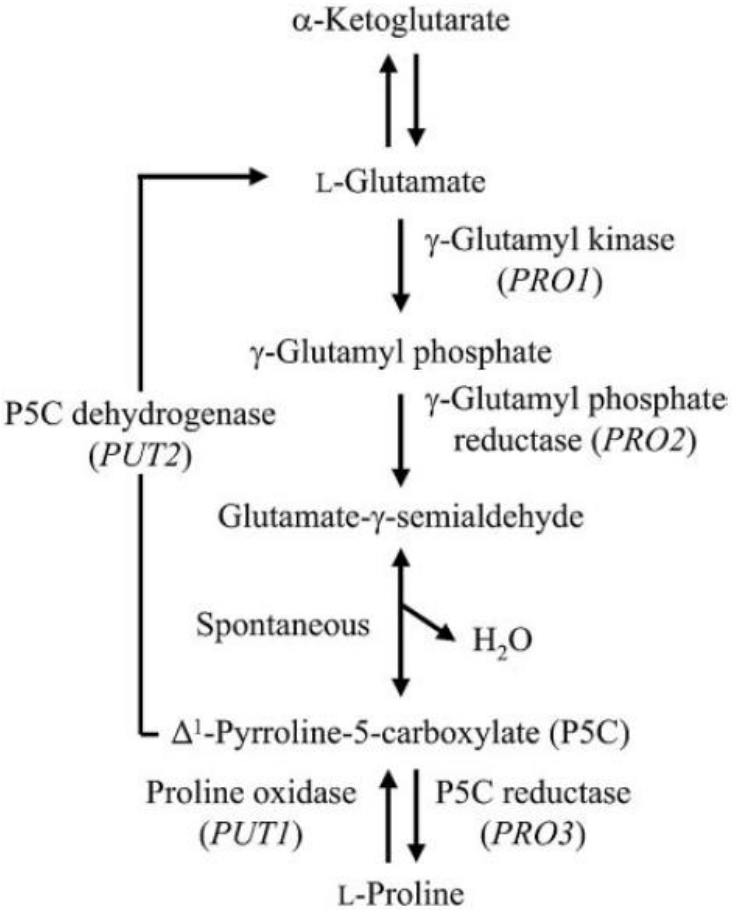


Figure 1.8 Biosynthesis and metabolism of L-proline in *S. cerevisiae*. Genes encoding enzymes are shown in parentheses (from Takagi *et al.* 2005).

Figure 1.8 shows that L-proline is synthesized from L-glutamate through a four step process in which three steps are enzyme catalysed reactions and one step is a spontaneous reaction. The enzymes of the biosynthesis of L-proline from L-gutamate are γ-glutamyl kinase (the *PRO1* gene product), γ-glutamyl phosphate reductase (the *PRO2* gene product) and Δ^1-pyrroline-5-carboxylate (P5C) reductase (the *PRO3* gene product). Two enzymes are involved in converting L-proline back to L-glutamate; these are proline oxidase (the *PUT1* gene product) that converts L-proline to P5C, and P5C dehydrogenase (the

PUT2 gene product) that converts P5C to L-glutamate, in mitochondria (Terao, Nakamori & Takagi 2003).

Increased intracellular L-proline content of yeast can be achieved by increasing the activity of one of the three enzymes involved in the synthesis pathway of L-proline or decreasing the activity of one of the two enzymes that convert L-proline in to L-glutamate.

Takagi *et al*. (2005) enhanced intracellular L-proline content of two different yeast strains (laboratory and sake strains) by substituting aspartic acid in position 154 of γ-glutamyl kinase that encoded by *PRO1* gene. This mutation increases the activity of the γ-glutamyl kinase and γ-glutamyl phosphate reductase (which are proposed to form a complex) and causes accumulation of L-proline inside the yeast cell. This accumulation resulted in increased ethanol tolerance of the yeast cells (Figure 1.9). To determine whether the effect was due to the increased L-proline content or other substances, the authors also tested the intracellular content of L-proline, total amino acids and trehalose and found that the L-proline content differed significantly between wild type and mutant strains (Figure 1.10), while there were no significant differences in other substances tested. This finding suggests that the improved ethanol tolerance was due to increased intracellular L-proline content (Takagi *et al.* 2005).

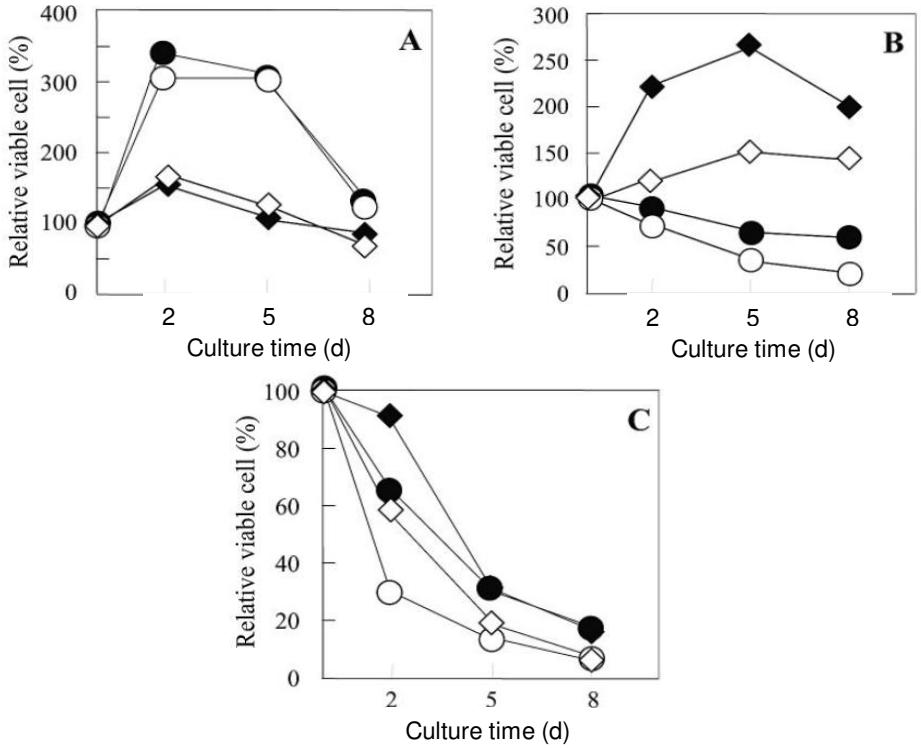


Figure 1.9 Relative numbers of viable cells of laboratory and sake strains grown in SD medium without (A) or with 9% (B) or with 18% (C) ethanol and incubated under static conditions. The *S. cerevisiae* strains used were the parent laboratory strain (○) and L-proline accumulating laboratory mutant strain (●) and control strain (◇) and L-proline accumulating sake strain (◆) (from Takagi *et al.* 2005).

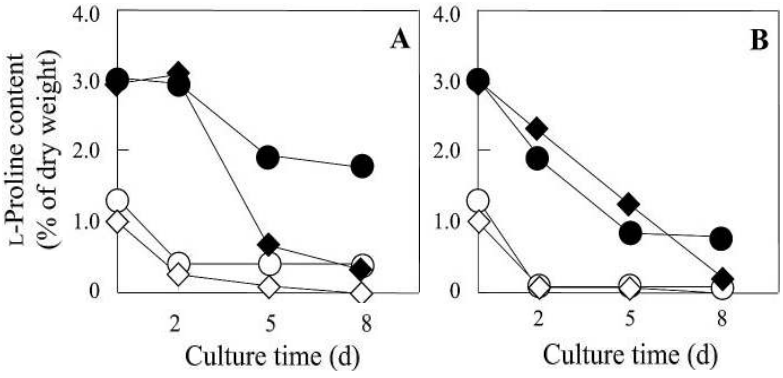


Figure 1.10 Intracellular L-proline content of laboratory and sake yeast strains grown in SD medium without (A) or with (B) 9% ethanol and incubated under static conditions. The *S. cerevisiae* strains used were the parent laboratory strain (○) and L-proline accumulating laboratory mutant strain (●) and control strain (◇) and L-proline accumulating sake strain (◆) (from Takagi *et al.* 2005).

Other studies showed that mutant yeast strains capable of accumulating intracellular L-proline were more tolerant to freezing (Sekine *et al.* 2007; Terao, Nakamori & Takagi 2003) and desiccation (Takagi *et al.* 2000). Results of these studies suggest that intracellular L-proline can act as a general stress protectant, not only for stress induced by a high ethanol concentration. This property is important for application of the yeast in various industries such as bread production, brewing, winemaking and bioethanol production.

Even though previous studies of L-proline focused on engineered yeast strains that accumulate intracellular L-proline, yeasts can also utilize extracellular L-proline as a sole carbon source (Lasko & Brandriss 1981). Transport of L-proline into the yeast cell is facilitated by at least four permeases, two of which are nitrogen regulated permeases (*PUT4* and *GAP1* encoded proteins), and two permeases that are regulated by the SPS (Ssy1-Ptr3-Ssy5) sensor (*AGP1* and *GNP1* encoded proteins) (Andreasson, Neve & Ljungdahl 2004).

The environmental conditions under which the yeasts are grown may affect the accumulation of extracellular L-proline in the yeast cell. Horak & Kotyk (1986) found that yeast cells grown at different pH and preincubated under aerobic or anaerobic conditions, followed by incubation in aerobic or anaerobic conditions showed different degrees of L-proline accumulation. Under anaerobic conditions, accumulation of L-proline was lower compared to yeast grown under aerobic conditions. This result showed that oxygen availability markedly influences L-proline uptake by yeast (see Table 1.3). Ingledew, Magnus & Solsuski (1987) also found that proline uptake in wine must requires oxygen.

Table 1.3 Effect of anaerobiosis and pH on the intracellular accumulation of L-proline (from Horak & Kotyk 1986)

Preincubation	Incubation	pH	Maximal theoretical accumulation ratio[#]	Experimental accumulation ratio	R*
Aerobic	aerobic	4.5	271	64	0.24
		7.0	45	72	1.60
Aerobic	anaerobic	4.5	28	9	0.32
		7.0	22	8	0.36
anaerobic	aerobic	4.5	178	69	0.39
		7.0	30	67	2.23
Anaerobic	anaerobic	4.5	25	7	0.28
		7.0	17	6	0.35

[#] The ratio was calculated for tight coupling with energy source as $[proline]_{in}/[proline]_{out}$ = exp (protonmotive force/60).
* experimental accumulation ratio / maximal theoretical accumulation ratio.

As noted above, yeast cells are capable of accumulating L-proline from their environment and therefore accumulation of intracellular L-proline can be achieved by supplementing the growth medium with L-proline, without the need for genetic engineering of the metabolic pathways. Therefore, in the present study, we anticipated that higher ethanol tolerance may be achieved via supplementation of the yeast growth medium with L-proline.

1.4.2 Inositol

Previous studies confirmed that inositol is important for cell growth. Yeast grown in media lacking inositol showed a decrease in PI synthesis, cell wall development, protein and ribonucleic acid synthesis and cell division that eventually led to loss of cell viability (Hanson & Lester 1980). It was also found that inositol deficiency can cause membrane damage and lead to loss of fermentation, respiration and sugar transport activity (Ulaszewski, Woodward & Cirillo 1978).

Supplementation of the growth medium by inositol can alter inositol synthesis and change the phospholipid profile of the plasma membrane (Carman & Han 2011). Inositol supplementation increases PI synthesis and eventually increases the PI content of the plasma membrane (Kelley *et al.* 1988). Synthesis of PI and PS are regulated by exogenous supply of inositol. It was found that the Km value of PI synthase for inositol was 9 fold higher than the cytosolic concentration of inositol. This result indicates that inositol supplementation, leading to higher intracellular inositol concentrations, can lead to greater synthesis of PI (Kelley *et al.* 1988).

Other investigations suggested that in the presence of inositol, synthesis of PI was increased while synthesis of PE and PC decreased and PA and PS remained similar (Chi, Kohlwein & Paltauf 1999). The differences in phospholipid composition in the presence and absence of inositol in the growth media is presented in Figure 1.11.

It is well known that increasing the PI content of the plasma membrane can increase ethanol production due to increasing ethanol tolerance (Chi, Kohlwein & Paltauf 1999). Chi, Kohlwein & Paltauf (1999) were the first to report that a high content of PI in the plasma membrane is related to high ethanol tolerance. The authors proposed a possible mechanism for the ethanol tolerance of high PI content yeast. They observed that, membrane proteins, especially the plasma membrane H^+-ATPase, and membrane integrity are protected from damage by ethanol when the cells contain a high proportion of PI. Therefore, they concluded that the conformation of membrane depends on the lipid composition of the membrane (Chi, Kohlwein & Paltauf 1999).

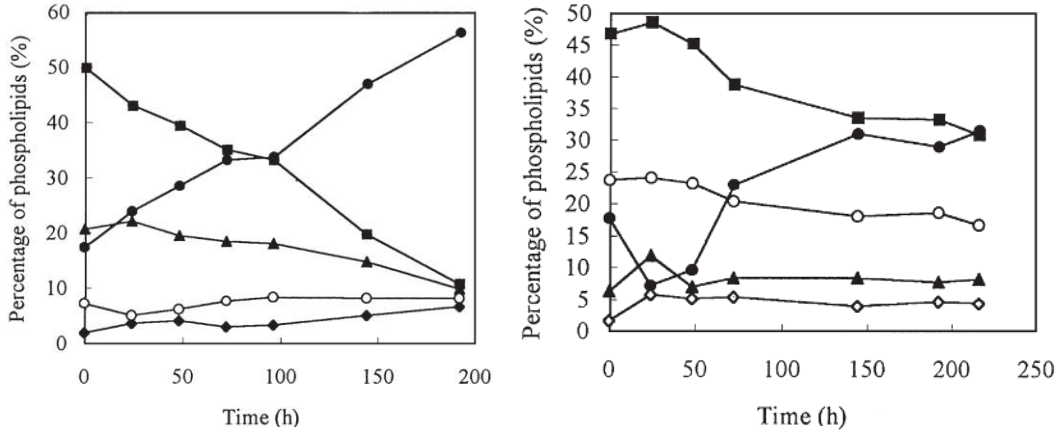


Figure 1.11 Changes in percentage of phospholipid species in the yeast cells grown in the presence and absence of inositol in the medium during fermentation. (A) 100 µg/mL added to fermentation medium (legend: PA (○), PC (■), PE (▲), PI (●), PS (◆)) or (B) no added inositol (legend: PA (▲), PC (■), PE (○), PI (●), PS (◇)). (from Chi, Kohlwein & Paltauf 1999).

Increasing ethanol tolerance has been related to increased activity of the plasma membrane H^+-ATPase (Rosa & Sa Correira 1992). Ethanol affects the permeability of the plasma membrane by perturbing the hydrophobic region, causing increasing passive influx of protons into cells (Leão & Van Uden 1984), leakage of intracellular components out of the cell and eventually decreased cell viability (Furukawa *et al.* 2004). Membrane fluidity (Learmonth 2011) and permeability to protons strongly influence ethanol tolerance; proton balance is maintained by the plasma membrane H^+ transporting ATPase pumping protons out of the cell (Mizoguchi & Hara 1998). The plasma membrane H^+-ATPase activity may be enhanced by inositol supplementation; plasma membrane inositol levels and H^+-ATPase activity were both found to approximately double when medium inositol levels were increased from 10 µM to 90 µM (Furukawa *et al.* 2004). The enhanced H^+-ATPase activity is considered to result from changes in the lipid environment of the plasma membrane, specifically increasing level of inositol-containing phospholipids (Furukawa *et al.* 2004).

Supplementation with inositol has been found to increase the saturated fatty acid content, especially C16:0, of the plasma membrane (Chi, Kohlwein & Paltauf 1999), as can be seen in Figure 1.12. The underlying increase in saturated C16:0 and C18:0 and concomitant decrease of C16:1 and C18:1 is due to the anaerobic condition of the fermentation process which precludes desaturase activity, however the increase in C16:0 and C18:0 is higher in inositol-supplemented cultures (Chi, Kohlwein & Paltauf 1999).

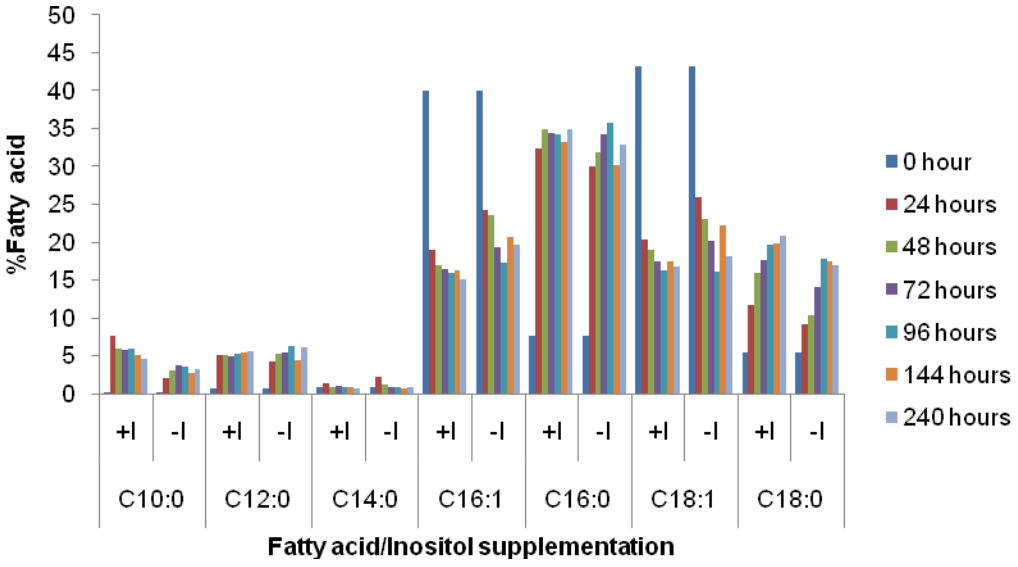


Figure 1.12 Changes in fatty acid composition during ethanol production in the presence and absence of inositol (adapted from Chi, Kohlwein & Paltauf 1999)

Inositol also affects the viability of yeast when exposed to increasing concentrations of ethanol (up to 20% v/v), but not the fermentation rate (Furukawa *et al.* 2004). Increasing medium inositol levels from 10 µM to 90 µM led to higher cell viability, although the fermentation rates were similar. This indicates that either in low or high inositol supplementation, enzymes of glycolysis are denatured and/or inhibited to the same extent by the increasing levels of ethanol (up to 18% v/v) produced by the cultures (Furukawa *et al.* 2004).

The viability increase upon higher inositol supplementation correlated with decreased leakage of intracellular components e.g. nucleotide, K^+ and phosphate, leading to the conclusion that the ability of inositol to improve viability may be via decreasing membrane permeability (Furukawa *et al.* 2004).

Interestingly, a more recent study on another yeast species, *Pachysolen tannophilus*, showed that even though inositol supplementation can increase ethanol production and cell growth, excess inositol can decrease the cell growth and ethanol production of the yeast (Ji *et al.* 2008). Cultures showed the highest cell density and ethanol production when the media were supplemented by 0.15 g/L and 0.1 g/L of inositol, respectively, as can be seen in Figure 1.13. The cell growth and ethanol production were observed to start to decline when the inositol supplementation was increased further (Ji *et al.* 2008).

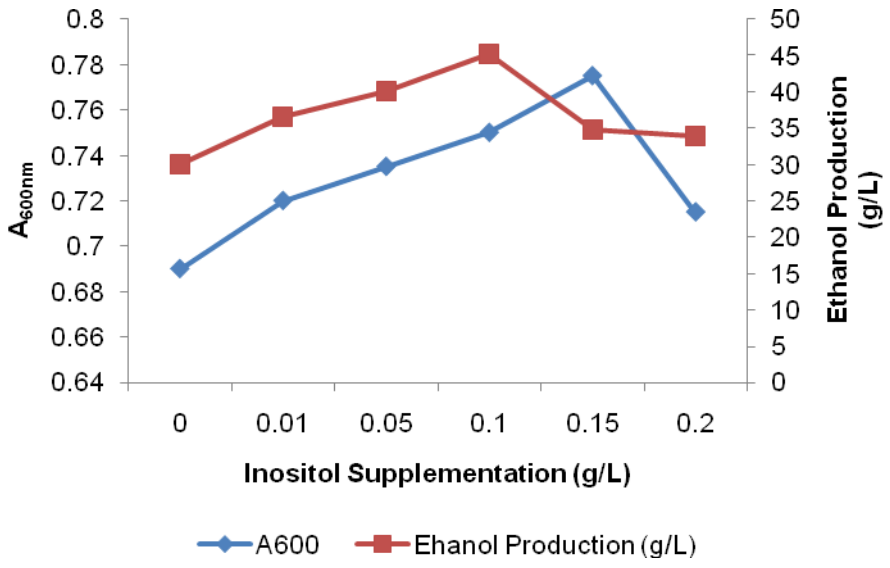


Figure 1.13 Effect of different levels of inositol supplementation on cell growth and ethanol production of the yeast *P. tannophilus* (adapted from Ji *et al.* 2008)

These authors also found that inositol supplementation led to increased tolerance of *P. tannophilus* to high ethanol concentration stress, as can be seen

in Figure 1.14. When subjected to initial ethanol concentrations of 10% to 14%, supplementation with inositol at levels of up to 0.15 g/L maintained the level of growth, although increasing the inositol concentration to 0.2 g/L led to decreasing growth of the yeast (Ji *et al.* 2008). Therefore, in the present study, the optimum concentration of inositol to use for supplementation will be determined in order to get the best ethanol fermentation result.

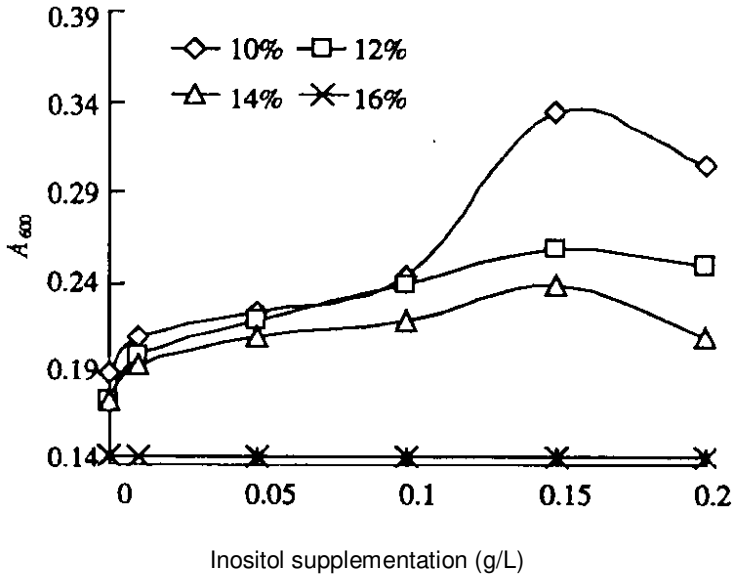


Figure 1.14 Effect of different levels of inositol supplementation on cell growth of *P. tannophilus* with initial ethanol concentration as indicated in the figure legend (from Ji *et al.* 2008)

Increasing intracellular inositol also has been achieved by means of genetic modification. Krause *et al.* (2007) developed a *S. cerevisiae* strain that lacked *OPI1* (which encodes a negative regulatory factor of the *INO1* structural gene, that encodes the enzyme catalysing the limiting step of inositol biosynthesis). By deleting *OPI1*, the cell synthesises inositol constitutively. The result of the study showed that a combination of inositol supplementation and genetic modification can lead to higher ethanol tolerance; the combination approach resulted in yeast cells tolerant to up to 18% ethanol, while the wild type yeast cells were only

tolerant to 15% ethanol in the presence of inositol supplementation (Krause *et al.* 2007).

1.5 Yeast Fermentation

Microorganisms have different routes of metabolism of sugars. Depending on the route, which depends on cellular conditions as well as the presence of specific enzymes for the metabolism, the final product of the metabolism can be ethanol, lactate, acetate, CO_2, succinate, formate, or H_2. The different metabolic pathways of sugar dissimilation (as described in Figure 1.15) are obvious at three levels of reactions (Pronk, Steensma & van Dijken 1996), which are:

1. Sugar transport into the cell and, in the case of a disaccharide, its hydrolysis followed by formation of sugar phosphates
2. Conversion of sugar phosphates to pyruvate by cleavage and oxidation
3. Further metabolism of the pyruvate

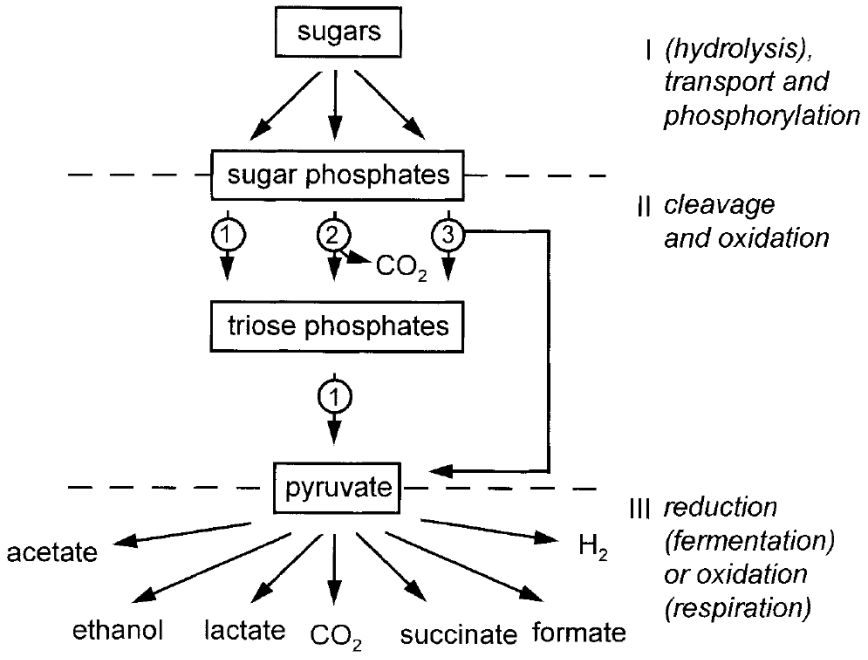


Figure 1.15 Scheme representing the diversity of sugar dissimilation pathways in microorganisms, which each consist of three levels of reactions. Numbers in circles represent (1) the Embden-Meyerhof pathway otherwise known as glycolysis, (2) the hexose monophosphate pathway and (3) the Entner-Doudoroff pathway (from Pronk, Steensma & van Dijken 1996).

Sugar in monosaccharide form may be transported into cells via facilitated diffusion, active transport or group translocation. In the case of a disaccharide, it can be directly transported into cells and hydrolysed inside the cells (intracellularly) to form monosaccharides or, alternatively, it can be hydrolysed outside the cell (extracellularly) and transported into the cell in the form of a monosachharide. The transported monosaccharide is then available for further metabolism (Pronk, Steensma & van Dijken 1996).

Even though there are three different metabolic pathways, all sugar metabolizing microorganisms share a common pathway in the lower part of the Embden-Meyerhof (glycolysis) pathway, which is the conversion of triose phosphate to pyruvate. The most obvious differences among microorganisms are in the further metabolism of pyruvate with the product as noted above (Pronk, Steensma & van Dijken 1996).

According to their ability to produce ethanol in aerobic conditions (i.e. in the presence of oxygen), yeast are divided into two groups; Crabtee-positive and Crabtee-negative yeasts. The former are yeasts that are capable of accumulating ethanol even in the presence of oxygen, while the latter only degrade sugars to CO_2 under aerobic conditions (Piskur *et al.* 2006; Pronk, Steensma & van Dijken 1996). *S. cerevisiae* belongs to the Crabtee-positive yeast type, since it has the capability to degrade six carbon (6C) sugars, especially glucose, to two carbon (2C) molecules, in particular ethanol, in the presence of oxygen without completely oxidizing them to CO_2. Another yeast which belongs to this group is the fission yeast *Schizosaccharomyces pombe*. Other yeasts, such as *Kluyveromyces lactis* and *Candida albicans*, are Crabtee-negative yeasts (Piskur *et al.* 2006). A scheme showing possible different metabolic pathways in Crabtee-positive and Crabtee-negative yeasts is presented in Figure 1.16.

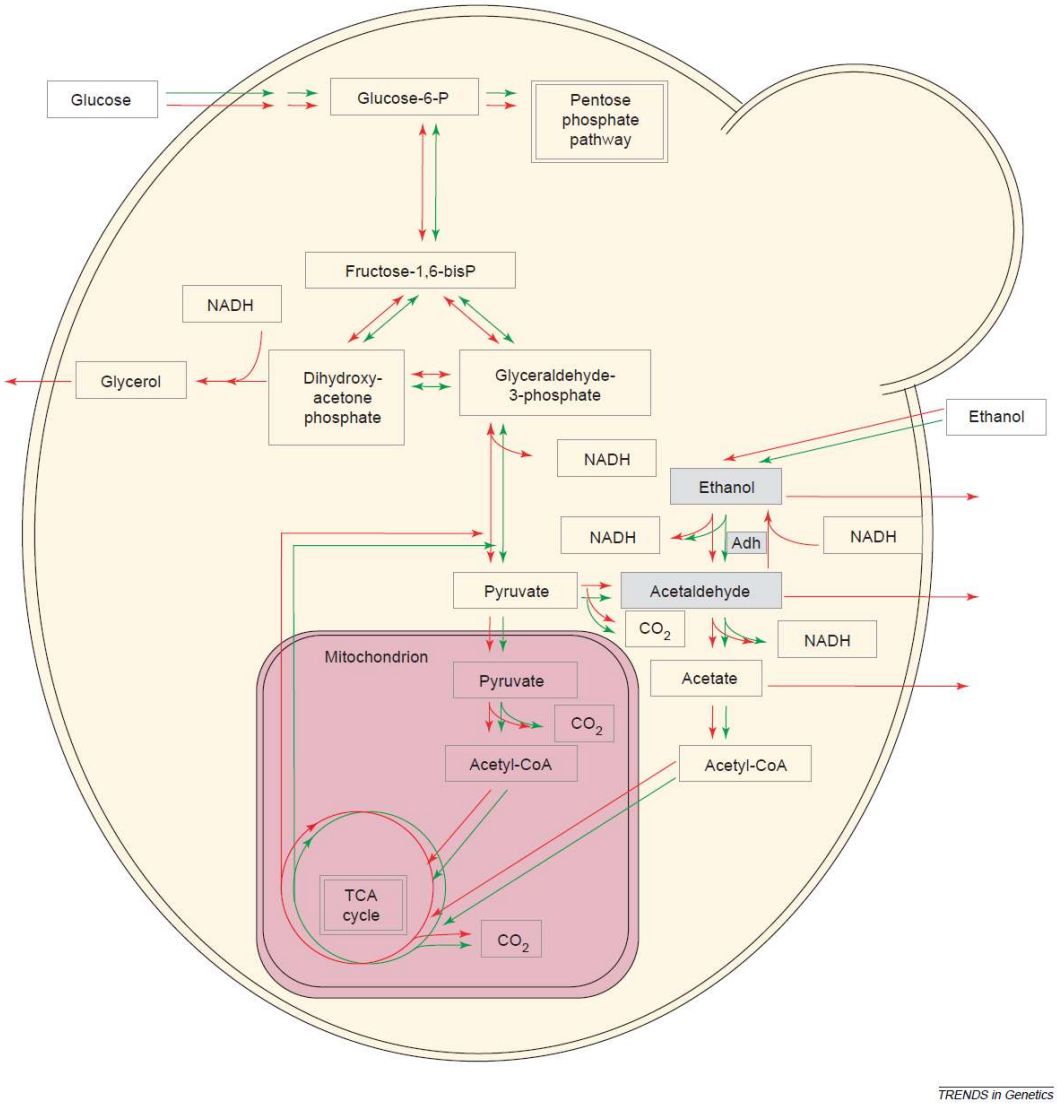


Figure 1.16 Scheme representing different pathways involved in dissimilating glucose under aerobic conditions in Crabtee-positive yeasts (represented by red arrows) and Crabtee-negative yeasts (represented by green arrows) (from Piskur *et al.* 2006).

In ethanol fermentation by *S. cerevisiae*, energy is provided by two events; the glycolysis pathway (i.e. conversion of glucose to pyruvate) and the fermentation pathway (i.e. conversion of pyruvate to ethanol) rather than the oxidative respiration pathway (Piskur *et al.* 2006). Glucose was found to have significant effects on regulation of oxidation and fermentation pathways. There are two major effects of glucose with regard to gene expression. The first effect of glucose is that it represses expression of many genes, including those that have roles in respiratory pathways (e.g. cytochromes) and enzymes for utilization of alternative sugars (e.g. galactose or maltose). The second effect is activation of expression of genes that encode proteins with roles in the glycolytic pathway and also glucose transporters (Johnston 1999). The activation of fermentation and repression of respiration in the presence of glucose is considered to offer advantages in terms of inhibiting competing microorganisms (Verstrepen *et al.* 2004).

After glucose depletion and ethanol accumulation, the metabolism of Crabtee-positive yeasts changes. The ethanol may become a substrate and is degraded as an energy source in the presence of oxygen. This change is known as diauxic shift (Lewis *et al.* 1993). This event does not occur in Crabtee-negative yeasts, since the initial metabolism does not produce ethanol (Piskur *et al.* 2006). The differences in metabolic pathways between Crabtee-positive and Crabtee-negative yeasts are presented in Figure 1. 17.

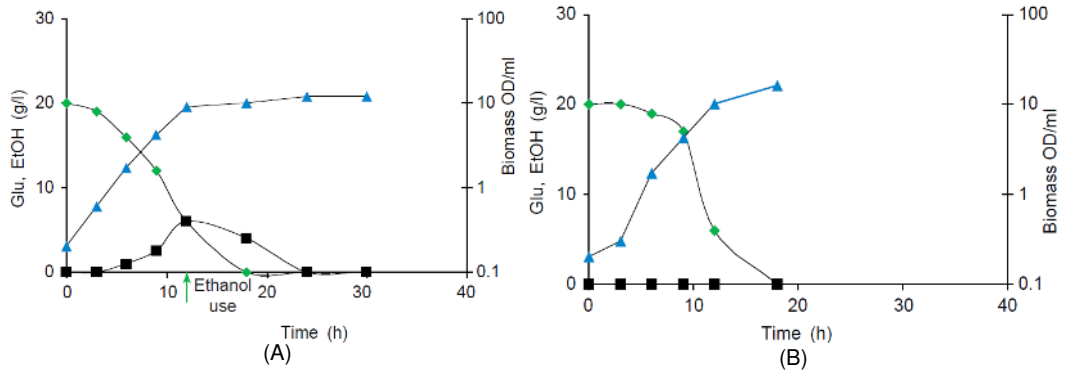


Figure 1.17 Consumption of glucose (♦) and appearance of ethanol (■) and biomass (▲) when yeast are grown in the presence of glucose under aerobic conditions. (A) *S. cerevisiae* (Crabtee-positive yeast) and (B) *K. lactis* (Crabtee-negative yeast). The diauxic shift is indicated by the green arrow in (A) (from Piskur *et al.* 2006).

As shown in Figure 1.15, in the ethanol fermentation process, pyruvate produced by glycolysis is first converted to acetaldehyde by pyruvate decarboxylase activity and subsequently reduced to ethanol by alcohol dehydrogenase activity (McKee & McKee 2003; Nelson & Cox 2008). In ethanolic fermentation each glucose molecule will produce two molecules of ethanol and CO_2. However, the theoretical maximum production by the ethanol fermentation process, i.e. 180 g glucose leading to 92 g ethanol and 88 g CO_2 cannot be achieved since there may be some possible contamination, production of other metabolites and cell mass or evaporation of ethanol. In a typical fermentation process, 95% of the sugar is converted to ethanol and CO_2, 1% to cellular components and the rest to other products such as glycerol (Beltran 2005).

1.6 Measurement of Membrane Fluidity

Since membrane fluidity is very important for cell health, measurement of membrane fluidity is also important in investigations of the effects of a particular stress. Methods that can be used to determine fluidity of cell membranes include various spectroscopies such as 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 measured using fluorescence spectroscopy, more specifically by measuring the generalized fluorescence polarization of the membrane probe laurdan.

When a molecule is exposed to electromagnetic radiation with an appropriate frequency, the molecule can absorb a photon, causing excitation of an electron from the ground electronic state (S_0) to a higher energy electronic state (S_1, S_2, etc.), termed an "excited" state. This process is illustrated in Figure 1.18, which is known as a Perrin-Jablonski diagram. The absorption of electromagnetic radiation and electron excitation take place on a time scale of ~10^{-15} s (Croney, Jameson & Learmonth 2001; Learmonth, Kable & Ghiggino 2009). In the excited state, the structure of the molecule is changed due to redistribution of its electron cloud. This in turn results in altered vibrational levels, altered dipole moment and a change in molecular shape (Learmonth, Kable & Ghiggino 2009).

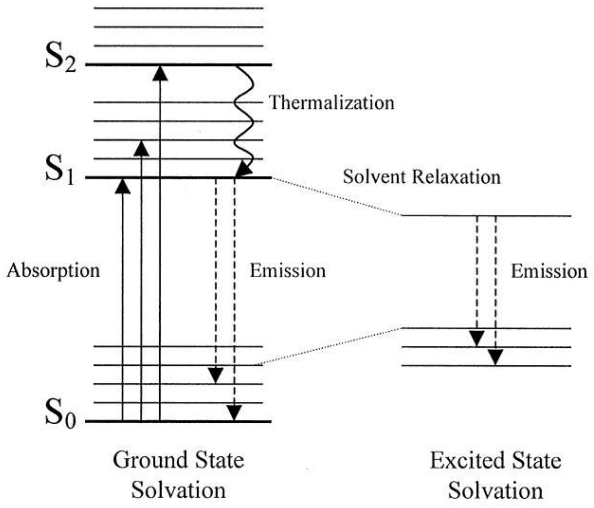


Figure 1.18 Simplified Perrin-Jablonski energy level diagram showing absorption (—) and emission (---) process as well as thermalization and solvent relaxation (from Croney, Jameson & Learmonth 2001)

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).

The excited state is an unstable condition and therefore, as illustrated in Figure 1.18, it may relax back to the ground state by emitting a photon. The emitted photon will have energy corresponding to the difference between the final and initial energy state of the molecule. This photon emission can be seen as fluorescence or phosphorescence (Learmonth, Kable & Ghiggino 2009; Nipper 2007). It is worth noting that the emitted fluorescence is not dependent on the wavelength absorbed nor subsequent energy level reached in the excitation event, i.e. fluorescence always occurs from the lowest vibrational level of the first excited state (S_1) to the ground state (S_0) (Croney, Jameson & Learmonth 2001).

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, named after Sir G.G. Stokes who discovered this phenomenon (Learmonth, Kable & Ghiggino 2009). An example of the Stokes shift of a fluorophore is presented in Figure 1.19.

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.

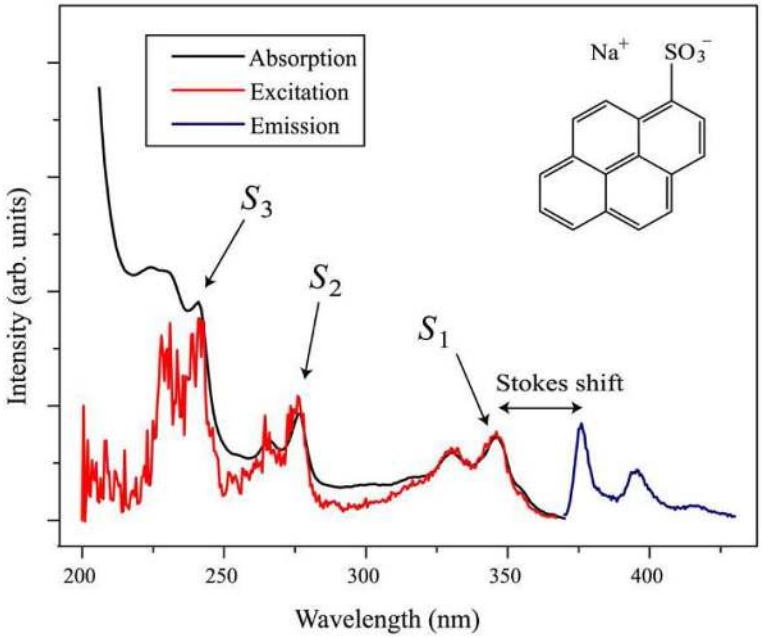


Figure 1.19 Absorption, excitation and emission spectrum of pyrene sulfonic acid (pictured top right). Three excitation states are observed for the molecule. Fluorescence occurred when the molecule shifted from the lowest excitation state (S_1) to the ground state, resulting in a Stokes shift (from Learmonth, Kable & Ghiggino 2009)

Fluorophores such as DPH (Carratu *et al.* 1996; Najjar, Chikindas & Montville 2007) and laurdan (Learmonth & Gratton 2002; Parasassi *et al.* 1998) are widely used in membrane fluidity investigation. Laurdan has an advantage over DPH in that it exhibits less cell density-dependent scattering of polarized light during measurements of yeast membrane fluidity (Learmonth & Gratton 2002).

The excitation and emission spectra of laurdan in membranes are very sensitive to the extracellular environment and this has lead 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).

Laurdan fluorescence can be used to distinguish whether a membrane is in a gel or liquid-crystalline state (Parasassi, Conti & Gratton 1986). When laurdan was embedded in a lipid bilayer and its emission spectrum recorded, it was noted that when the lipid bilayer changes from a gel to a liquid-crystalline state the laurdan emission spectrum exhibits a 50 nm red shift (Parasassi, Conti & Gratton 1986; You, Rosenfield & Knipple 2003). The shifted emission spectrum and changes in colour of membrane-embedded laurdan due to changes in temperature and membrane fluidity are presented in Figure 1.20.

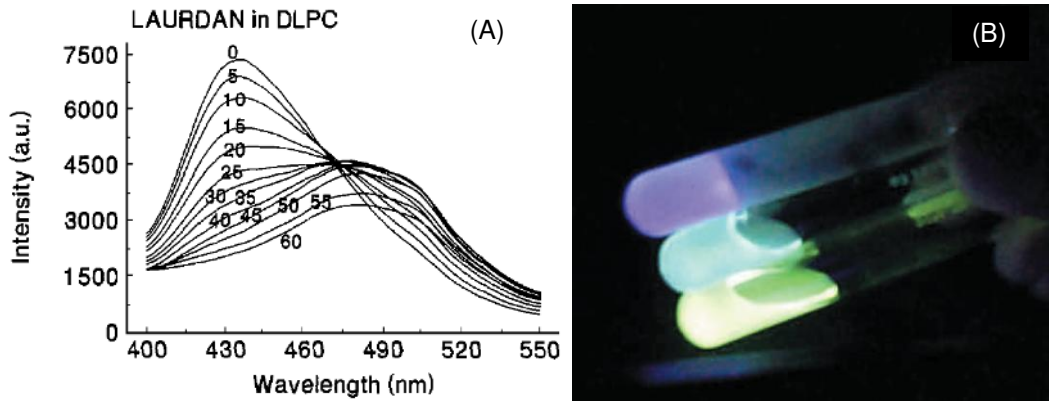


Figure 1.20 (A) Emission spectrum of laurdan in dilauroyl-phosphatidylcholine (DLPC) vesicles as a function of temperature from 0 to 60°C (from Parasassi *et al.* 1998). (B) Colour changes of laurdan dissolved in glycerol. The mixture of laurdan and glycerol are frozen to -70°C (top), kept at on room temperature (middle) and heated to 80°C (bottom) (from Croney, Jameson & Learmonth 2001).

Another important approach to determining membrane fluidity utilises polarized light and assesses polarization/depolarization using fluorescent membrane soluble probes such as laurdan or DPH. Light is a form of electromagnetic radiation, consisting of an oscillating electric wave and an oscillating magnetic wave perpendicular to it (Learmonth, Kable & Ghiggino 2009). Normal ambient light is known as unpolarised light and can be visualised as a wave with half of its vibration in the horizontal plane and the other half of its

vibration in the vertical plane (Butcher 2008). It is possible to make polarised light from unpolarised light using polarising optics such as filters and prisms. After passing through the polarising optics, the light will be linearly polarised, i.e. vibration in only one plane (plane of polarisation) for horizontal plane and vertical plane (Learmonth, Kable & Ghiggino 2009).

Based on fluorescence measurements with excitation using polarised light, membrane fluidity can be determined by calculating the polarization (or alternatively the anisotropy value) of the emitted light. If a molecule is illuminated with polarised light and is able to rotate during the excited state lifetime, then the emitted light will be depolarised relative to the absorbed light. The degree of polarisation depends on the mobility of the emitting species; higher mobility leads to lower polarisation. The same is also true for anisotropy (Learmonth, Kable & Ghiggino 2009). The polarisation value can be calculated using an equation as follows:

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

where P : Polarization value

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

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

or, alternatively, the anisotropy can be calculated using the following equation:

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

where r : anisotropy value

It should be noted that polarisation and anisotropy values need to be corrected using an instrument grating correction factor (G), which is expressed as:

$$G = \frac{I_{VH}}{I_{HH}}$$

Anisotropy and polarisation are both expressions of the same phenomenon and can be easily interconverted as follows:

$$r = \frac{2P}{3 - P}$$

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

A simpler approach, using the spectral sensitivity of laurdan to its environment, has been developed. This approach is based on the concept of the spectroscopic property known as generalized polarization (GP). GP is calculated based on the shift between gel and liquid-crystalline phase and can also be used as a membrane fluidity index (Learmonth & Gratton 2002; Yu *et al.* 1996). The GP value is calculated from the relative fluorescence intensities at wavelengths at the blue (~440 nm) and red (~490 nm) edges of the spectrum that represent gel and liquid-crystalline states, respectively (Learmonth & Gratton 2002). GP can be calculated using the following equation:

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

where I_{gel} is the fluorescence intensity at the blue edges (440 nm) and I_{lc} is the fluorescence intensity at the red edges (490 nm). The GP value theoretically may vary from -1 to +1 and it is inversely related to membrane fluidity, i.e. a high GP

value means low fluidity (Butcher 2008; Learmonth & Gratton 2002; Yu *et al.* 1996). Due to the advantages noted above as well as facilitation of simpler and more rapid measurements, laurdan GP was used as the measure of membrane fluidity in this study.

1.7 Membrane Fluidity and Yeast Adaptation to Environmental Stress

Los & Murata (2004) defined fluidity of a membrane as the degree of molecular disorder and molecular motion within a lipid bilayer. The fluidity of a biological membrane has been used to explain qualitative characteristics of the lipid bilayer. The fluidity of a membrane influences the rate of movement of intramembrane particles in the plane of the bilayer. Movement of these particles, such as membrane proteins, plays an intrinsic role in their function (Nipper 2007).

The lipid composition of a membrane is very important and is a major determining factor in membrane fluidity and phase (Turk *et al.* 2004). Several other factors may contribute to maintenance of membrane fluidity, as discussed in Section 1.2.3, including temperature, phospholipid class distribution, phospholipid fatty acyl saturation, ionic strength, pH, protectant molecules, membrane proteins, sterols and cellular metabolic status (Learmonth 2011). Cells must maintain their membrane fluidity in order to survive in severe conditions.

Changes in fluidity have been reported when microorganisms are subjected to ethanol (Alexandre, Rousseaux & Charpentier 1994), hyperosmotic conditions, salt, freezing, heat (Learmonth 2011) and organic solvents (Gutiérrez *et al.* 2003). Generally, when subjected to various stresses, the membrane fluidity will decrease or increase (depending upon the particular stress), and eventually the severity of the change may lead to disruption of the plasma membrane. For example, at low temperatures, the membrane becomes more rigid whereas at high temperatures, fluidization may occur. The same phenomena are also

observed when cells are subjected to high or low osmolarity conditions (Los & Murata 2004). A schematic diagram showing changes in the lipid bilayer under stress conditions is given in Figure 1.21.

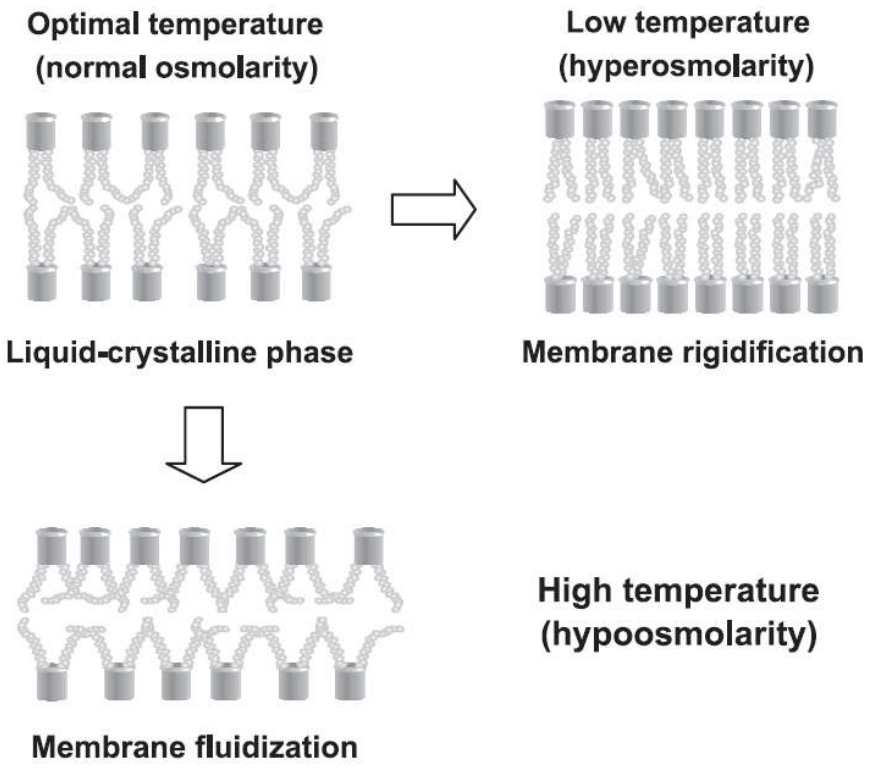


Figure 1.21 Diagram showing changes in membrane order when cells are subjected to stress (from Los & Murata 2004).

S. cerevisiae cells grown in increasing ethanol concentrations showed lowered DPH (1,6-Diphenyl-1,3,5-hexatriene) anisotropy values, which is indicative of increasing membrane fluidity, as can be seen in Table 1.4 (Alexandre, Rousseaux & Charpentier 1994). These authors also showed that in the presence of 10% ethanol, the unsaturation index was increased, correlating with the anisotropy decrease which in turn indicates increasing membrane fluidity (Alexandre, Rousseaux & Charpentier 1994). Lowering of DPH anisotropy and

laurdan generalized polarization have also been shown in other studies of yeast membranes under heat and high ethanol stress conditions (Learmonth 2011).

Table 1.4 Anisotropy values for the plasma membranes of yeast cells subjected to ethanol shock with or without prior culture in the presence of 10% ethanol measured using DPH as a membrane probe (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% ethanol.

Microorganisms, including yeast, have mechanisms to adapt to environmental stresses. Previous studies have reported that changes in membrane fluidity brought about by changing the membrane fatty acid composition can alter the viability of yeast cells when subjected to high ethanol concentrations (Kajiwara *et al.* 2000), freezing (Rodrıguez-Vargas *et al.* 2007), heat and oxidative stress (Steels, Learmonth & Watson 1994).

Yeast mutant strains have been developed that have an increased capability to synthesize monounsaturated fatty acids (by overexpression of *OLE1*) or to synthesize dienoic fatty acids (by expression of the *Arabidopsis thaliana FAD2* gene) or a combination of both capabilities (Kajiwara *et al.* 2000). Analysis of total fatty acids revealed that the mutant overexpressing both *FAD2* and *OLE1* had a high proportion of unsaturated fatty acids (82%), with ~54% of the total as dienoic fatty acids. This double mutant strain also had higher tolerance to 15% (v/v) ethanol (Kajiwara *et al.* 2000). This finding suggests indirectly that increasing membrane fluidity (by increasing phospholipid fatty acid unsaturation) can enhance ethanol tolerance of yeast.

Another study by Rodriguez-Vargas *et al.* (2007) investigated the influence of membrane fluidity on the tolerance of the yeast cells. These authors developed a mutant with the capability to synthesize dienoic fatty acids. The authors expressed two *Helianthus annuus* desaturase genes, *FAD2-1* and *FAD2-3*, in wild type *S. cerevisiae* (strain W303) cells and determined the effect on membrane fluidity and freezing tolerance. Expression of these genes increased the unsaturation index and increased the membrane fluidity of the cells (indicated by decreasing DPH polarization values). The mutant strains were found to have lower polarization values, 0.116 ± 0.033 and 0.125 ± 0.020 for W303*FAD2-1* and W303*FAD2-3* strains, respectively, compared to control W303 cells strain (0.160 ± 0.016). The mutant strains had higher tolerance than the wild type to freezing stress.

1.8 Outline of Investigations in this Project

Ethanol tolerance is considered to be one of the most important factors in producing maximal amounts of ethanol and increasing efficiency in the fuel ethanol industry, along with improving metabolic flux and fermentation rate. There are many factors affecting yeast ethanol tolerance, including the genetic makeup of the yeasts, culture conditions (e.g. temperature, pH) and composition of nutrients and other beneficial molecules in the culture medium. This study was designed to primarily investigate the latter factors and commenced with laboratory scale investigations using defined laboratory media. Several yeast strains with differing intrinsic ethanol tolerance were investigated. Components of the yeast growth medium were modified in order to determine conditions that favour the highest ethanol tolerance. In particular, following up on recent publications, investigations focused on the effects of the sugar inositol (Chi, Kohlwein & Paltauf 1999) and the amino acid L-proline (Takagi 2008), which

have shown some promise as stress tolerance enhancers. Despite this positive role, excess of these substances in the growth medium may be detrimental to the yeast. Therefore, it was planned to vary the inositol and L-proline content of growth media in order to define concentrations that potentiate the highest ethanol tolerance of the yeast. The aim was also to compare these findings to other known tolerance-enhancing components such as unsaturated fatty acids (You, Rosenfield & Knipple 2003) and mineral ions (Xue *et al.* 2008).

1.8.1 Objectives

The objectives of this research were to:

1. determine (amongst the novel yeast strains studied) the most appropriate yeast strain and culture condition to improve the efficiency of bioethanol production, considering ethanol tolerance and ethanol production
2. determine the most appropriate concentrations for supplementation of growth media with L-proline or inositol to improve ethanol tolerance and/or production.
3. investigate the effect of addition of L-proline or inositol to growth media on membrane fluidity and to relate this to ethanol tolerance.

Chemically defined media were used in the laboratory experiments to minimize the effect of variability of medium components that might affect the ethanol tolerance or fermentation productivity. The experimental design included several yeast strains with different levels of ethanol tolerance. The parameters measured included fermentation rate, yeast cell count, cell division rate and viability, membrane fluidity and ethanol tolerance.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Yeast strains and maintenance

The following *Saccharomyces cerevisiae* strains were initially selected as candidates for this study. Selection was based on known characteristics. They were selected as promising candidates for use in ethanol production and also on the basis of suitability for investigating a range of ethanol tolerances and heat tolerances. Heat tolerance was considered due to its overlap with ethanol tolerance (Piper 1995).

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

Yeast strain	Type	Ethanol tolerance	Heat tolerance	Notes
A12	Baker's	65 ± 2%[a,b]	4 ± 2%[a,c]	Noted for good growth at high temperatures. Generally tolerant, most tolerant to ethanol, H_2O_2, slow freeze, acid[a].
A14 (Y271)		13-14%[d]	N/A	ATCC 26603 – high alcohol production from cane sugar, isolated from Jamaican cane sugar. Grows in up to 46.5% (w/v) sugar
PDM	Wine	15-17%[e]	Fermentation range to 30°C	Mauri Yeast Australia commercial wine strain. Efficient ethanol conversion: 16 g/L sugar for 1% ethanol
K7	Sake	~ 17.5%[b]	~ 65%[c]	ATCC 26422

[a]data from Lewis *et al.* (1997)
[b]Ethanol tolerance - % survivors of 20% ethanol, 1h, room temp (~25°C)
[c]Heat tolerance - % survivors of 52°C, 5 min with growth temp 25°C
[d]Reported by Pierce, Litchfield & Lipinsky (1981). Figures represent % ethanol tolerated, not % survivors of 20% ethanol.
[e]Reported in Mauri Yeast Australia documentation for PDM. Figures represent % ethanol tolerated, not % survivors of 20% ethanol.

As noted in Chapter 3, the growth characteristics of some of these strains were found to be problematic in initial experiments, and it was decided to conduct inositol or L-proline supplementation experimentation on A12, PDM and K7.

Yeast strains were maintained on slopes of a complete medium, yeast extract peptone (YEP), containing (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 2% glucose and 0.67% Yeast Nitrogen Base (Difco). In initial experiments, L-proline and inositol were added to experimental cultures with various concentrations as follows:

- L- Proline: 0, 0.1, 0.5, 1, 2, and 3 g/L
- *myo*- Inositol : 0, 0.005, 0.05, 0.1, 0.15 and 0.2 g/L

As the YNB medium already had inositol as a component, the 0 g/L inositol supplementation actually had about 0.002 g/L inositol.

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 were prepared on a monthly basis or as required. Supplements were freshly prepared and sterilized by filtering through 0.22 μm pore size sterile syringe filters.

Starter cultures were inoculated from slopes and grown overnight (~16 h) at 30°C and 180 opm in an orbital shaker (Paton). For L-proline and inositol addition experiments, L-proline and inositol were added to the experimental culture at a final concentration as mentioned above 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 4:1 to ensure adequate oxygen mixing.

Samples from the cultures were aseptically removed by drawing off with a micro pipette every 6 hours from 0 to 30 hours. Examination of the samples included measuring growth rate by measuring optical density, viable cell numbers, % budding and glucose and ethanol concentrations. Detailed analysis including ethanol tolerance and membrane fluidity was performed at 6 and 24 h.

2.4 Growth Rate

Yeast growth was monitored by measuring optical density of the culture at 600 nm (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).

2.5 Viable Cell Numbers

Viable cell numbers were assessed using the methylene violet staining method and light microscopy (400x 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, 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. The number of viable cells in 1 mL of cultures was then calculated as follows:

The cells in 5 of the small squares in the centre 5 x 5 grids were counted and counts averaged. Each of the 25 squares is divided into 16 sub squares. Each of the 16 sub squares is 1/400 mm^2 in area. The depth is 0.1 mm.

So, if 1 of these 25 small squares were counted:

Volume counted = 16/400 mm^2 x 0.1 mm (depth)

So cells/mL = 400/16 x 10 (per mm^3) x 1000 (mm^3 to cm^3 = mL)

i.e. cells counted x 2.5 x 10^5 cells/mL.

So for 5 squares counted: Cells/mL = Cell count ÷ 5 x 2.5 x 10^5

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 were then divided by the total cells and multiplied by 100 to give the percentage of viable cells.

2.7 Percent Budding

When counting, both budding and non-budding cells were recorded as an indicator of cell growth rate. The number of budding cells was then divided by the total number cells and multiplied by 100 to give the percentage of budding cells.

2.8 Determination of membrane fluidity by spectrofluorometric analysis

2.8.1 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 plasma membranes, as outlined by Learmonth (2011).

For labelling, an aliquot of washed cells was standardized by diluting with centrifuged (8800 *g*) supernatant to an OD_{600nm} of 0.4 and a volume of 3 mL 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.

2.8.2 Protocol for setting up PC1 to conduct spectrofluorimetric analysis

Fluorescence measurements were taken with a PC1 photon-counting spectrofluorometer (ISS Inc., Illinois USA). The illuminator current dial was first turned anticlockwise to its lowest setting (10 amps) and then the instrument was switched on. The lamp was allowed to warm up for 10 minutes, after which the amperage was increased to 18 amps. It should be noted that it was important to turn on the lamp 30 minutes prior to use, to allow the lamp to stabilize. The heater/circulator button on the thermocirculator was turned on and the temperature dial was set to the required temperature. The thermocirculator regulates the temperature of the temperature-controlled stirrer of the cuvette holder. The PC1 spectrofluorometer main power was then switched on and the fan checked to make sure it was functioning. The computer was turned on and the Vinci software started. The calibration of the instrument slits and excitation and emission monochromators were checked prior to experimentation.

2.8.3 Measurement of Generalized Polarization of laurdan localized in yeast membranes

In this study, the fluorescent probe laurdan was used to measure Generalized Polarization, as described by Parasssi *et al*. (1990) and applied to

yeast by Learmonth & Gratton (2002) and Butcher (2008). After calibrating the PC1 spectrofluorometer as described previously, 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.

When laurdan is in a lipid membrane, it exhibits a 50 nm red shift of the emission spectrum as the membrane changes from the gel to liquid-crystalline phase. Thus, by measuring the relative emission intensities at wavelengths at the blue and red edges of the spectrum, representing gel (440 nm) and liquid crystalline (490 nm) phases, membrane fluidity may be inferred. The results were expressed as Generalized Polarization (GP) determined using equation 3.

Equation 3

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

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

I_{490nm} : Emission intensity at 490 nm

2.9 Ethanol tolerance

2.9.1 Sample preparation

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 culture were centrifuged at 1500 *g* for 2-3 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.

2.9.2 Ethanol tolerance test

The concentration of ethanol and time 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 410 µL sample cells 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 two methods which were methylene violet staining and total plate count.

For the methylene violet staining method, the stress was relieved by making a five- (for 6 h culture) or ten-fold (for 24 h culture) dilution in MilliQ water. The percentage of viable cells was then calculated as described in section 2.5. The result of this viability calculation was then expressed as “viability reduction” (referred as viability reduction by methylene violet staining (VR MVS) in this thesis) as calculated using the following equation:

$$\text{VR MVS}\,(\%) = \frac{\text{viability control - viability test}}{\text{viability control}} \times 100\%$$

For the total plate count method, after the stress period the samples were diluted using YNBNG to give serial ten-fold dilutions. Then, 100 µL of each diluted sample was spread on YEP agar plates, and incubated for about 72 hours at room temperature before counting the resultant colonies. The result was also expressed as “viability reduction” (referred to as viability reduction by total plate count (VR TPC) in this thesis) as calculated using the following equation:

$$\text{VR TPC (\%)} = \frac{\text{total viable cell control - total viable cell test}}{\text{total viable cell control}} \times 100\%$$

The extra 18% dilution of the cells resulting from the addition of ethanol was taken into account during the calculation of the viable count. The tubes with 90 µL of ethanol were prepared immediately before the experiment as the solvent may evaporate quickly and the small volume could allow substantial changes in the final concentration.

2.10 Measurement of glucose, ethanol, L-proline and inositol using HPLC

The amount of glucose, ethanol, L-proline and inositol was determined by measuring the compounds in the growth media compared to the initial concentration at the beginning of the fermentation (0 h) using HPLC.

2.10.1 Instrumentation

The HPLC system (Shimadzu) consisted of 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 PC with a CBM 20A communication bus module. The spectrum was scanned for 195, 200, 210 and 220 nm using PDA (Photodiode array) detector. RID (refractive index detector) spectrum was also recorded. After comparison of PDA and RID spectra, RID was preferred for the present series of experiments as it produces more reliable spectra and can detect all the desired compounds.

2.10.2 Column

A Waters Sugar-Pak I HPLC column (part no. 85118) with dimensions of 6.5 × 300 mm was used for the separation of analytes. The stationary phase of the

column is sulfonated styrene divinylbenzene resin in calcium form. The column was maintained at 80°C. A guard column was used to prevent column damage.

2.10.3 Mobile phase

The mobile phase was deionized MilliQ water (resistivity ~ 18 Mohm) containing 5 mg/L $CaNa_2$-EDTA (Sigma Aldrich) 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. Prior to the initial use, and after running about 150 samples, the column was reconditioned by passing through a 500 mg/L $CaNa_2$-EDTA solution at 80°C at a 0.5 mL/min flow rate for at least 2 hours in the reverse direction.

2.11 Determination of total sugar concentration by the phenol-sulphuric acid method

While the HPLC analytical methods were being worked up, in the initial experiments (Chapter 3) determination of glucose was performed using the phenol-sulphuric acid method as described by Dubois *et al.* (1956). To prepare a standard curve a series of glucose solutions were prepared to give final concentrations of 0, 20, 40, 60, 80, and 100 µg/mL. A volume of 150 µL of either standard or sample solution was then added to a reaction tube, to which 150 µL of 5% phenol solution in water was added. When necessary, samples were diluted to ensure experimental readings fell within the standard curve. The tubes were then mixed and stood at room temperature for 5 minutes. Then 750 µL of concentrated sulphuric acid was added rapidly; the tubes were then vortexed and placed on a hot plate (100°C) for 10 minutes. After cooling to room temperature, the absorbance of the standards and samples were read at 490 nm with a Beckman DU650 spectrophotometer, using the reagent blank to zero the

spectrophotometer. A standard curve was plotted and this was used to calculate the concentration of glucose in each of the samples.

2.12 Statistical Analysis

Raw data was initially compiled into Minitab 15® for Windows®. This software package was then used to perform one way analysis of variance (one way ANOVA) which compared the variance of each parameter (i.e. GP, viability reduction) between the three strains with the variability within each replicate experiment of the same strain. Significant differences between the data were determined based on the p value. When $p < 0.05$, the null hypothesis was rejected, which means there is a significant difference between the data. When significant differences were detected in the one way ANOVA, the test was followed by the Fisher's LSD (least significant difference) test to determine which data differed significantly.

CHAPTER THREE: INITIAL EXPERIMENTS ON FERMENTATION PERFORMANCE OF *Saccharomyces cerevisiae* IN MEDIA WITH HIGH SUGAR CONCENTRATIONS

3.1 Introduction

3.1.1 General Introduction

In industrial processes, it is common to use feedstocks with high sugar concentration. A high sugar concentration at the initial step of fermentation may lead to higher amounts of ethanol produced at the end of the fermentation. This will lower production costs as it will reduce the water and energy processing requirements for distillation. However, when a high sugar concentration is initially present in fermentation media, yeast cells are exposed to high osmotic stress. This is not ideal for yeast cells, and the fermentation may become stuck (Bafrncová *et al.* 1999). When basal media were used in fermentation experiments, sugar was not fully utilized by yeast cells, leading to high residual sugar at the end of the fermentation (Bafrncová *et al.* 1999; Reddy & Reddy 2006; Thomas, Hynes & Ingledew 1994). Cell viability, sugar uptake and ethanol productivity have been enhanced by supplementation of media with excess assimilable nitrogen in the form of yeast extract, casamino acids and other supplements such as glycine, glycine betaine, proline, finger millet flour, soya flour and yeast cell walls (Bafrncová *et al.* 1999; Reddy & Reddy 2006; Thomas, Hynes & Ingledew 1994; Thomas & Ingledew 1990).

Most experiments with high sugar concentration have used complex medium such as yeast extract, peptone, casamino acids, wheat hydrolysate or corn hydrolysate (Bafrncová *et al.* 1999; Reddy & Reddy 2006; Thomas *et al.* 1993; Wang *et al.* 2007). However, these media components include fluorophores, and

in the current project it was planned to utilise fluorescence spectroscopy to assess yeast membrane fluidity. Therefore, complex media could not be used as high fluorescence background interferes with the spectroscopic techniques.

The initial experiments in this study were designed to model industrial conditions which use high sugar concentrations, up to 22 % (w/v), with many studies utilising 16% (w/v) sucrose (Andrietta, Steckelberg & Andrietta 2008; Caylak & Sukan 1998; Cazetta *et al.* 2007). In addition to mimicking industrial conditions, we also proposed to assess yeast plasma membrane fluidity *in situ* during fermentation. Therefore, background fluorescence intensity of fermentation medium had to be considered.

Figure 3.1 shows the relative fluorescence of YNB and YEP media upon excitation at 340 nm, the wavelength of excitation of the laurdan probe. Critical emission wavelengths for analysis of emission are 440 and 490 nm, and it can be seen that YEP has high emission at those wavelengths. Such high emission precludes analysis using laurdan.

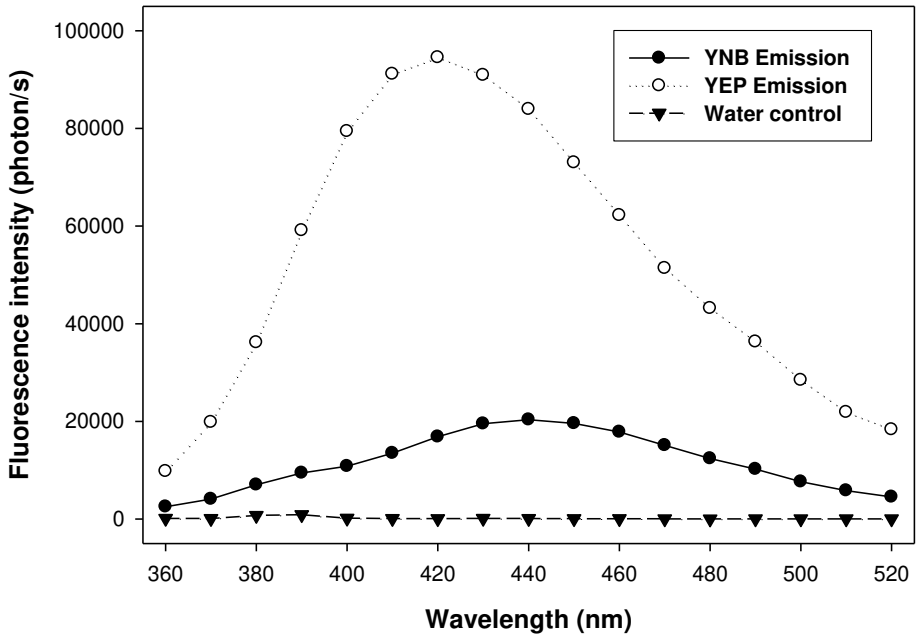


Figure 3.1 Fluorescence emission spectra of YEP and YNB, excited at 340 nm. Personal communication, R. P. Learmonth (2011).

Thus for the initial stage of the present experiment, we examined fermentation performance of the yeast in defined media with different sugar concentrations, in order to determine the best media for subsequent experimentation. The experiments commenced utilising Yeast Nitrogen Base (YNB) medium containing 16% (w/v) sucrose as the carbon source. It was considered that the medium composition provided sufficient nutrition to sustain fermentation of this level of sugar.

3.1.2 Yeast strains and culture condition

Initial experiments with YNB containing 16% sucrose utilised the yeast strains A12, A14, K7 and PDM. A12 is an ethanol tolerant baker's yeast according to a previous study (Lewis 1993), A14 is a yeast strain used in industrial production of bioethanol, PDM is an industrial wine strain (Mauri Yeast) which usually can produce up to 17% (v/v) ethanol, and K7 is a sake yeast strain

(ATCC 26422) that can produce up to 17.5% ethanol. After obtaining variable data in initial experiments (data not shown), it was decided to follow up using only one strain (A12), the best growing strain, to simplify the investigation of fermentation performance with different media and sugar concentrations. For this experiment the following media were used:

1. YEP with 16% Sucrose (YEP16S)
2. YEP with 16% Glucose (YEP16G)
3. YNB with 16% Glucose (YNB16G)
4. YNB with 1.5% ammonium sulphate and 2% glucose (YNBAS2G)
5. YNB with 2% glucose (YNB2G)
6. YNB with 1.5% ammonium sulphate and 16% glucose (YNBAS16G)

3.1.3 Specific growth conditions and experimental design

For growth analysis, batch culture experiments were set up, and samples were aseptically removed from the culture by drawing off with a sterile pipette at 0, 4, 8, 24, 30, 48, 72, 96, 120, 144 and 168 hours. Samples were then analysed for optical density at 600 nm (Section 2.4), viable cell count (section 2.5), percent cell viability (section 2.5), percent budding rate (section 2.6), and glucose concentration by phenol sulphuric acid method (Section 2.11). Data presented in the following results were drawn from single exploratory experiments.

3.2 Results

3.2.1 Growth parameters of different yeast strains grown in YNB with a high sugar concentration

Growth parameters of PDM, A12 and A14, cultured in YNB medium with high initial sugar concentration (16% glucose), are presented in Figure 3.2. Either OD_{600nm} or total cell counts indicated that among these three strains, PDM had the highest cell number (Figure 3.2 (A) and (B)). A12 and A14 did not show any

differences. Respiro-fermentative (exponential) phase growth was observed during the first 24 hours, however growth and viability decreased after this time, and ferments seemed to have stuck at around 8-12% sucrose (Figure 3.3). The sugar was rapidly taken up by all strains during the first 24 hours. A14 maintained a sugar utilization rate relatively higher than the other strains up to 72 hours. After the rapid sugar utilization, the level of sugar in the medium was relatively constant until the end of the experiment. The residual sugar concentrations at 168 hours were 6.9, 7.7 and 6.0% (w/v) for PDM, A12 and A14, respectively.

While A14 achieved the highest viable cell number at 48 hours as indicated by total viable cell number, PDM and A12 showed more rapid growth earlier, with the highest viable cell numbers at 8 hours (Figure 3.2 (C) and (D)). It is also noteworthy that the total viable cell number of A14 was similar at the 8 h time point to A12, while PDM has the highest total viable number. All strains showed their highest cell viability at 8 hours, and at this time point A14 had the highest cell viability of all strains,

This initial experiment utilised YNB medium with 16% glucose. YNB contains 0.5% ammonium sulphate as the primary nitrogen source. On determination that all ferments were becoming stuck at high residual sugar concentrations and cells were dying off, we considered that the level of nitrogen may have been insufficient, so further experimentation was conducted with inclusion of additional nitrogen sources. The media were supplemented with 1% (w/v) ammonium sulphate as additional nitrogen source, to give a final ammonium sulphate concentration of 1.5%. However, no significant differences in sugar utilization were observed when the media was supplemented with 1% ammonium sulphate as can be seen on Figure 3.5 (A). This will be further discussed in section 3.2.3.

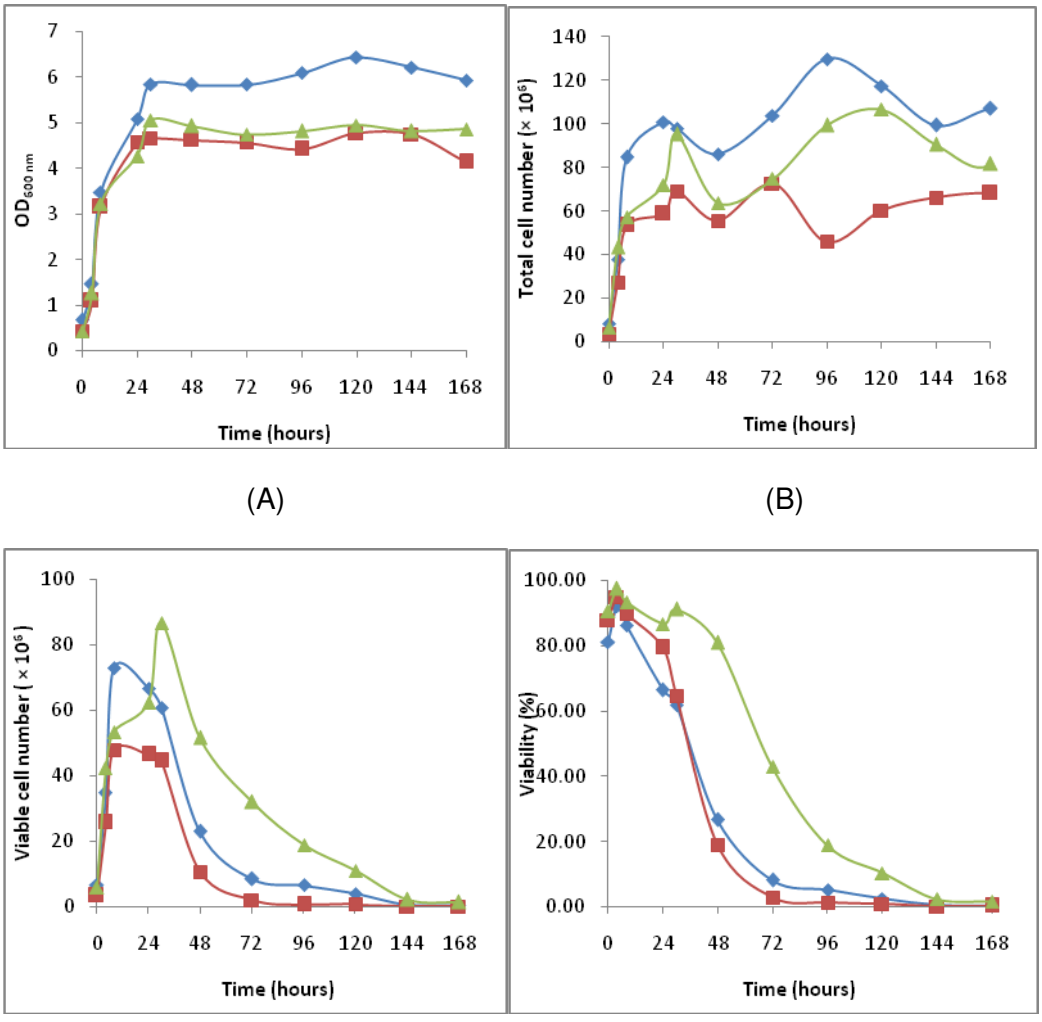


Figure 3.2 Growth parameters of PDM (♦), A12 (■) and A14 (▲) yeast strains grown on YNB media with 16% sucrose. (A) OD_{600nm} (B) total cell number (C) viable cell number (D) cell viability.

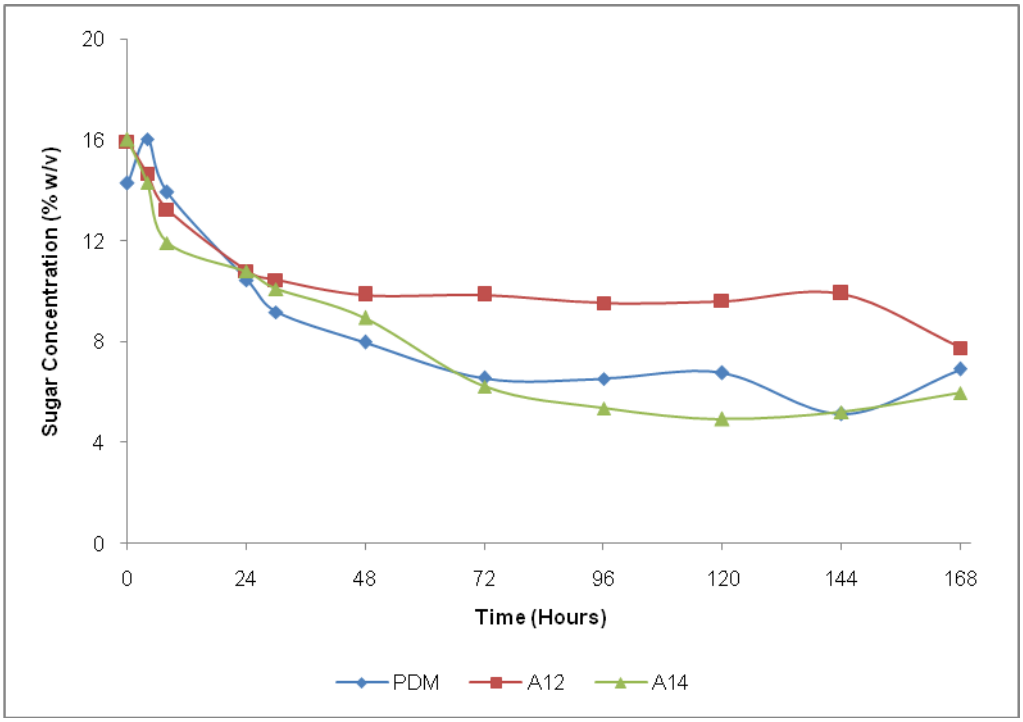


Figure 3.3 Sugar utilization by the three different yeast strains. Cultures were grown in YNB medium with 16% (w/v) glucose as carbon source under aerobic conditions at 30°C.

3.2.2 Growth of A12 in different media

To determine optimum conditions for sugar utilization, further experimentation with alternative media was conducted with strain A12. These experiments investigated a complex medium (YEP) in comparison to the defined medium (YNB), each with 16% sucrose. In addition, in case of a problem with glucose / fructose assimilation after sucrose hydrolysis, cultures were set up with either 16% glucose or 16% sucrose. As a further comparison, cultures were also set up with a lower (2% w/v) sugar concentration, as was used in previous studies of L-proline or inositol supplementation (see Sections 1.4.1 and 1.4.2). Strain A14 was initially used in this experiment. However, when this yeast strain was grown in YEP media, the cells flocculated markedly into large clumps, making it difficult to count the total cells and assess viability using methylene violet staining, as well as to determine OD, as the cells tended to precipitate very fast. Therefore, A14 was omitted from the present experiment. While the other

strains maintained essentially unicellular cultures, A12 was considered the best strain for further experimentation.

Based on OD data, yeast grown in YNB media, either supplemented or not supplemented by an additional 1% ammonium sulphate, showed respiro-fermentative (exponential) growth up to 8 hours, and started to slow at 24 hours. In contrast, yeast grown in YEP media still showed high cell growth rates up to 96 hours. OD data clearly indicate that yeast cells grown in YEP can reach higher cell densities. Yeast grown in YEP with glucose showed the highest OD followed by cells grown in YEP with sucrose.

In Figure 3.4 it can be seen that yeast cells grown in YEP with glucose showed the highest total cell number, confirming the OD data. However, the total cell number for yeast cells grown in YEP with sucrose was not different to that of yeast grown in YNB media. All cultures grown in YNB media showed a similar pattern. During the first 8 hours, cells grown in YNB or YEP with sucrose showed respiro-fermentative (exponential) growth, with growth starting to decline at 24 hours. However, cell counts of yeast grown in YEP with sucrose (or glucose, to a lesser extent) were moderately increased again at about 120 hours, indicating a small secondary fermentation.

Viable cell numbers and cell viability data showed similar patterns in all medium compositions. The number of viable cells increased rapidly during the first 8 hours. Yeast cells grown in YEP maintained a higher viable cell number and % viability than cells grown in YNB.

3.2.3 Sugar utilization by the A12 yeast strain in different media

Sugar was rapidly assimilated during the first 24 hours of fermentation (Figure 3.5 A) in all media (either YEP or YNB-based media, and either sucrose or glucose as sugar source). After 24 hours, yeast grown in YNB-based media

seemed to stop utilizing sugar, as indicated by relatively constant sugar levels throughout the remainder of the experiment. Supplementation of YNB containing 16% glucose with 1.5% ammonium sulphate did not ameliorate the stuck fermentation, indicating that something additional to nitrogen nutrition was problematic. However, yeast grown in the nutritionally rich YEP media still assimilated the sugars up to 168 hours of fermentation and therefore the sugar was almost completely utilized, with only 0.5% w/v sucrose or 0.3% w/v glucose remaining at 168 hours. In contrast to the results with 16% sugar, in Figure 3.5 B it can be seen that YNB, with or without added 1% ammonium sulphate, was nutritionally sufficient to sustain complete utilisation of 2% glucose.

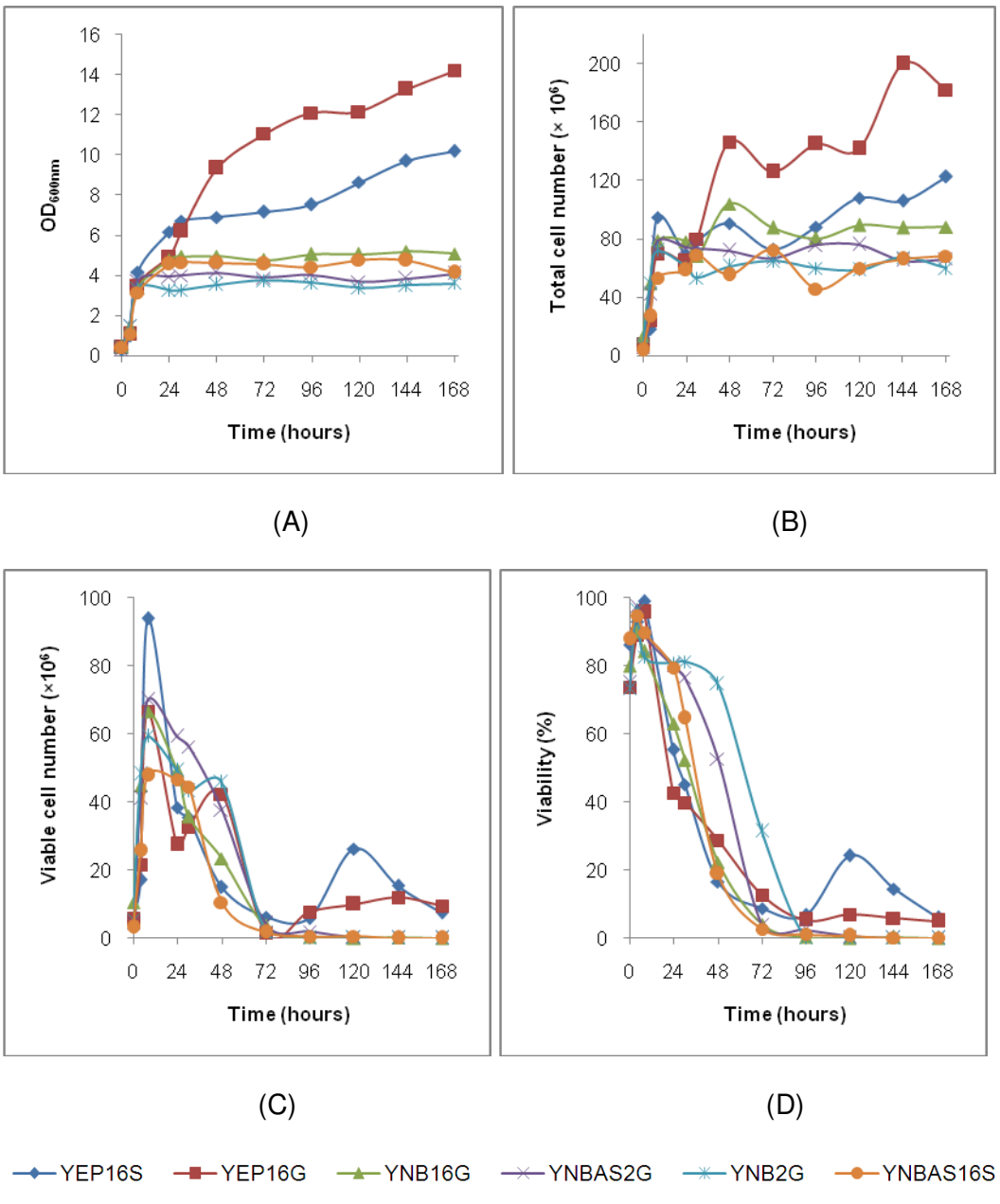
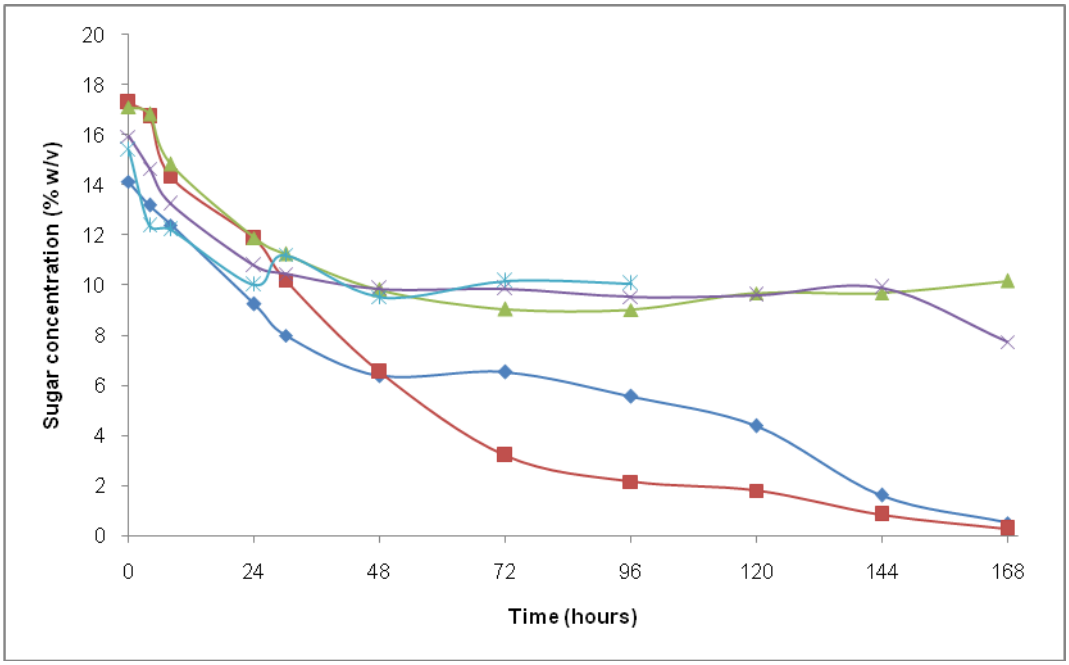
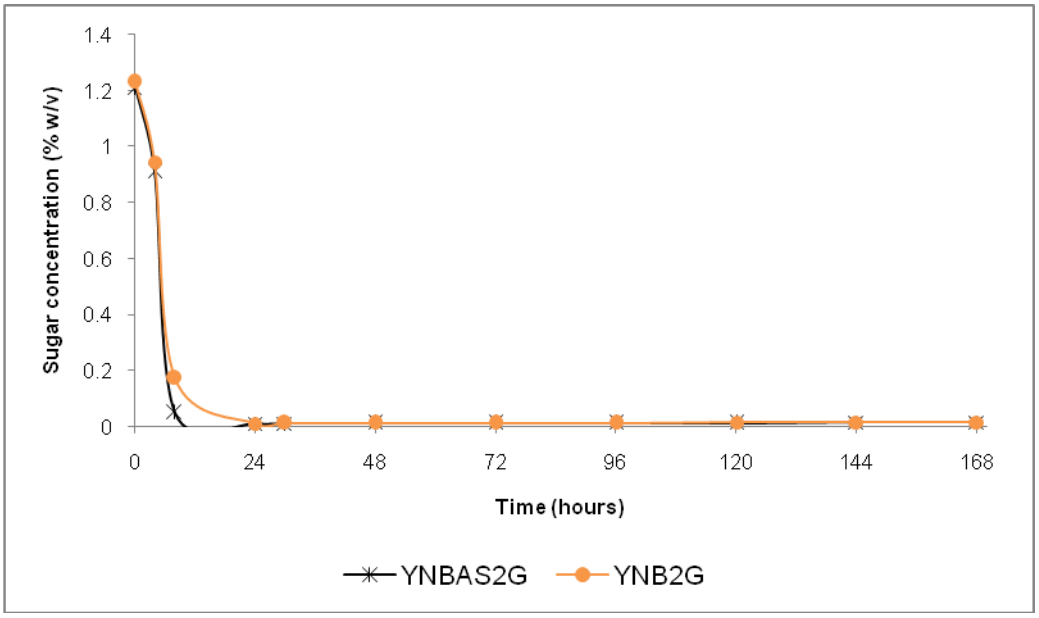


Figure 3.4 Growth parameters of the A12 yeast strain grown on different media as indicated. Cultures were grown under aerobic conditions at 30°C (A) OD_{600nm}, (B) total cell number, (C) viable cell number, (D) cell viability.



(A)



(B)

Figure 3.5 Sugar utilization of A12 strain grown in different media. Cultures were grown in different media as indicated on figure legend. Cultures were grown under aerobic conditions at 30°C. Cells grown in YNB16S were only monitored for up to 96 h, as this experiment was performed at the initial stage of experimentation, and as a result it was decided to follow cultures for a longer time in subsequent experiments.

3.3 Discussion

As the initial objective of the present study was producing high quantities of bioethanol under modelled industrial conditions, a high initial sugar concentration was used in the initial stage of the experiments. Three yeast strains were used initially; A12, A14 and PDM. The medium used was YNB with 16% sucrose. Growth parameters of the three yeast strains were similar, with exponential growth up to 24 hours and thereafter loss of cell viability. A12 showed the highest rate of cell viability decline followed by PDM and A14. This result indicates that even though the total cell number of A14 was lower than PDM, its viability was better. This property is required for lengthy fermentations, as the cells maintain their activity for a longer period. .This property might be an advantage for high sugar concentration fermentation which requires long fermentation times. Further investigation comparing yeast cell viability and fermentation performance of different yeast strains is required to test this hypothesis.

When the yeast was grown in YNB with high sucrose concentration, the growth and fermentation performance was poor. Sugar utilization was monitored as a fermentation performance indicator. All strains showed rapid sugar utilization during the first 24 hours, after which fermentation virtually ceased, leaving 6% to 8% (w/v) residual sugar. This could have been due to insufficient nutrition available in the media. YNB was used as the base medium in this experiment so that analysis of membrane fluidity by fluorescence spectroscopy of laurdan-labelled cells would be possible *in situ*. This medium has been used successfully in the past for ferments with moderate sugar levels (e.g. 2 to 5%), however its nutritional value seems to be limited for high sugar ferments. Nitrogen insufficiency is a common cause of stuck ferments, so we investigated whether increasing the level of bioavailable nitrogen could resolve this problem. Therefore, we added an additional 1% ammonium sulphate to provide a final

concentration of 1.5%, as has been applied in previous studies (Chi, Kohlwein & Paltauf 1999). However, when the YNB was brought up to 1.5% ammonium sulphate, utilisation of 16 % glucose could still not be completed and ferments still stopped at high residual sugar levels. In order to confirm the feasibility of high gravity (16 % sugar) ferments with the chosen yeast strains, we investigated the nutritionally richer (although compositionally relatively undefined) YEP-based media, as has been applied in previous studies (Thomas & Ingledew 1990).

The growth pattern of the yeast varied depending on the medium. In YEP media the yeast grew more rapidly compared to the YNB media. The highest cell numbers were achieved for yeast grown in YEP with glucose as the carbon source as can be seen in Figure 3.4 (A) and (B). However, in maintaining the cell viability, sucrose tended to give a better result as can be seen in Figure 3.4 (C) and (D) only due to secondary ferments at later time points. In general, viability .decreased faster with sucrose compared to glucose in YEP-based media. The higher nutrition in YEP appeared to promote cell growth as evidenced by higher OD and total cell numbers of cultures grown in YEP.

In the last part of the fermentation process it can be seen that the viable cell number was increased after 72 hours for yeast cells grown in YEP medium either with sucrose or glucose as the carbon source (Figure 3.4(C)). However, the viable cell number dropped again after 120 and 144 hours for YEP cultures with sucrose and glucose, respectively. Cells grown in YEP also showed better viability at the end of fermentation. This result indicates that YEP is better for maintaining viability of the yeast cells compared to YNB.

When the performance of cultures grown in media with only 2% sugar were analysed, a different pattern was observed. Yeast grown in YNB tended to have higher viability (Figure 3.4(D)). Addition of ammonium sulphate to the YNB,

seemed to marginally change the growth pattern, however YNB alone is sufficient to sustain complete fermentation of 2% glucose.

The findings in this chapter indicate that for high sugar fermentation, the nutritionally rich but relatively undefined medium YEP is preferred over the defined medium YNB, as YEP can promote high sugar utilization and therefore increase fermentation performance. Furthermore, YNB cannot be used as a medium for fermentation of high levels of sugar. However, as the aim of this study was to use spectrofluorometry for membrane fluidity determination, the emission spectrum of the media used must be taken into consideration. YEP shows very high emission intensity at the wavelengths used to analyse laurdan emission. An additional aim was to measure laurdan fluorescence *in situ* during fermentation experiments, thus removal of cells and resuspension in a non-fluorescent medium was not an option to be considered. YNB has relatively low emission at these wavelengths, and therefore for further experiments in the present study we decided to use YNB.

Having established that YNB had to be used rather than nutritionally rich media, the problem of stuck fermentation had to be resolved. We had hoped to extend the previous studies on possible effects of inositol or L-proline supplementation on ferments of media containing 16% sucrose. The previous studies (Krause *et al.* 2007; Takagi *et al.* 2005) upon which our aims were based used media with relatively low levels (2%) of glucose as the carbon source. At this stage of the project we decided to continue exploratory studies with YNB containing 2% glucose, to test any beneficial effects of supplementation with inositol or L-proline. It was anticipated that after these confirmatory explorations had been conducted, we would then resolve the problems noted above and could then extend the analyses to high sugar ferments.

3.4 Conclusions

The present study confirmed that nutrient availability is very important for fermentation performance. Comparison of YNB and YEP media indicates that nutritionally insufficient media are not suitable for high sugar fermentation. When fermentation was performed in YEP media, rapid and complete sugar utilization was observed. Therefore, we recommend a nutritionally rich medium such as YEP for high sugar fermentations.

However, one of our objectives in the present study was to assess the effects of L-proline and inositol supplementation on membrane fluidity using spectrofluorometry techniques and we considered the fluorescence background and the undefined chemical composition of YEP media as unfavourable factors for our further experiments. Therefore, for our present study, we chose YNB with low sugar concentration for further study.

CHAPTER FOUR: COMPARISON OF MEMBRANE FLUIDITY AND ETHANOL TOLERANCE OF DIFFERENT YEAST STRAINS

4.1 Introduction

4.1.1 General introduction

Ethanol tolerance and membrane fluidity have been shown to have a strong correlation. These properties can be different from one strain to another, and depend upon the intrinsic properties of the particular yeast strain investigated.

Indirect determination of membrane fluidity by measuring the unsaturation index of the yeast plasma membrane has indicated that increasing the unsaturation index of the yeast membrane can improve the ethanol tolerance of the yeast cell. Introduction of genes responsible for unsaturated fatty acid synthesis reportedly improve ethanol tolerance (Kajiwara *et al.* 2000; You, Rosenfield & Knipple 2003). However, membrane fluidity is not only influenced by the fatty acid composition, but also by other component(s) of the yeast plasma membrane, such as proteins and sterols (Alexandre, Rousseaux & Charpentier 1994; Learmonth 2011). Therefore, this finding needs more confirmation in terms of how the unsaturation index may influence the actual plasma membrane fluidity under physiological conditions.

Another investigation in which DPH anisotropy was measured to determine the membrane fluidity revealed that high membrane fluidity is related to higher ethanol tolerance (Alexandre, Rousseaux & Charpentier 1994). This conclusion was drawn from study of two different yeast species, *S. cerevisiae* and *Kloeckera apiculata*. Assessment of different yeast strains from the same species might

reveal different results. Therefore, in the present study we compared the membrane fluidity of different strains of *S. cerevisiae*.

4.1.2 Yeast strains and culture conditions

Yeast strains used in this experiment were A12, PDM and K7. A12 is an ethanol tolerant baker's yeast according to previous studies (Lewis 1993), PDM is an industrial wine strain (Mauri Yeast) which can produce up to 17% (v/v) ethanol, and K7 is a sake strain (ATCC 26422) that can produce up to 17.5% ethanol. These strains were chosen for their high ethanol production in nature.

4.1.3 Specific growth conditions and experimental design

For growth analysis, batch culture experiments were set up, and samples were aseptically removed from the culture by drawing off with a sterile pipette every 6 hours from 0 to 30 hours. Samples were then analysed for optical density at 600 nm (Section 2.4), viable cell count (section 2.5), percent cell viability (section 2.5), percent budding rate (section 2.6), and glucose and ethanol concentration (section 2.9). Data presented in the following results were drawn from four independent experiments.

Ethanol tolerance (section 2.8) and GP (section 2.7) were measured at 6 and 24 hours, representing respiro-fermentative (exponential) and respiratory phases of the cell growth, respectively.

The GP data for each strain was drawn from eight replicates. Ethanol tolerance by methylene violet staining was obtained from four independent measurements. Ethanol tolerance by TPC at the 6 hour time point was drawn from four independent experiments, while TPC at the 24 hour time point was taken as average of six independent experiments.

The medium used in this experiment was YNB with 2% (w/v) glucose, prepared as a 10× stock solution and diluted to the required concentration prior to use.

Where appropriate, the experimental data were analysed for statistical significance using a one way ANOVA, with *post hoc* comparison using the Fisher's LSD (least significant difference) test to determine the specific differences (section 2.10). Differences were considered significant at the level of $p < 0.05$.

4.2 Results

As mentioned earlier, there are strong indications of a correlation between membrane fluidity and ethanol tolerance of a particular microorganism. Therefore, as the first step of our experiments, we examined membrane fluidity (by laurdan GP) of the yeast strains to verify whether there were any differences among the strains used. We also observed the ethanol tolerance of each strain (represented as viability reduction), and correlated its value with the laurdan GP.

4.2.1 Growth parameter comparisons

Optical density at 600 nm was used to measure cell density, allowing comparison of relative growth rate of the three strains used in this experiment (Figure 4.1). Generally, during the lag (0-6 hours) and respiro-fermentative phase (6-12 hours), the $OD_{600\ nm}$ values were comparable, the only significant difference was found at the 6 hour time point where A12 had a significantly higher OD_{600nm} compared to PDM ($p = 0.028$). After 12 hours, A12 and PDM started to enter respiratory phase, whereas K7 was still in the respiro-fermentative phase. At 18 hours, K7 started to enter its respiratory phase and interestingly, starting from this time point, the OD_{600nm} value for K7 was significantly higher than that for each the other strain ($p = 0.003$). At 18 hours, K7 had the highest OD_{600nm} value followed

by PDM and A12. The cell density of K7 remained higher at 24 ($p < 0.001$) and 30 hours ($p < 0.001$). This data indicates the different cell growth patterns of the three yeast strains used in this experiment. The optical density of the three strains during fermentation is presented in Figure 4.1.

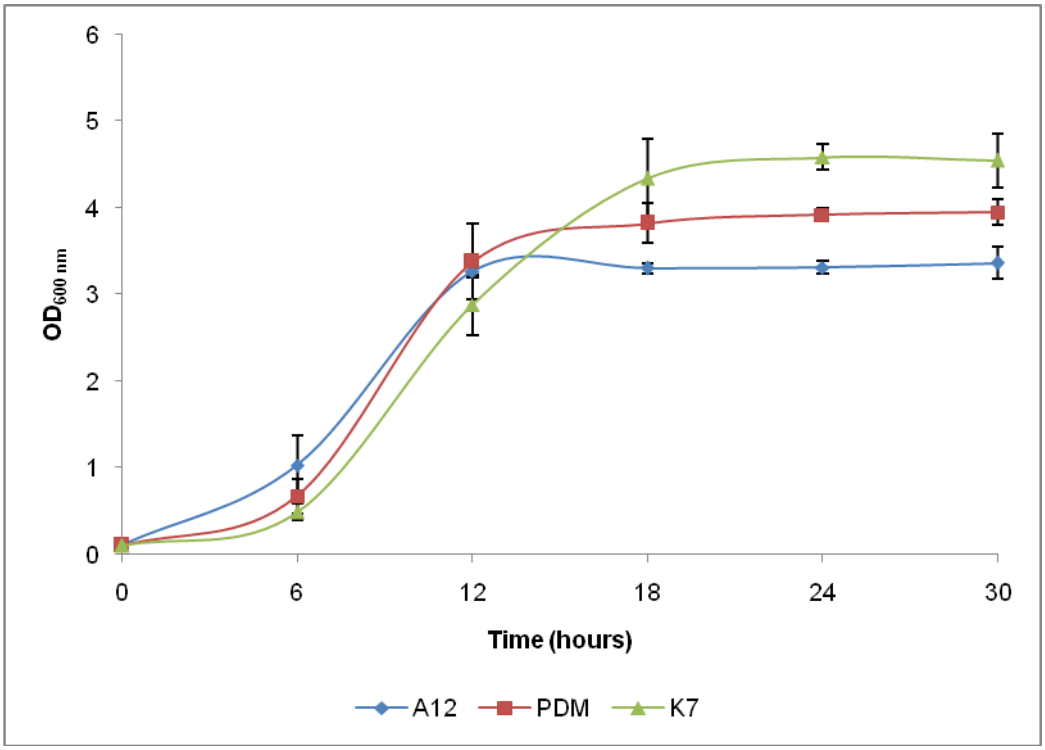


Figure 4.1 Optical densities of three yeast strains during fermentation. Cultures of the strains indicated were grown in YNB medium with 2% (w/v) glucose as carbon source under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviation.

In addition to optical densities, cell density was also assessed by light microscopy with counting. The cells were examined under a light microscope after staining by methylene violet solution. From this method total cell counts, viable cell counts, cell viability percentage and budding rates can be determined. Figure 4.2 shows the cell counts of the three yeasts during the fermentation. Unlike the OD_{600nm} data, total cells measured by light microscopy did not show

any significant differences, neither did budding rate nor total viable cell counts. However, total cell counts provided similar indications to OD_{600nm} data.

Budding rate data indicated a trend in that the budding rates decreased during the lag phase (0-6 hours) and reached their lowest value at 6 hours. At 12 hours the budding rate was increased and stayed more or less the same throughout the fermentation. No significant differences were detected for these parameters for any of the strains used in the present experiment.

Interestingly, cell viability data showed some significant differences between the strains. At the beginning of the fermentation, K7 started with significantly higher viability compared to the others ($p = 0.022$). At 6 hours, all the strains reached their highest cell viability values, although no significant differences were observed at this time point. At the 12 hour time point, the cell viability of A12 and PDM were drastically reduced, while K7 maintained a significantly higher viability ($p < 0.001$); this higher viability was maintained throughout the remaining fermentation time. It seems that A12 and PDM reach the respiratory phase faster than K7, as can be seen on Figure 4.1. While K7 started the respiratory phase at 18 h, A12 and PDM started their respiratory phase at 12 h. As we used ~18 h culture as starter, this mean that some of A12 and PDM cells started to enter death phase, while K7 starter cultures still had better viability.

Glucose utilization and ethanol production rates of all strains did not show any significant differences (Figure 4.3). Generally, glucose was exhausted between 12 and 18 hours. Even though not statistically significant, there was a trend in which ethanol was rapidly produced after 6 hours and reached its highest concentration at 18 hours. The highest ethanol concentration at 18 hours was recorded for PDM with an ethanol concentration of 0.75 ± 0.24 % (v/v), however this concentration was not significantly different from those of the other strains. After glucose was exhausted by 18 hours, the ethanol concentration started to

decrease due to respiratory growth. Taken together, the growth parameters measured indicate that the cultures were in lag phase from 0-6 hours, respiro-fermentative phase from 6-18 hours (whereupon glucose was exhausted and ethanol levels peaked) and thereafter in respiratory growth phase as evidenced by declining ethanol levels.

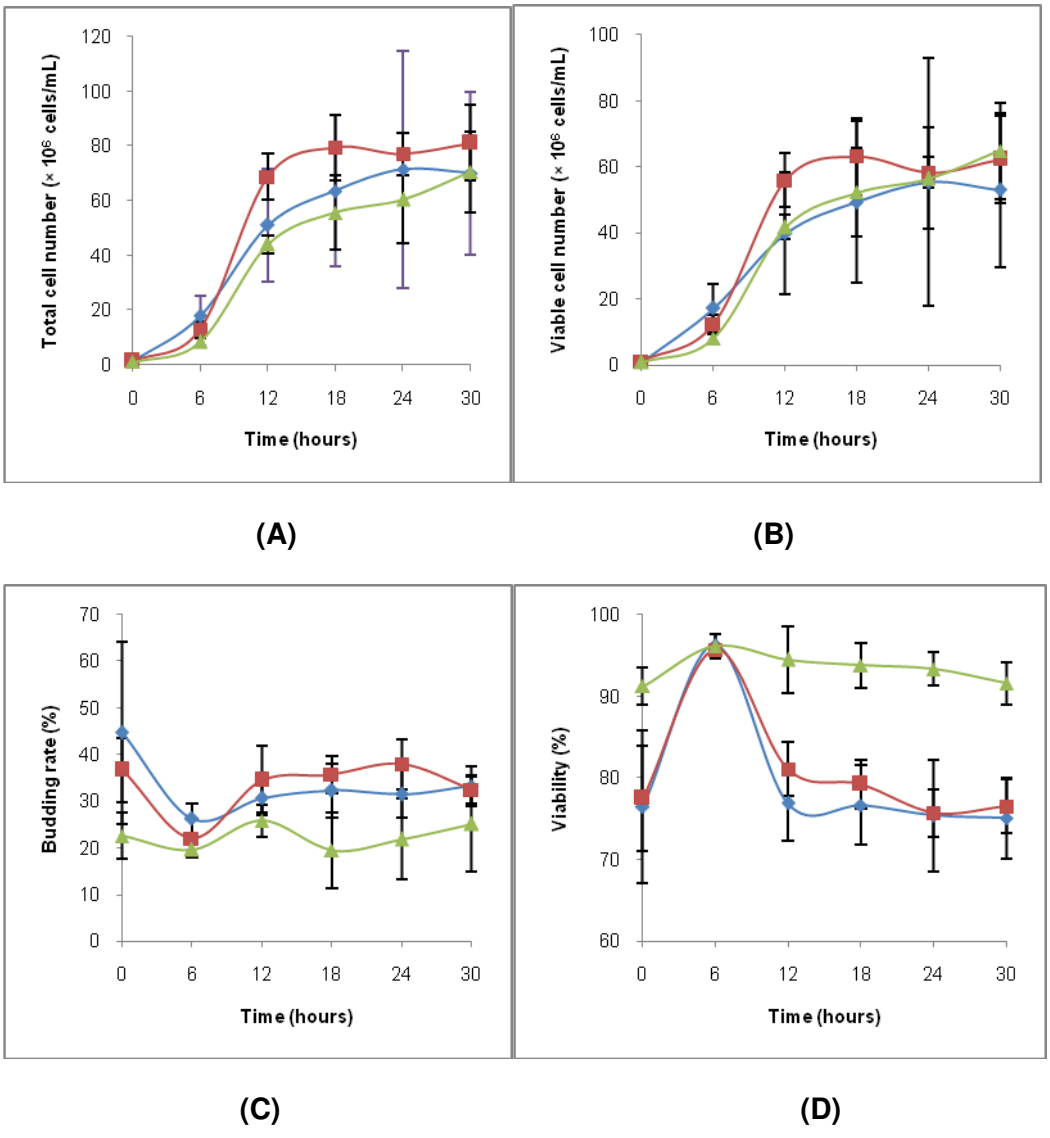


Figure 4.2 Comparison of total cell number (A), viable cell number (B), budding rate (C) and cell viability (D) determined by light microscopy for three different yeast strains, A12 (♦), PDM (■) and K7 (▲). Cultures were grown in YNB medium with 2% (w/v) glucose as carbon source under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviation.

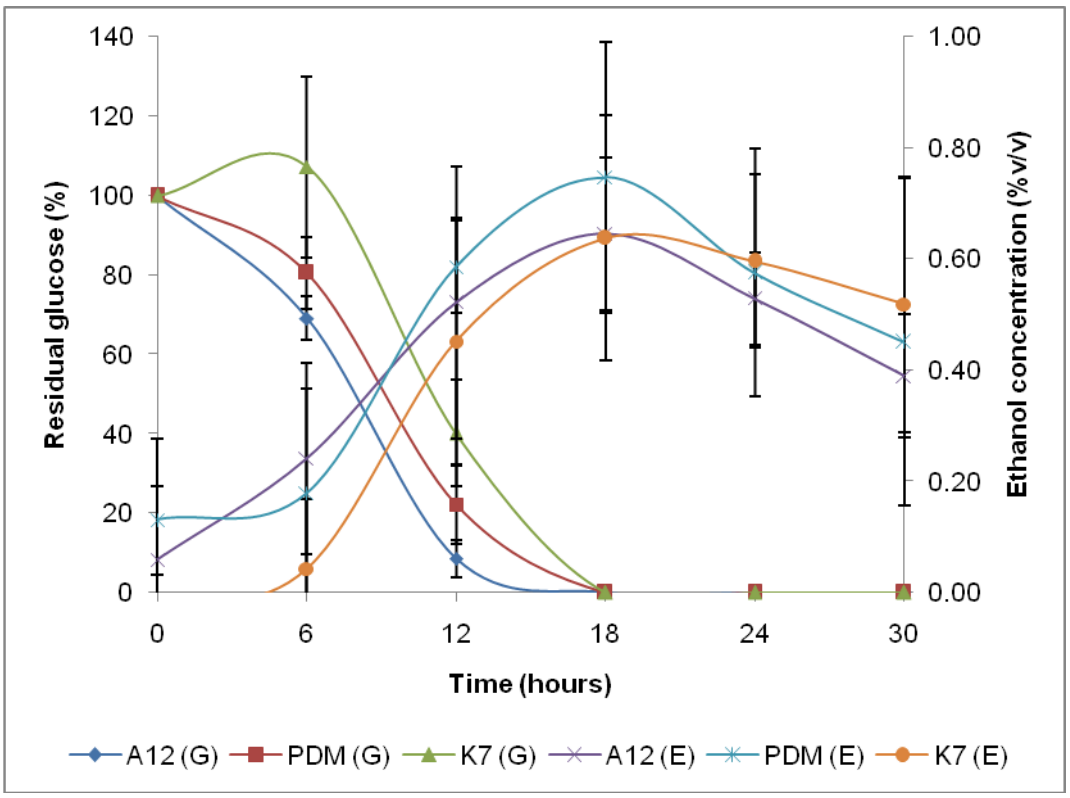


Figure 4.3 Glucose utilization and ethanol production by three different yeast strains. Cultures of the yeast strain indicated were grown in YNB medium with 2% (w/v) glucose as carbon source under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviation.

4.2.2 Membrane fluidity comparisons

Membrane fluidity was assessed by generalized polarization (GP) of laurdan fluorescence emission (Parasassi *et al.* 1998), as applied to yeast cell plasma membranes by Learmonth & Gratton (2002) and Butcher (2008). This method is simpler and more robust compared to other methods for determining membrane fluidity such as polarization and anisotropy measurements, and therefore was preferred in the present experiments.

Figure 4.4 shows GP the values of the three yeast strains. Significant differences between GP values are marked using the same letter above the error bars. Statistical analysis using one way ANOVA showed that at 6 hours of

culture, GP values differed significantly between the yeast strains tested. The highest GP value was recorded for A12, followed by K7 and PDM. This result showed that A12 had the lowest membrane fluidity, followed by K7 and PDM. Unlike the 6 hour results, the 24 h cultures did not show any significant differences in GP value between the strains tested. This result showed that there were no differences in membrane fluidity at 24 hours of culture.

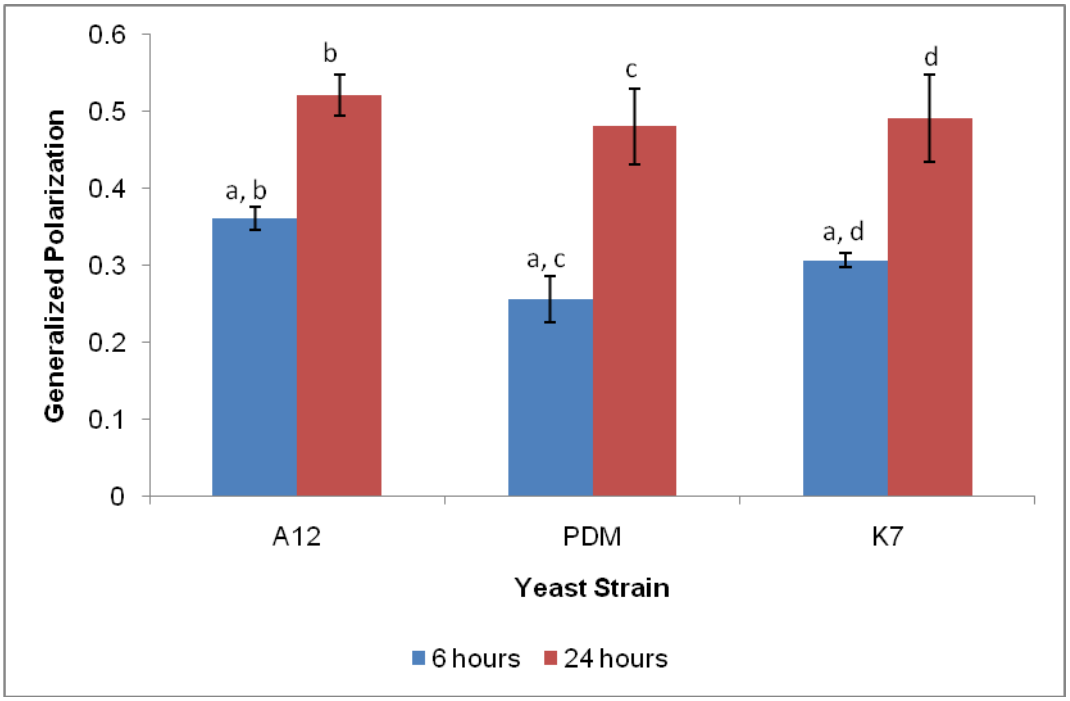


Figure 4.4 Generalized polarization of the three yeast strains at 6 and 24 hours of culture. Cultures of the yeast strain indicated were grown under aerobic conditions in YNB medium with 2% glucose as carbon source. Statistically significant differences are indicated by the same letters above the bars. Error bars represent standard deviation.

Comparison of the GP values at 6 and 24 hours of culture showed that the GP of each strain was significantly higher after 24 hours of culture compared to 6 hours of culture ($p < 0.001$). The increasing GP value with increasing culture time showed that the membrane fluidity decreased. Similar results were observed in previous studies by Learmonth & Gratton (2002), Butcher (2008) and Learmonth (2011).

4.2.3 Ethanol tolerance comparisons

Figure 4.5 shows viability reduction upon ethanol stress, tested using TPC. The results show that there were no significant differences in viability reduction between strains at 6 or 24 hours of culture. However, even though not statistically significant, the viability reduction of PDM was slightly more than for the other strains at 24 hours. This result was confirmed by methylene violet staining and will be discussed in the following section.

The TPC test showed that the viability reduction caused by ethanol stress was significantly decreased ($p < 0.05$) from 6 to 24 hours of culture. This result suggests that ethanol tolerance was increased in respiratory phase cells as compared to respiro-fermentative cells, for each strain. However, the TPC test failed to distinguish any difference in ethanol tolerance between the strains either at 6 or 24 hours of culture.

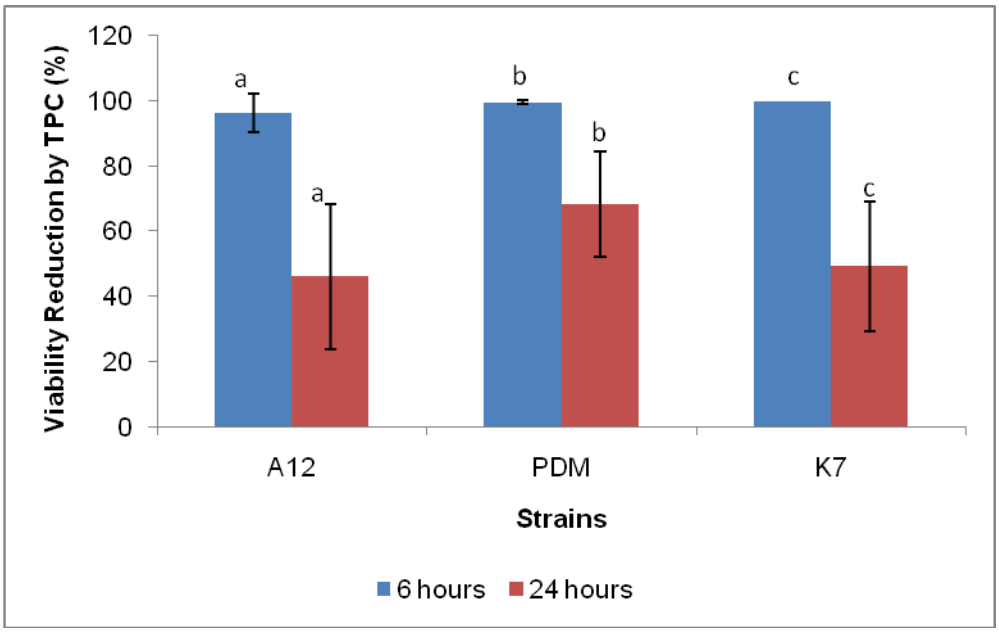


Figure 4.5 Viability reduction induced by ethanol stress as determined by the total plate count (TPC) method. Cells of the yeast strains indicated were grown in YNB medium with 2% glucose under aerobic conditions at 30°C until the indicated time points. Then, they were exposed to 18% v/v ethanol, and subsequently diluted and grown on agar plates. Statistically significant differences are indicated by the same letters above the bars. Error bars represent standard deviation.

Methylene violet staining was used as an alternative method for determining ethanol tolerance (Figure 4.6). Statistical analysis of the data showed that the viability reduction values of different yeast strains were significantly different at 6 ($p = 0.021$) and 24 ($p = 0.008$) hours of cultures. Further testing by Fisher's LSD test showed that PDM had significantly higher viability reduction compared to A12 and K7, at either 6 or 24 hours of culture. This suggests that PDM has lower ethanol tolerance compared to the other strains, but the viability reduction for A12 and K7 did not differ significantly. However, unlike the TPC method, MVS failed to detect any differences in viability reduction between 6 and 24 hours of culture, likely due to a high variability in the counts.

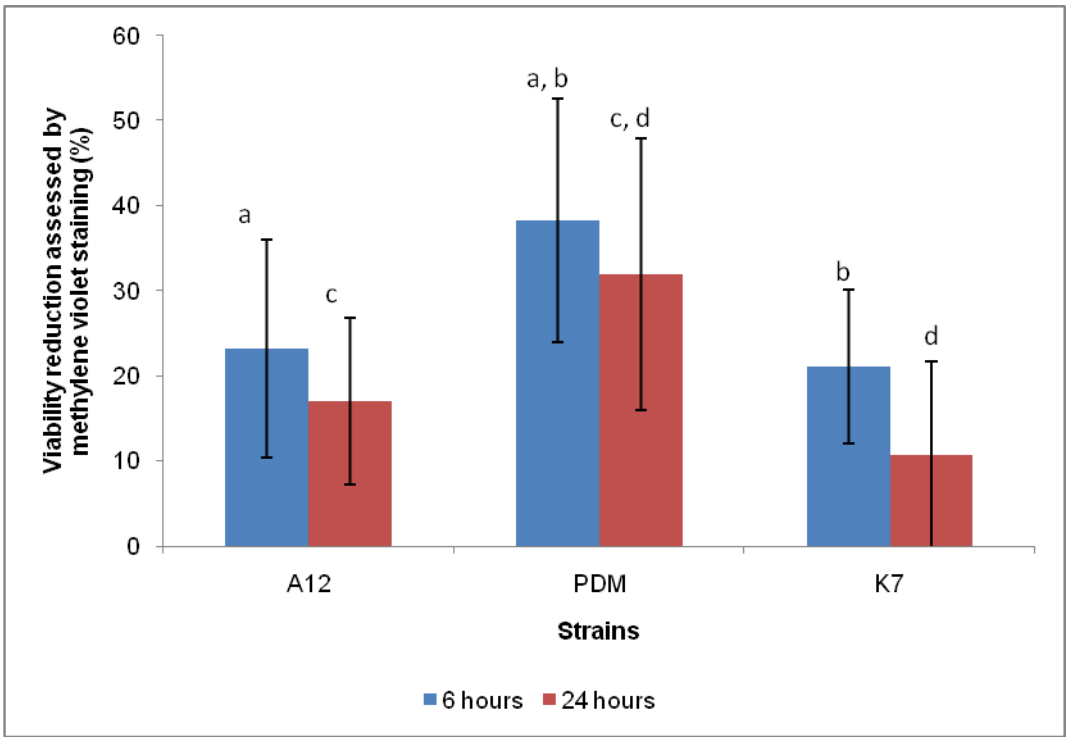


Figure 4.6 Viability reduction induced by ethanol stress as determined by the methylene violet staining method. Cells of the yeast strains indicated were grown in YNB medium with 2% glucose under anaerobic conditions at 30°C until the indicated time points. Then they were exposed to 18% v/v ethanol. Cells were counted under microscope (400 × magnification) after exposure to 18% v/v ethanol and staining using methylene violet in sodium citrate solution. Statistically significant differences are indicated by the same letters above the bars. Error bars represent standard deviation.

4.2.4 Correlation between test parameters for different yeast strains

Correlation between parameters tested was evaluated using the Pearson correlation coefficient. The results of correlation analysis are presented in Table 4.1. The scatter plot matrix for each correlation is presented in Figures 4.7 to 4.12. Solid lines on the scatter plot matrices represent the linear regression line for each individual strain (indicated by the same colour, as described in the legend). Dashed lines represent the linear regression line for the overall data.

Correlation analysis for the overall data showed that there is a very strong positive correlation between time and GP value ($r = 0.899$; $p < 0.001$). The same result was also observed for individual strains with $r > 0.900$ and $p < 0.001$. This result confirmed the findings above and showed that the GP value increases with increasing culture age, which indicates that membrane fluidity of all strains is lower in cells in the respiratory growth phase.

A strong negative correlation was observed between time and viability reduction by TPC ($r = \sim 0.8$; $p < 0.05$) and GP with viability reduction by TPC ($r = 0.65$-0.79; $p < 0.05$) either for the overall data or for the data for individual strains, which shows that viability reduction by TPC decreases with increasing time and GP value. Since viability reduction is inversely related to ethanol tolerance, these results suggest that ethanol tolerance of all yeast strains is higher in respiratory phase cells which also have a lower intrinsic membrane fluidity.

Viability reduction by methylene violet staining correlated poorly with either culture time, GP or viability reduction by TPC. A weak correlation ($r \sim 0.500$) between viability reduction by methylene violet staining and other parameters was observed for the A12 strain, but not for the other strains. Significant, but weak correlation was also observed between viability reduction and methylene

violet staining with viability reduction by TPC for the overall strain analysis (r = 0.370; p = 0.044).

Table 4.1 Pearson correlation coefficients between time, GP, viability reduction by TPC and viability reduction by Methylene violet staining for all strains.

	Culture Time* (hours)		Generalized Polarization		Viability Reduction by TPC (%)	
Generalized Polarization	A12	r = 0.970 p < 0.001				
	PDM	r = 0.927 p < 0.001				
	K7	r = 0.939 p < 0.001				
	Overall	r = 0.899 p < 0.001				
Viability Reduction by TPC (%)	A12	r = - 0.873 p < 0.001	A12	r = - 0.788 p = 0.007		
	PDM	r = - 0.804 p < 0.001	PDM	r = - 0.654 p = 0.040		
	K7	r = - 0.870 p = 0.001	K7	r = - 0.687 p = 0.028		
	Overall	r = - 0.805 p < 0.001	Overall	r = - 0.681 p < 0.001		
Viability Reduction by Methylene Violet Staining (%)	A12	r = - 0.515 p = 0.128	A12	r = - 0.575 p = 0.082	A12	r = 0.588 p = 0.074
	PDM	r = - 0.175 p = 0.628	PDM	r = - 0.314 p = 0.378	PDM	r = 0.135 p = 0.710
	K7	r = - 0.221 p = 0.540	K7	r = - 0.100 p = 0.783	K7	r = 0.394 p = 0.260
	Overall	r = - 0.196 p = 0.299	Overall	r = - 0.301 p = 0.106	Overall	r = 0.370 p = 0.044

* 6 hour (respiro-fermentative) culture vs. 24 hour (respiratory) culture.

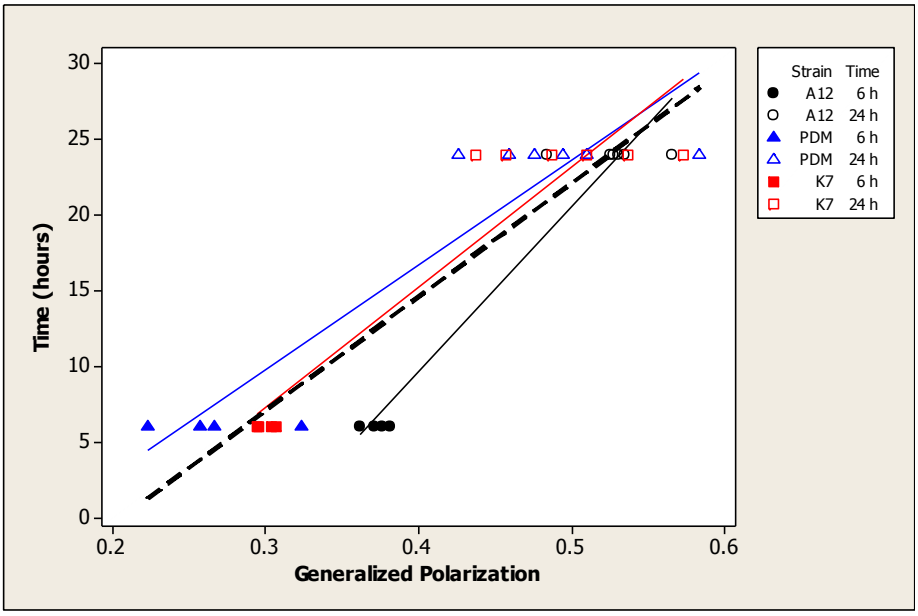


Figure 4.7 Scatter plot matrix correlating culture time and GP values of three different yeast strains. Strains are represented by different shapes and colours. Black circles represent A12, blue triangles represent PDM and red squares represent K7. Filled and empty shapes represent data for 6 and 24 hours of culture, respectively. Straight lines with corresponding colours represent the individual linear correlation for each strain, and the dashed line represents the total linear correlation for all the data on the graph.

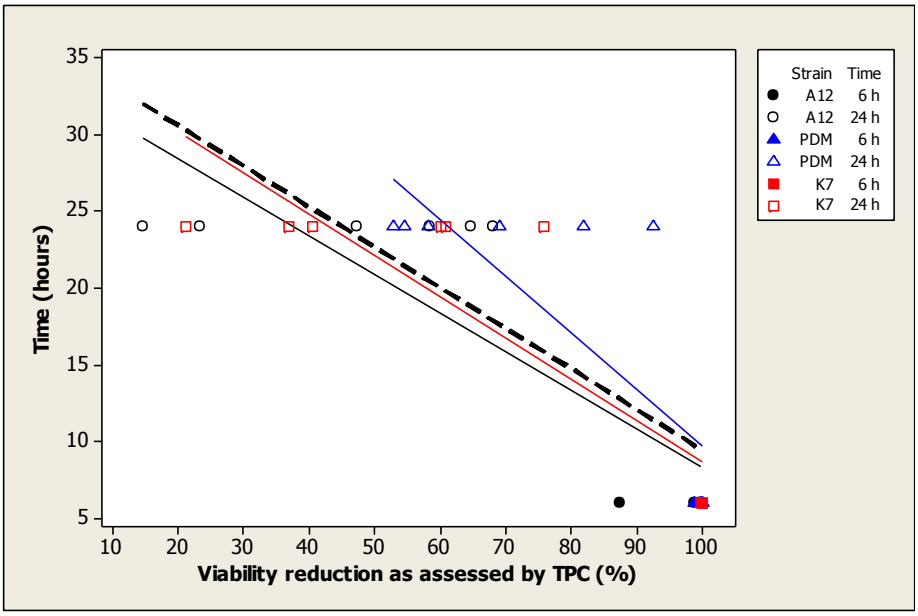


Figure 4.8 Scatter plot matrix correlating culture time and viability reduction (as assessed by the total plate count (TPC) method) values of three different yeast strains. Strains are represented by different shapes and colours. Black circles represent A12, blue triangles represent PDM and red squares represent K7. Filled and empty shapes represent data for 6 and 24 hours of culture, respectively. Straight lines with corresponding colours represent the individual linear correlation for each strain, and the dashed line represents the total linear correlation for all the data on the graph.

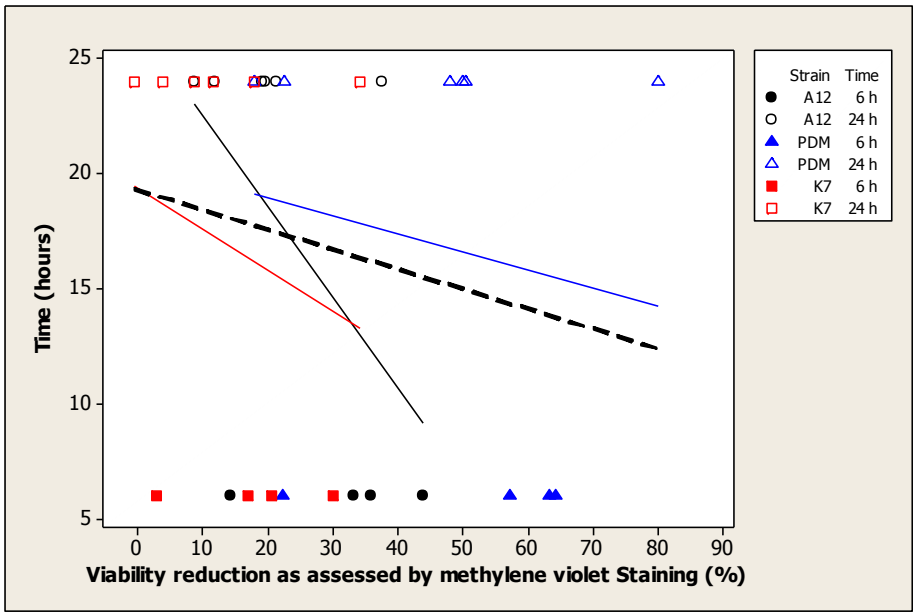


Figure 4.9 Scatter plot matrix correlating culture time and viability reduction (as assessed by the methylene violet staining method) values of three different yeast strains. Strains are represented by different shapes and colours. Black circles represent A12, blue triangles represent PDM and red squares represent K7. Filled and empty shapes represent data for 6 and 24 hour culture, respectively. Straight lines with corresponding colours represent the individual linear correlation for each strain, and the dashed line represents the total linear correlation for all the data on the graph.

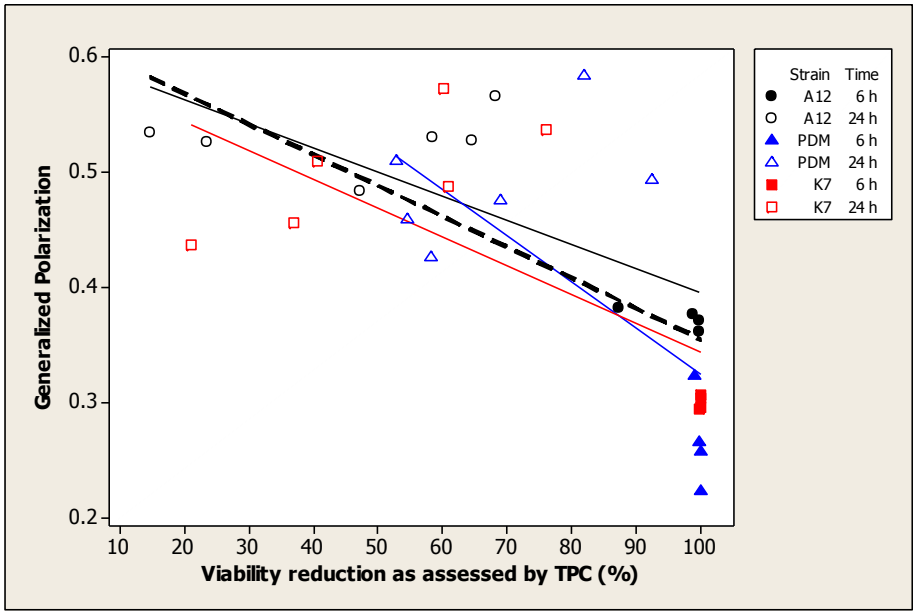


Figure 4.10 Scatter plot matrix correlating GP and viability reduction (as assessed by the total plate count (TPC) method) values of three different yeast strains. Strains are represented by different shapes and colours. Black circles represent A12, blue triangles represent PDM and red squares represent K7. Filled and empty shapes represent data for 6 and 24 hours of culture, respectively. Straight lines with corresponding colours represent the individual linear correlation for each strain, and the dashed line represents the total linear correlation for all the data on the graph.

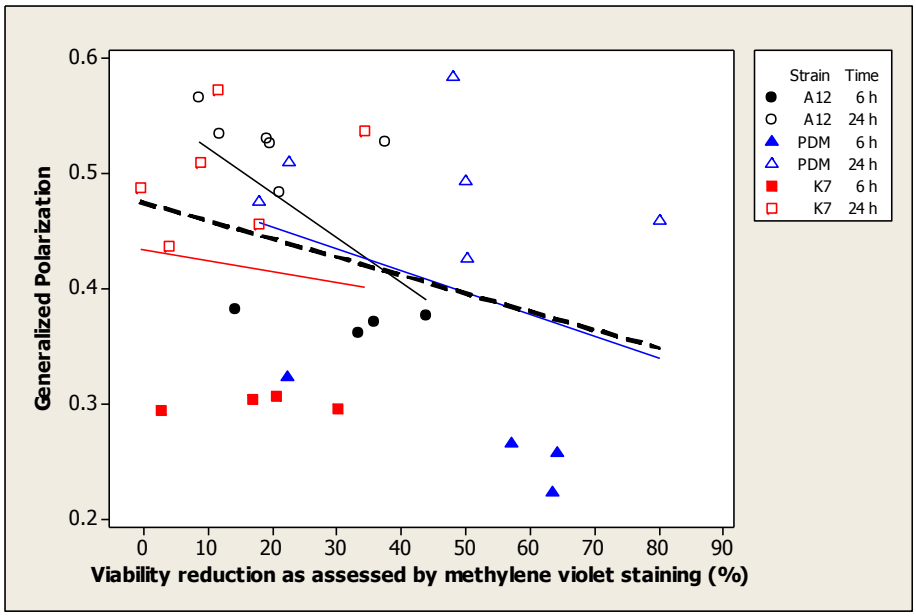


Figure 4.11 Scatter plot matrix correlating GP and viability reduction (as assessed by the methylene violet staining method) values of three different yeast strains. Strains are represented by different shapes and colours. Black circles represent A12, blue triangles represent PDM and red squares represent K7. Filled and empty shapes represent data at 6 and 24 hours of culture, respectively. Straight lines with corresponding colours represent the individual linear correlation for each strain, and the dashed line represents the total linear correlation for all the data on the graph.

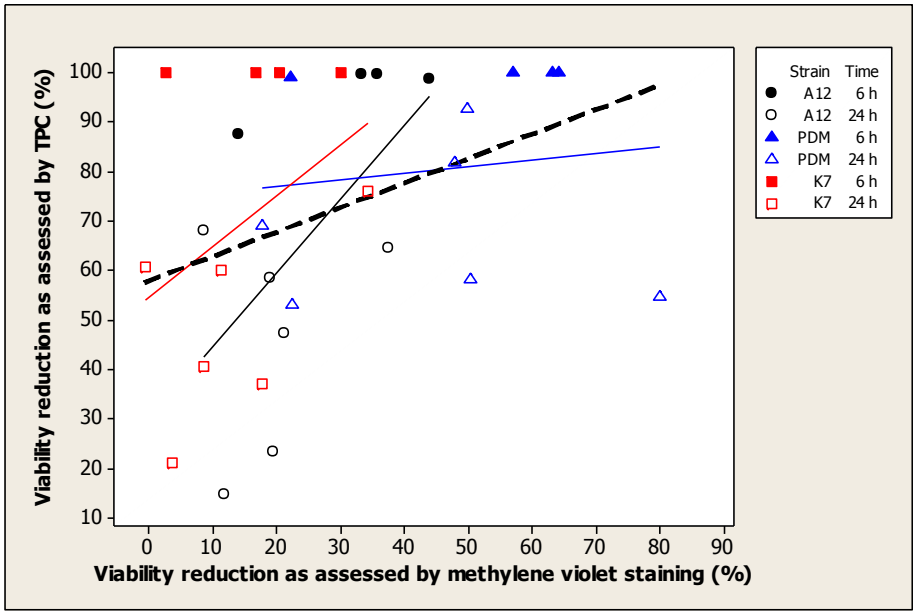


Figure 4.12 Scatter plot matrix correlating viability reduction (as assessed by TPC method) and viability reduction (as assessed by methylene violet staining method) values of three different yeast strains. Strains are represented by different shapes and colours. Black circles represent A12, blue triangles represent PDM and red squares represent K7. Filled and empty shapes represent data at 6 and 24 hours of culture, respectively. Straight lines with corresponding colours represent the individual linear correlation for each strain, and the dashed line represents the total linear correlation for all the data on the graph.

4.3 Discussion

4.3.1 Comparison of growth parameters

Either OD_{600nm} or total cell counts demonstrated that during the first 6 hours, all yeast strains were in their lag phase, followed by respiro-fermentative growth. Interestingly, OD_{600nm} data showed that K7 had a longer respiro-fermentative phase of up to 18 hours compared to 12 hours for A12 and PDM. Starting at the 18 hour time point, the OD_{600nm} for K7 was significantly higher than the other strains, whereas before that, the OD_{600nm} for K7 was lower than the others. This indicates that initially K7 grows slower than the other strains, but in the end it has the highest cell number.

While OD_{600nm} data indicated significant differences between the strains, no significant differences were found between strains for total cell counts. The total cell count data showed very high variability. This might be because the total cell number was calculated manually by counting under a microscope, and manual counting may be associated with high error rates. Another possibility is uneven distribution of the cells in sampling (i.e. sampling error).

The cell viability data were interesting. The data showed that the viability of A12 and PDM strains increased during the first 6 hours of fermentation. The highest cell viability was recorded at 6 hours and after that time the viability declined rapidly to relatively constant levels at about 75% of the 6 hour viability level. Interestingly, cell viability of K7 was very high throughout the fermentation, never less than 91%. This indicates that K7 had better cell survival during the fermentation period. This would be an advantage over the other strains if this property is also exhibited when the yeast grows in media with high sugar concentrations as it would prolong the time during which viable cells can ferment the sugars. Therefore, of the strains trialled K7 seems to have the greatest potential for further exploration.

There were no significant differences between the yeast strains tested in glucose utilization during the fermentation. Ethanol production also did not differ significantly between strains. The highest ethanol concentration was detected at 18 hours of fermentation, and ethanol levels started to decrease after that time point, probably due to respiratory growth. Previous studies (Lewis *et al.* 1993) showed that the decrease in ethanol level was due to metabolic activity and not simply due to evaporation. Glucose was exhausted between 12-18 hours. As *S. cerevisiae* belongs to the Crabtee-positive yeast group (Piskur *et al.* 2006), after glucose depletion at 18 hours, the yeast start to use ethanol as a carbon source. This can be noted from the decrease in ethanol concentration after 18 hours of fermentation. Therefore, when fermentation is conducted with the batch method under aerobic conditions and high ethanol concentration is desired, the fermentation should be stopped before the yeast start to use the product. In the present experiment, with reduced nutrition and low glucose concentration, 18 hours is the right time to stop the fermentation. Higher glucose and richer media may change this time point. Another option would be to ensure that after they start, the fermentations are conducted under anaerobic conditions, as oxygen is required for the respiratory assimilation of ethanol.

4.3.2 Membrane fluidity and ethanol tolerance

Previous published works showed that the stress tolerance of yeast cells is related to the fluidity of the yeast cell plasma membrane. Alexandre, Rousseaux & Charpentier (1994) and Alexandre, Berlot & Charpentier (1994) compared membrane fluidity by measuring anisotropy of two different yeast species with different degrees of ethanol tolerance, and found that yeast species with higher membrane fluidity had higher ethanol tolerance. However, statistically,

comparison of two yeast strains is not sufficient for drawing a valid conclusion, therefore more yeast strains need to be investigated.

Modification of the cell plasma membrane by incorporating more unsaturated fatty acids, thereby changing the unsaturation index of the plasma membrane has also been found to increase tolerance of the yeast cell to high ethanol concentrations (Kajiwara *et al.* 2000; You, Rosenfield & Knipple 2003), freezing and also salt stress (Rodrıguez-Vargas *et al.* 2007). Dinh *et al.* (2008) also found that yeast cells that developed tolerance to high ethanol concentrations have a higher composition of unsaturated fatty acids, particularly oleic acid (C18:1).

However, Swan & Watson (1999) found slightly different results. They enriched mutant yeast cells with different unsaturated fatty acids, namely oleic (C18:1), linoleic (C18:2) and linolenic acid (C18:3). They observed that yeast cells enriched with oleic acid are more tolerant to ethanol and heat, followed by cells enriched with linoleic and linolenic acids (Swan & Watson 1999). These experiments followed on from previous experiments where heat and oxidative stress tolerance were studied in yeast cells grown under aerobic or anaerobic conditions and then supplemented with various specific lipids (Steels *et al.* 1994). In the latter experiments, unsupplemented anaerobically-grown cells with largely saturated fatty acid profiles were more resistant to heat stress than C18:1 or C18:3 supplemented cells. These experiments suggested that increasing fluidity by introducing more unsaturation to fatty acyl chains leads to more susceptible cells. However, direct measurement of membrane fluidity was not reported in their study, and therefore we cannot compare our membrane fluidity data.

In this study ethanol tolerance of the yeast cell was monitored using two different methods, total plate count (TPC) and methylene violet staining (MVS) after exposing the cells to 18% ethanol. The total plate count is widely used for ethanol tolerance determination of yeasts (Chi, Kohlwein & Paltauf 1999; Krause

et al. 2007), while methylene violet staining is rarely used. As these two methods can be used to distinguish live and dead cells, we tried to apply the methylene violet staining to calculate the viability reduction of ethanol stressed cells, and investigate whether it can be used in an ethanol tolerance test.

In the present study, considering GP measurements and viability reduction as assessed by methylene violet staining, we found that at 6 hours of culture, A12 and K7 strains which have significantly higher GP values than PDM showed lower viability reduction. This indicates that strains with lower membrane fluidity have higher tolerance against ethanol, which acts by fluidising membranes. However, viability reduction as assessed by TPC failed to distinguish the ethanol tolerances of the strains at 6 hours culture. Similar phenomena were also observed of 24 hours of culture, in which PDM, which has the lowest GP, tended to have a higher viability reduction, either as determined by total plate count or methylene violet staining. However, at 24 hours of culture the differences were not statistically significant.

The results of the present study contradict most of the previously published works which indicate that higher membrane fluidity tends to lead to higher ethanol tolerance (Alexandre, Rousseaux & Charpentier 1994; Dinh *et al.* 2008; You, Rosenfield & Knipple 2003). Previous studies used different approaches to determine the membrane fluidity of the yeast cell. They mainly measured increasing levels of unsaturated fatty acid, expressed as the unsaturation index (Alexandre, Rousseaux & Charpentier 1994; Dinh *et al.* 2008; Rodrıguez-Vargas *et al.* 2007; You, Rosenfield & Knipple 2003) or direct measurement of membrane fluidity by measuring DPH anisotropy (Alexandre, Rousseaux & Charpentier 1994). Studies which simply infer fluidity from lipid unsaturation data do not account for the impacts of the many other factors that can modulate membrane fluidity (Learmonth 2011; Learmonth & Gratton 2002).

Alexandre, Rousseaux & Charpentier (1994) also stressed that membrane fluidity as deduced from unsaturation index does not always reflect the real condition of the membrane fluidity, therefore they used DPH anisotropy to directly measure membrane fluidity. They reported that ethanol tolerance is connected with higher membrane fluidity. However, their data showed that *S. cerevisiae* which had higher ethanol tolerance did not always show higher membrane fluidity compared to *Kloeckera apiculata* which had lower ethanol tolerance. For example, when the cells were grown in the presence of ethanol and further exposed to 12% (v/v) ethanol, *K. apiculata* showed a lower anisotropy value (0.133 ± 0.002) compared to *S. cerevisiae* (0.138 ± 0.003). Even though not statistically significant, there is a possibility which indicates that *K. apiculata* has higher membrane fluidity, while this yeast is more susceptible to high ethanol concentration. This finding is consistent with the results of this study and also with a hypothesis that lower intrinsic membrane fluidity helps to minimise the fluidising impact of high ethanol concentrations.

While Alexandre, Rousseaux & Charpentier (1994) used anisotropy of the fluorescent probe DPH, the present work uses a different method for determination of membrane fluidity. We used generalized polarization of laurdan for determining membrane fluidity. Parasassi *et al.* (1990) explained that laurdan is very sensitive to the polarity of the environment, and further investigation by the same group also revealed that the spectrum is red shifted with decreasing membrane fluidity (Parasassi *et al.* 1998). The different methods used by Alexandre, Rousseaux & Charpentier (1994) and the present experiment may lead to different results, although in general the studies report consistent trends.

Another thing to note is that the GP significantly increased from 6 to 24 hours of culture for all strains. These results indicate that membrane fluidity is changing during the cell growth, which means that membrane fluidity decreases

with time course. Specifically, membrane fluidity is relatively higher in respiro-fermentative cells which are also less tolerant to a range of stresses (Learmonth 2011; Learmonth & Gratton 2002; Lewis, Learmonth & Watson 1993; Lewis *et al.* 1993). As for the ethanol tolerance, it increases with time course. This result is in agreement with previous observations in which lower membrane fluidity led to better ethanol tolerance (Learmonth 2011). Similar phenomena were also observed by Swan & Watson (1999) and Steels *et al.* (1994) which suggested that ethanol tolerance is related to lower membrane fluidity.

Correlation analysis for all parameters tested suggests that there are strong correlations between culture time, viability reduction as assessed by TPC and GP. The correlations with culture time are essentially reporting the difference between respiro-fermentative (6 hour) and respiratory (24 hour) cells. It is well known that the former cells are less stress tolerant and have higher membrane fluidity (Learmonth 2011).

Poor correlation was detected for viability reduction as assessed by methylene violet staining with other parameters. However, as described earlier, viability reduction as assessed by methylene violet staining succeeded in distinguishing the ethanol tolerance of different yeast cells at a particular time point (6 hours or 24 hours of culture), which the TPC method failed to achieve. In contrast, viability reduction as assessed by TPC can distinguish ethanol tolerance of yeast cells from different time points of cultures, which methylene violet staining failed to distinguish. Therefore, each method has its own limitation and advantage, and these should be considered when using them as an ethanol tolerance determination method. Generally, from our observations, we could use methylene violet staining when we intended to discriminate ethanol tolerance of different yeast strains at a particular time point and the TPC method is better for

discriminating ethanol tolerance of a particular yeast strain at different time points.

The lack of correlation between viability assessment by methylene violet staining and total plate count is particularly troubling. It is likely that the high variability observed in the cell counts led to the lack of correlation and sampling techniques could be further refined. In addition, the methods report different findings, in that methylene violet staining may report live cells that have lost the ability to replicate; only cells able to divide will be counted after growth on plates. Additionally, the methylene violet approach was established to provide a more reliable assessment of viability than methylene blue staining, although it may suffer from a similar problem when used to assess the viability of highly stressed cell populations.

4.4 Conclusions

This study provides a comparison of the membrane fluidity and ethanol tolerance of different yeast strains. Three yeast strains were used in this experiment and it was found that they have different properties.

Unlike most previously published studies, the present study revealed that yeast strains with lower membrane fluidity have higher ethanol tolerance. This conclusion is supported by two data, i.e.:

1. At 6 hours of culture, the PDM strain which had the lowest GP value, i.e. highest membrane fluidity, showed the lowest ethanol tolerance, indicated by the highest viability reduction compared to the A12 and K7 strains.
2. Increasing GP values from 6 to 24 hours of culture, which indicated decreasing membrane fluidity, occurred along with significantly decreasing viability reduction values, which shows that ethanol tolerance increases with decreasing membrane fluidity.

Correlation analysis showed strong correlations between growth phase (respiro-fermentative, 6 hours, vs. respiratory, 24 hours), membrane fluidity and ethanol tolerance.

Even though viability reduction as assessed by methylene violet staining did not show any strong correlation with the other parameters measured, it could be useful in some circumstances. Therefore, we suggest that these two methods can be used for different purposes.

Finally, among the three strains used in the present experiment, A12 and K7 had better performance than PDM in terms of ethanol tolerance. Growth parameter comparison revealed that K7 had better properties compared to the other strains. Therefore, of the strains tested this strain has the best potential for further exploration.

CHAPTER FIVE: EFFECT OF PROLINE SUPPLEMENTATION ON ETHANOL TOLERANCE

5.1 Introduction

5.1.1 General Introduction

Proline has been widely documented to have a protective effect against various stress factors in many organisms (Chen & Dickman 2005; Graham & Wilkinson 1992; Takagi 2008; Verbuggen & Hermans 2008; Yamada *et al.* 2005). The exact mechanisms by which L-proline protects cells against stresses is not fully understood, but many published works indicate that L-proline is accumulated when cells are exposed to various stress factors, and that L-proline accumulation increases tolerance to stresses (Takagi 2008). Takagi (2008) outlined that L-proline has functions in osmoregulation, inhibition of dehydration and ice nucleation, lowering of nucleic acid Tm, and protecting cells from oxidative stress by acting as a scavenger for reactive oxygen species.

Yeasts with the ability to accumulate intracellular L-proline have been developed (by genetic modification of proline metabolic pathways) and tested against several stress factors. Enzymes which play important roles in proline metabolism were modified; either to suppress proline degradation (Takagi *et al.* 2000) or to promote proline synthesis (Morita, Nakamori & Takagi 2003; Takagi *et al.* 2005; Terao, Nakamori & Takagi 2003).

Proline accumulating yeast strains have been shown to have higher tolerance compared to parent strains, to freezing (Morita, Nakamori & Takagi 2003; Takagi *et al.* 2000), oxidative stress (Chen *et al.* 2006; Terao, Nakamori & Takagi 2003), and high ethanol concentrations (Takagi *et al.* 2005).

In the present study, we supplemented growth medium by L-proline, based on the findings of Horak & Kotyk (1986) and Lasko & Brandriss (1981) that *S.*

cerevisiae has the ability to utilize and accumulate proline. The yeast accumulate about 8 times more proline under aerobic conditions than under anaerobic conditions (Horak & Kotyk 1986). Therefore, we used increasing concentrations of L-proline under aerobic conditions to investigate whether extracellular L-proline may be assimilated and lead to protective effects against high ethanol concentrations.

5.1.2 Yeast strains and culture conditions

The yeast strains used were those investigated in Chapter 4, i.e. A12, PDM and K7.

5.1.3 Specific growth conditions and experimental design

For growth parameter analysis, batch culture experiments were set up and samples were aseptically removed from the culture by drawing off with a sterile pipette every 6 hours from 0 to 30 hours. Samples were then analysed for optical density at 600 nm (Section 2.4), viable cell count (section 2.5), percent cell viability (section 2.5), percent budding rate (section 2.6), and glucose and ethanol concentration (section 2.9).

Ethanol tolerance (section 2.8) and GP (section 2.7) were only measured at two time points, 6 and 24 hours, representing respiro-fermentative and respiratory phases of the cell growth, respectively. The medium used in this experiment was YNB with 2% (w/v) glucose, prepared as a 10× concentration stock solution and diluted as required prior to use. The stock solution was filtered through a sterile 0.22 µm pore size membrane. L-proline was supplemented by sterile addition of appropriate amounts of freshly prepared L-proline stock solution (50 g/L) to give the desired final concentrations of 0.1, 0.5, 1.0, 2.0, and 3.0 g/L. Media without proline supplementation were used as control media.

Where appropriate the experimental data were analysed for statistical significance using a one way ANOVA, with *post hoc* comparison using the Fisher LSD test to determine the specific differences (section 2.10). Differences were considered significant at the level of $P < 0.05$.

5.2 Results

No significant differences were found between the three strains for the growth parameters assessed in this experiment. Generally, glucose was exhausted after 18 hours of fermentation and the highest ethanol concentration was detected at 18-24 hours. Similar to results presented in Chapter 4, the only significant difference in growth parameters was for cell viability, where K7 maintained higher viability throughout fermentation, and after 12 hours, the viability of K7 was significantly higher than the other strains ($p < 0.05$). L-proline supplementation did not lead to any significant effect on the growth parameters determined in this experiment.

5.2.1 Generalized polarization in supplemented and unsupplemented cultures

As noted above, L-proline was supplemented to growth medium at 6 concentrations. Membrane fluidity was determined by measuring laurdan GP. Samples were taken at 6 and 24 hours of culture for GP measurement, representing respiro-fermentative and respiratory growth phases, respectively. .

Figure 5.1 presents GP at 6 hours of culture for yeast cells grown in L-proline supplemented and unsupplemented media. A significant difference due to L-proline supplementation was only detected for PDM ($p = 0.040$), and not for A12 ($p = 0.058$) or K7 ($p = 0.631$). The results of a Fisher LSD test for PDM GP values are presented in Figure 5.2. Significant differences are indicated by the

same letters above the bars. From the figure, it can be seen that supplementation with 0.5 g/L proline generally leads to significantly higher GP compared to other levels of supplementation. The finding of statistical significance seems surprising given the high standard deviation of this point, which overlaps the standard deviations of other data. The statistical analysis was re-checked thoroughly, confirming the significance, although the possibility exists that this is a statistical artifact rather than a meaningful biological phenomenon. Also, other supplementation levels did not show any significant differences compared to control. This result indicates that supplementation with 0.5 g/L L-proline may increase the GP of the yeast membranes, and it means that at this level of supplementation the membrane fluidity may decrease. Overall observation of GP at 6 hours of culture indicates that L-proline supplementation does not have any other significant effect on membrane fluidity.

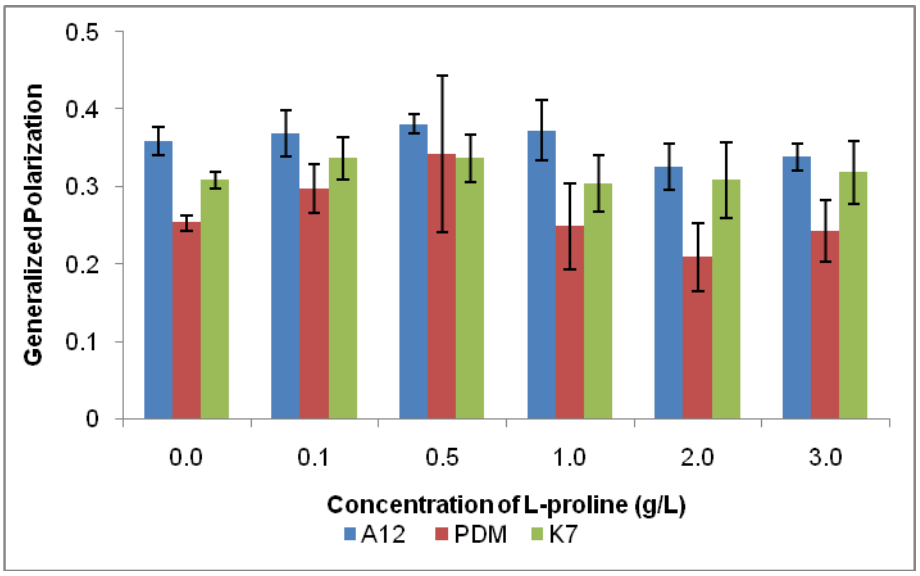


Figure 5.1 Generalized polarization of yeast strains supplemented with different levels of L-proline at 6 hours of culture. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviations.

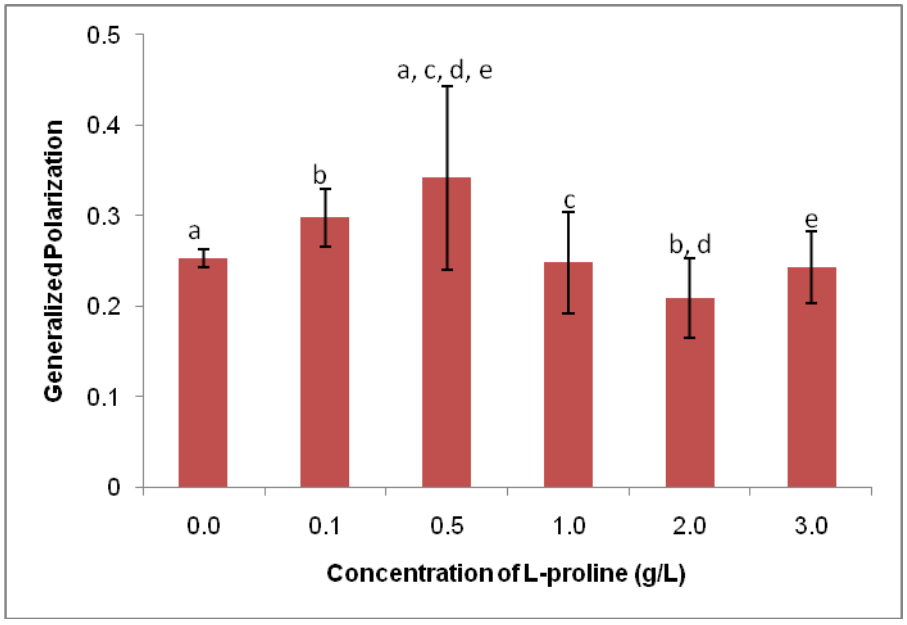


Figure 5.2 Generalized polarization of the PDM yeast strain at 6 hours of culture. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic condition at 30°C. Data presented on this graph are the same as Figure 5.1 for the PDM strain. Significant differences as revealed by the Fisher LSD test are indicated by the same letters above the bars. Data are the means of four independent experiments with. Error bars represent standard deviations.

Figure 5.3 presents the GP values of L-proline supplemented and unsupplemented cultures at 24 hours of culture. The GP was significantly higher at 24 hours than at 6 hours of culture ($p < 0.05$) for all supplemented and unsupplemented cultures. However, at 24 hours of culture no significant differences were found between L-proline supplemented and unsupplemented cells for any of the three stains tested. The GP changes observed are consistent with previous results, as described in Chapter 4, that GP increases in respiratory cells as compared to respiro-fermentative cells.

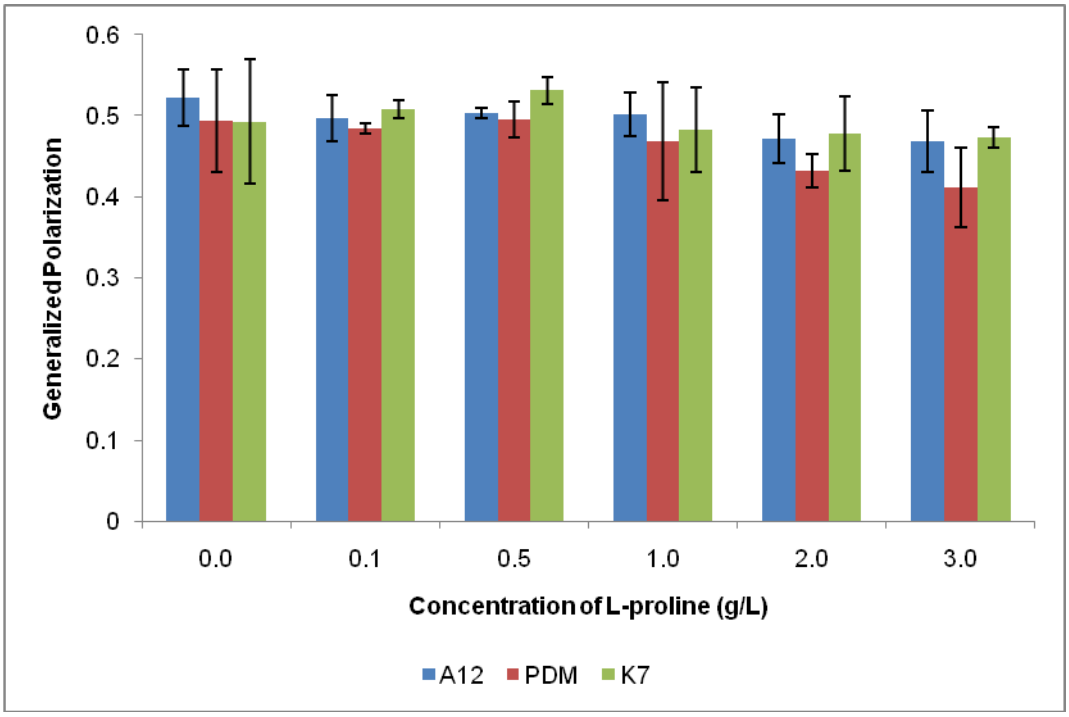


Figure 5.3 Generalized polarization of yeast strains supplemented with different levels of L-proline at 24 hours of culture. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviations.

5.2.2 Ethanol tolerance of yeast grown in supplemented and unsupplemented culture

Previous studies indicated that L-proline may have protective effects against some stress factors. The present study focused on ethanol tolerance of yeast grown in L-proline supplemented and unsupplemented cultures. As in Chapter 4 two methods were used to determine ethanol tolerance of the yeasts, namely total plate count (TPC) and methylene violet staining, after exposing the cells to 18% ethanol.

Figure 5.4 and 5.5 present viability reduction of yeast cultures, measured by TPC method at 6 and 24 hours, respectively. From the graphs presented, it can be noted that at 6 hours culture cells are very sensitive to high (18% v/v) ethanol levels, indicated by viability reduction by ~90%, in either the cells grown in L-proline supplemented or unsupplemented media.

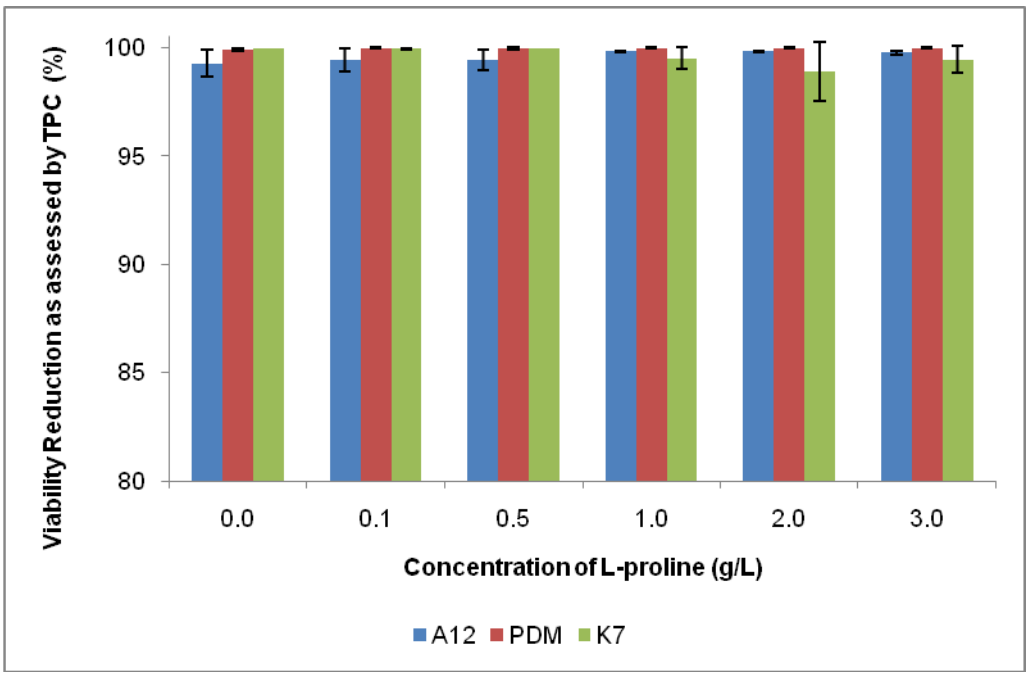


Figure 5.4 Viability reduction as determined by the total plate count method at 6 hours of culture L-proline-supplemented and -unsupplemented yeasts. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of two independent experiments. Error bars represent standard deviation.

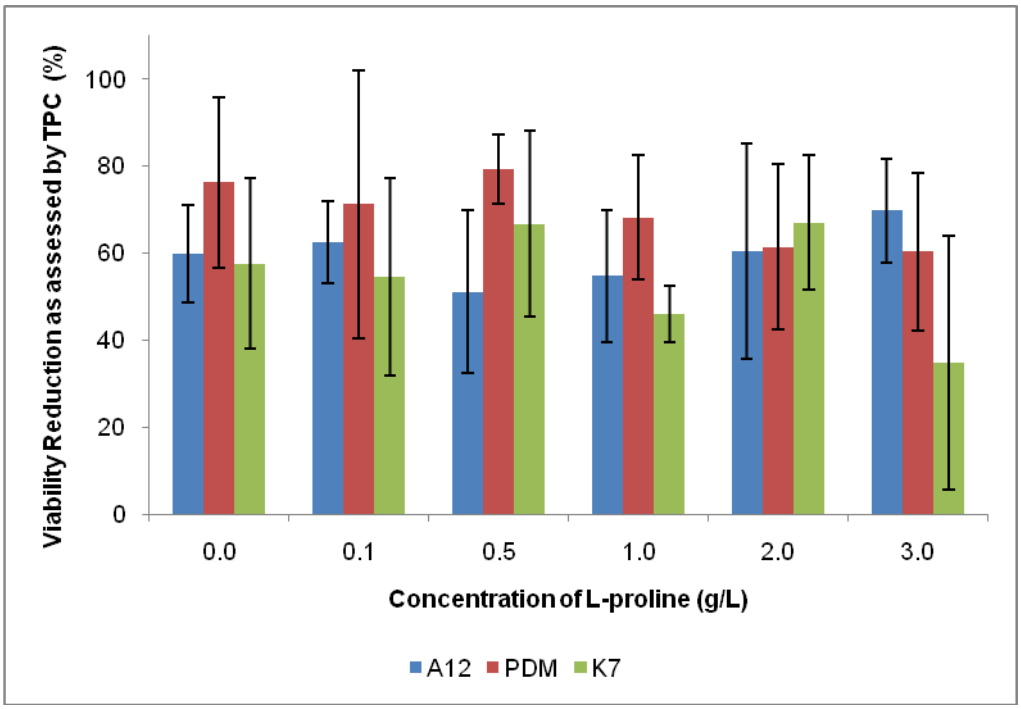


Figure 5.5 Viability reduction as determined by the total plate count method of 24 hours of culture for L-proline-supplemented and -unsupplemented yeasts. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of three independent experiments. Error bars represent standard deviation.

Compared to 6 hours of culture, the 24 hours of culture yeast generally had lower viability reduction values (i.e. greater tolerance to ethanol), even though the values are not significantly different for the three cultures at each time point.

Methylene violet staining was also used for determining viability reduction after exposing the cells to 18% ethanol. Viability reduction as determined by methylene violet staining is presented in Figures 5.6 and 5.7 for 6 and 24 hours of culture, respectively.

Methylene violet staining also did not indicate any effect of L-proline supplementation on ethanol tolerance either at 6 nor 24 hours of culture. Even though the viability reduction value did not differ significantly, generally viability reduction values at 24 hours of culture were lower than at 6 hours of culture, indicating greater tolerance to ethanol of respiratory phase cultures.

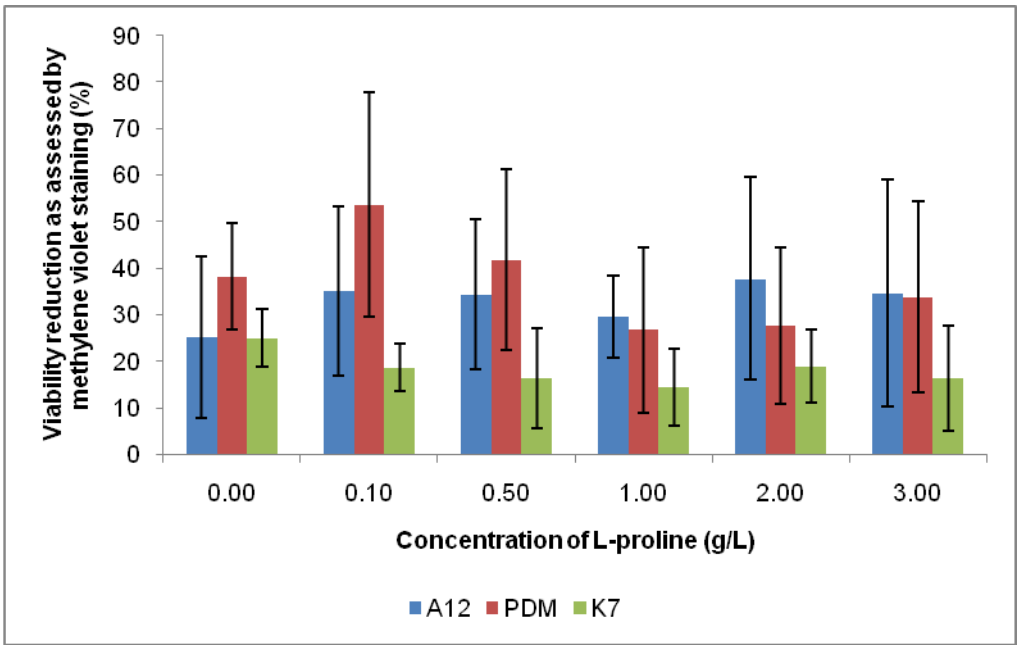


Figure 5.6 Viability reduction as determined by the methylene violet staining method at 6 hours of culture for L-proline-supplemented and -unsupplemented yeasts. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviation.

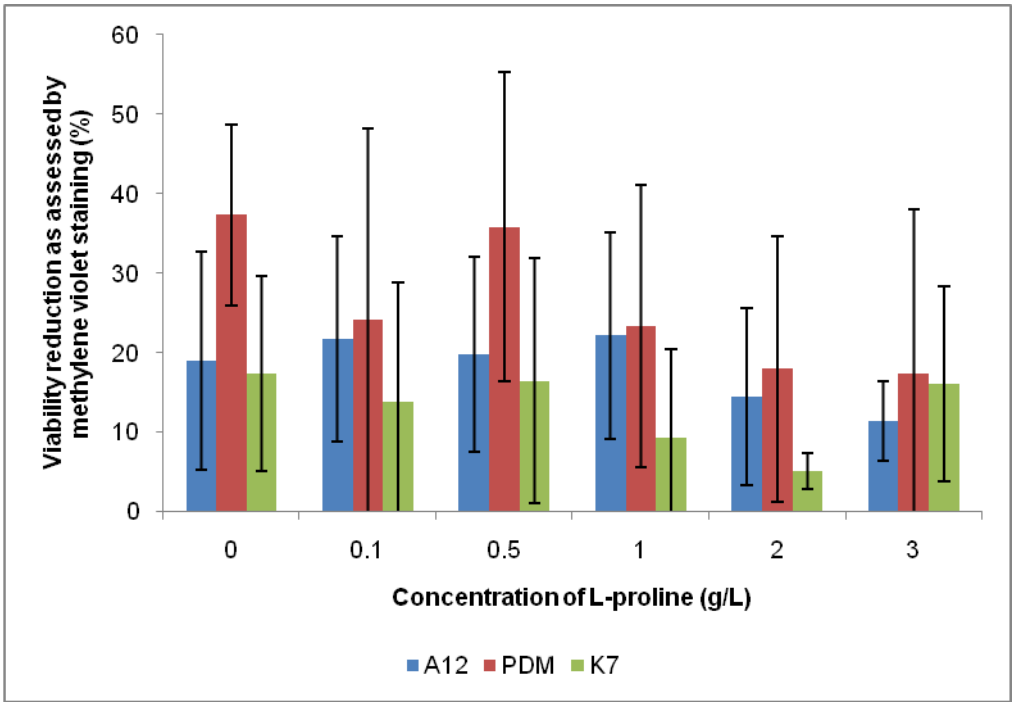


Figure 5.7 Viability reduction as determined by the methylene violet staining method at 24 hours of culture for L-proline-supplemented and -unsupplemented yeasts. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviation.

5.3 Discussion

5.3.1 Effect of L-proline supplementation on membrane fluidity

There is an indication that L-proline may have a role in membrane stabilization. Experiments conducted by Rudolph & Crowe (1985) and Rudolph, Crowe & Crowe (1986) found that L-proline can stabilize and preserve membranes upon freezing. Their results led to the suggestion that L-proline protects membranes by interacting with phosphatidylcholine, changing the physical properties of the membrane and broadening the transition temperature from gel to liquid crystalline phase (Rudolph, Crowe & Crowe 1986). Since there is a strong indication that L-proline interacts with and stabilizes membranes, we investigated whether L-proline supplementation affected membrane fluidity, by measuring GP of laurdan.

Our results indicated that L-proline supplementation did not have any strong effect in terms of changing the fluidity of yeast plasma membranes. The only significant difference was detected at 6 hours of culture for the PDM strain with 0.5 g/L L-proline supplementation, which showed a significantly higher GP value compared to other levels of supplementation ($p < 0.05$). Subject to the concerns raised above, this indicates that membrane fluidity for yeast supplemented with 0.5 g/L L-proline may be significantly lower than the other levels of supplementation. However, this phenomenon could not be related to ethanol tolerance; viability reduction as assessed by either TPC or methylene violet staining failed to identify any difference in ethanol tolerance associated with the lower membrane fluidity, as will be discussed later in this section.

Variation between GP values within the same treatment was very high as evidenced by the large standard deviations, which ranged from about 1.3% up to 29.5%. Thus, while it was conclusively demonstrated that membrane fluidity was lower in respiratory phase cells, no consistent differences in membrane fluidity resulted from L-proline supplementation in either respiro-fermentative or respiratory phase yeasts of the three strains tested.

5.3.2 Effect of L-proline supplementation on ethanol tolerance

Ethanol tolerance of the yeast cells was deduced from viability reduction values, as determined by either TPC or methylene violet staining methods. As expected from the Chapter 4 results, viability reduction as assessed by TPC at 6 hours of culture was very high (~99%) for both L-proline-supplemented and -unsupplemented cultures, which showed that the cells are very sensitive to a high ethanol concentration. However, methylene violet staining showed that some cells were still viable after the high ethanol stress, as indicated by the lower viability reduction compared to the TPC method. Viability reduction values as

assessed by methylene violet staining ranged from 53.7 ± 24.1% to 14.6 ± 8.2% for PDM grown in 0.1 g/L and K7 grown in 1 g/L L-proline supplemented media, respectively. However, even though the cells were not dead, they had lost the ability to divide, as indicated by low viability as assessed by the TPC method. Further study is required to investigate whether the viable cells detected by methylene violet staining are still capable of fermenting sugars. If so, they could still usefully contribute to the fermentation. Additionally, it could be determined whether the cells became non-viable (by methylene violet staining) during the incubation period of the agar plates or if there was an increase in clumpiness/floc.

Ethanol tolerance at 24 hours of culture was generally higher than at 6 hours of culture, even though the data did not indicate significant differences in viability reduction value as determined by either TPC or methylene violet staining. This result is in agreement with the results presented in Chapter 4, in which ethanol tolerance was increased in respiratory phase cells.

As at 6 hours of culture, at 24 hours of culture the cells did not show any significant differences in ethanol tolerance (as determined using either viability determination method) when the media were supplemented with various concentrations of L-proline. However, even though not statistically significant, the PDM strain tended to be the most susceptible to ethanol.

5.4 Conclusions

The present study failed to confirm the findings of previous studies that suggested that proline could enhance tolerance to stress (Takagi *et al.* 2005). However, it should be noted that the previous studies increased intracellular proline levels by genetic modification of cells, while our approach was to investigate whether a non-GMO approach of supplementation of media with L-proline could be a viable alternative strategy. However this study found that

supplementation with L-proline did not lead to any consistent effects in terms of the membrane fluidity or ethanol tolerance of the yeast strains investigated.

CHAPTER SIX: EFFECT OF INOSITOL SUPPLEMENTATION ON ETHANOL TOLERANCE

6.1 Introduction

6.1.1 General Introduction

Inositol has been documented to have protective effects against ethanol stress (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Ji *et al.* 2008; Krause *et al.* 2007). Supplementation of inositol was found to change phospholipid composition by increasing the phosphatidylinositol (PI) content of the plasma membrane, while phosphatidylcholine (PC) and phosphatidylethanolamine (PE) levels were decreased (Chi, Kohlwein & Paltauf 1999). Another study also revealed that inositol supplementation affects H^+-ATPase activity and protects yeast plasma membranes against leaking (Furukawa *et al.* 2004).

Mutant yeast strains capable of accumulating higher levels of intracellular inositol were also found to have better tolerance against high ethanol concentrations (Krause *et al.* 2007). Chi, Kohlwein & Paltauf (1999) found that inositol supplementation, not only increased tolerance to high ethanol concentrations, but also increased ethanol productivity. However, another study showed that while inositol supplementation improved viability in the presence of high ethanol concentrations, it did not affect the final ethanol production and fermentation rate (Furukawa *et al.* 2004).

Previous researchers used different concentrations of inositol in the growth media. Chi, Kohlwein & Paltauf (1999) used 0.1 g/L, Furukawa *et al.* (2004) used 10 mM (0.0018 g/L) and 90 mM (0.0162 g/L) and Krause *et al.* (2007) used 75 mM (0.0135 g/L) inositol.

Ji *et al.* (2008) investigated effect of inositol supplementation on a different yeast cell species, *Pachysolen tannophilus*, and used several inositol concentrations in their growth media. They found that inositol provided optimal effects at 0.15 g/L for cell growth and ethanol tolerance, and 0.1 g/L for ethanol productivity, while excessive inositol tended to have negative effects by lowering growth rate and ethanol productivity.

To the best of our knowledge, there are no published works reporting effects of more than two levels of inositol supplementation on cell health of *S. cerevisiae*. Therefore in the present study we investigated several levels of inositol supplementation to more closely investigate its effect on ethanol tolerance in *S. cerevisiae* strains.

As described previously (Chapter 1.4.2), inositol supplementation tends to increase PI and decrease PC and PE content of the yeast cell plasma membrane (Chi, Kohlwein & Paltauf 1999). Fatty acid compositions are different for each type of phospholipid component. The main fatty acid components varied from one study to another, but as a general picture, PI is mainly composed of C18:0 and C20:4, PE is mainly composed of C16:0, C18:0 and C20:4, PC is mainly composed of C16:0 and PS is mainly composed of C18:0 (Christie 2010). Therefore changes in phospholipid class composition might also change the membrane fluidity, since different degrees of fatty acid saturation will result. This led us to investigate the membrane fluidity in addition to the ethanol tolerance of yeasts grown in inositol supplemented media.

6.1.2 Yeast strain and culture condition

The yeast strains used were those investigated in Chapter 4, i.e. A12, PDM and K7.

6.1.3 Specific growth conditions and experimental design

For growth parameter analysis, batch culture experiments were set up, and samples were aseptically removed from the culture by drawing off with a sterile pipette every 6 hours from 0 to 30 hours. Samples were then analysed for optical density at 600 nm (Section 2.4), viable cell count (section 2.5), percent cell viability (section 2.5), percent budding rate (section 2.6), and glucose and ethanol concentrations (section 2.9).

Ethanol tolerance (section 2.8) and GP (section 2.7) were only measured at two time points, 6 and 24 hours, representing respiro-fermentative and respiratory phases of the cultures, respectively. The medium used in this experiment was YNB with 2% (w/v) glucose, prepared as a 10× concentration stock solution and diluted as required prior to use. The stock solution was filtered through a sterile 0.22 µm pore size membrane. Inositol was supplemented by adding an appropriate amount of freshly prepared, sterile inositol stock solution (3 g/L) to give the desired final inositol concentration of 0.005, 0.05, 0.1, 0.15, and 0.2 g/L. Media without inositol supplementation were used as control media. As the YNB medium formulation already contains 0.002 g/L inositol, the control media were not at zero, but at 0.002 g/L. Calculations for inositol supplementation considered the basal level in the media. Due to time and financial constraints it was not possible to construct or obtain an equivalent of YNB that lacked inositol.

Where appropriate the experimental data were analysed for statistical significance using a one way ANOVA, with *post hoc* comparison using the Fisher LSD test to determine the specific differences (section 2.10). Differences were considered significant different at the level of $p < 0.05$.

6.2 Results

Similar to the results presented in Chapters 4 and 5, no significant differences were found between the three strains for the growth parameters assessed in this experiment. Generally, glucose was exhausted after 18 hours of fermentation and the highest ethanol concentration was detected at 18-24 hours. Again, the only significant difference in growth parameters was for cell viability, where K7 maintained a high cell viability throughout the fermentation, and after 12 hours of culture, the cell viability of K7 was significantly higher than for the other two strains ($p < 0.05$). Also, similar to the findings in Chapter 5, inositol supplementation did not lead to any significant change in the growth parameters assessed in this experiment.

6.2.1 Effect of inositol supplementation on Generalized Polarization

It is important to note that YNB medium used in the present experiment contains 0.002 g/L inositol. Therefore, the basal (unsupplemented) medium already contains 0.002 g/L inositol. Figure 6.1 shows the GP value of yeast strains grown in inositol-supplemented or -unsupplemented media at 6 hours of culture. No significant difference were detected in this data, but a possible trend can be observed at 0.05 g/L inositol-supplementation for PDM which resulted in a higher GP value compared to other levels of inositol supplementation. This difference was not statistically significant due to the relatively high variability in these readings.

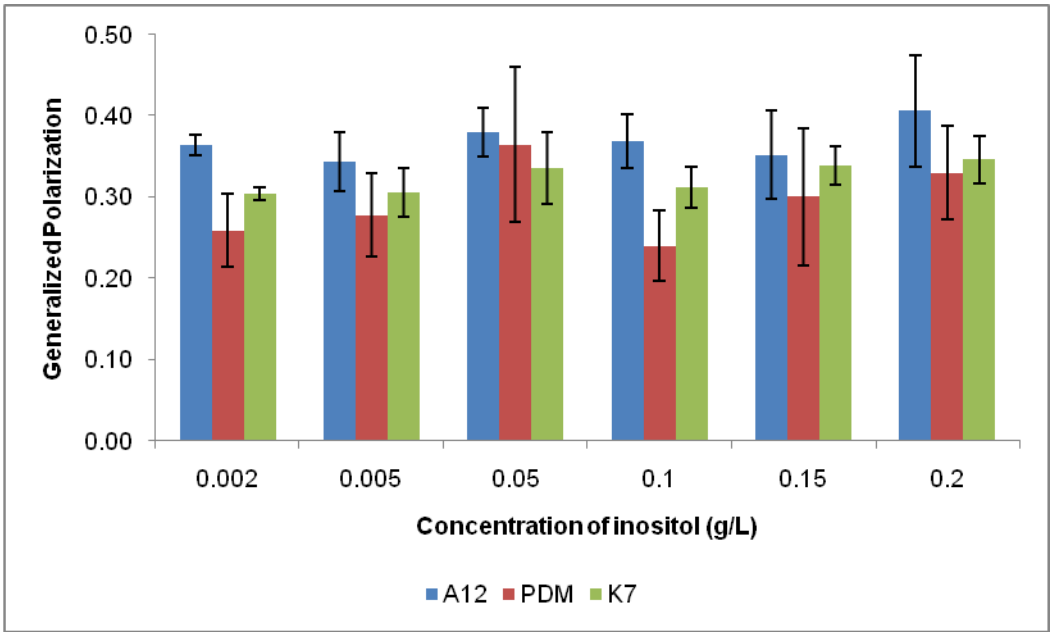


Figure 6.1 Generalized polarization of yeast strains grown in inositol-supplemented and -unsupplemented media at 6 hours of culture. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviations.

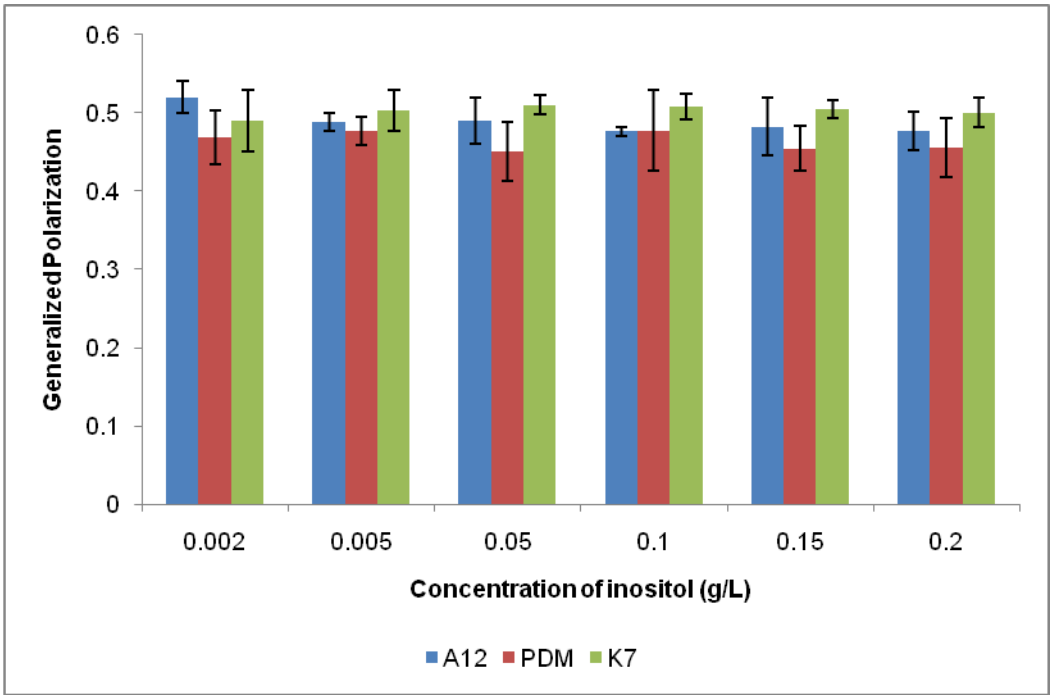


Figure 6.2 Generalized polarization of yeast strains grown in inositol-supplemented and -unsupplemented media at 24 hours of culture. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviations.

Generalized polarization values for 24 hours of cultures are presented in Figure 6.2. From this figure, it can be seen that there were also no significant differences between the generalized polarisation values for yeast strain with and without inositol supplementation at this time point. However, as expected and consistent with the results in the previous chapters, the GP values for 24 hours of culture were significantly higher than at 6 hours of culture, indicating lower membrane fluidity in respiratory phase cells.

6.2.2 Effect of inositol supplementation on ethanol tolerance

Figure 6.3 presents viability reduction induced by ethanol stress as determined by the TPC method. Similar results to those described in Chapter 5 were seen. Viability reduction was very high at 6 hours of culture indicating that respiro-fermentative cells are very sensitive to high concentrations of ethanol. No significant differences in viability reduction were observed for yeast strains with and without inositol supplementation at this time point.

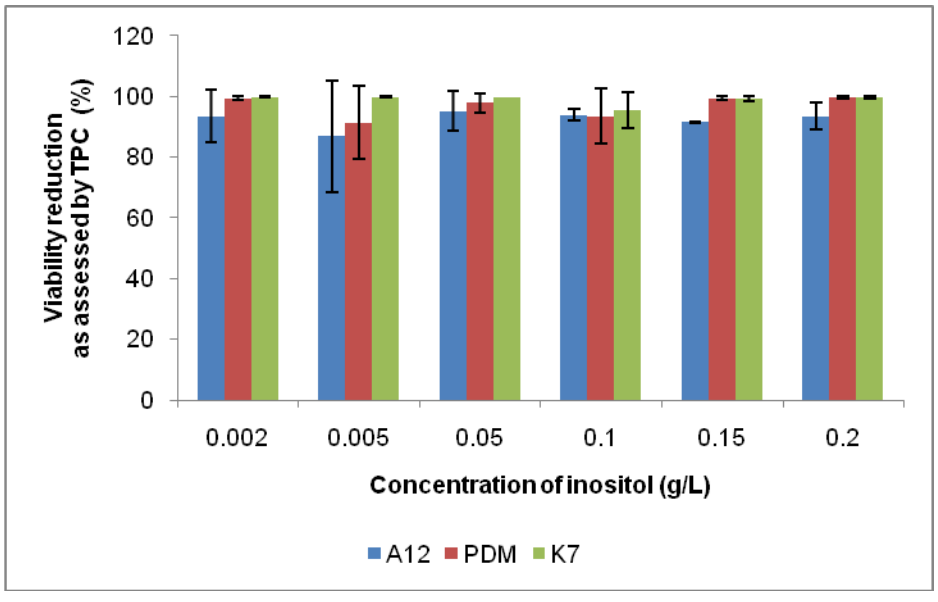


Figure 6.3 Viability reduction of yeast cells grown in inositol-supplemented and -unsupplemented media at 6 hours of culture. Viability reduction was determined by the TPC method after exposing yeast cells to 18% v/v ethanol. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of two independent experiments. Error bars represent standard deviations.

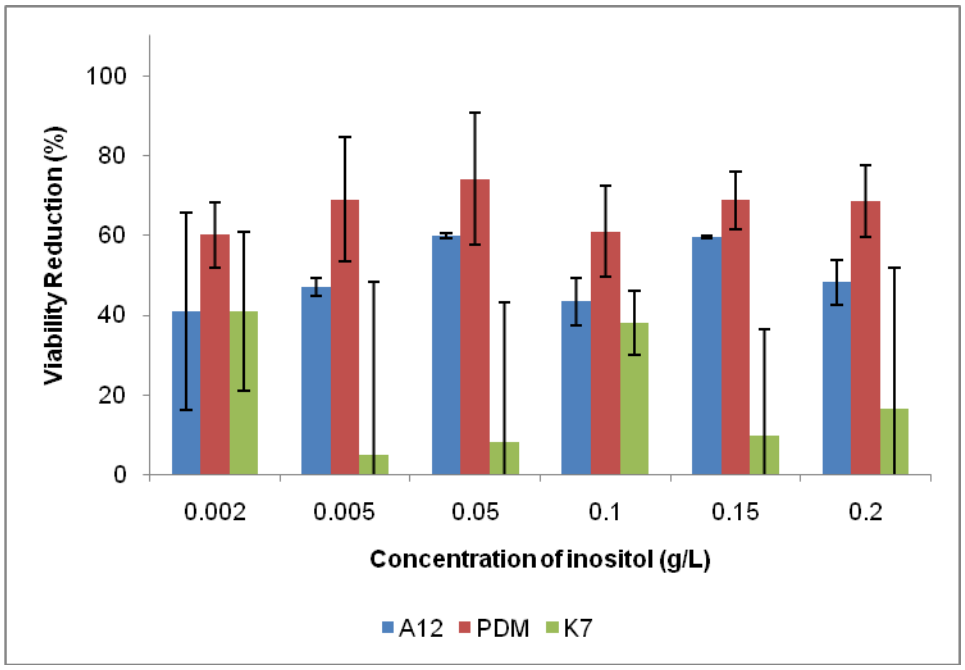


Figure 6.4 Viability reduction of yeast cells grown in inositol-supplemented and -unsupplemented media at 24 hours of culture. Viability reduction was determined by the TPC method after exposing yeast cells to 18% v/v ethanol. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of three independent experiments. Error bars represent standard deviations.

Viability reduction of 24 hours culture as determined by the TPC method was generally lower than at 6 hours culture, as expected (Figure 6.4). Again, no significant differences were detected between the cultures with or without inositol supplementation at this time point.

Furthermore, methylene violet staining did not show any significant differences in viability reduction between the cultures with or without inositol supplementation, either at 6 or 24 hours of culture (Figure 6.5 and Figure 6.6). However, like the TPC method, methylene violet staining also showed a decrease at viability reduction at 24 hours of culture, which indicates that ethanol tolerance is increased in respiratory phase cells. It should be noted that very high variability between replicate samples was observed for this data.

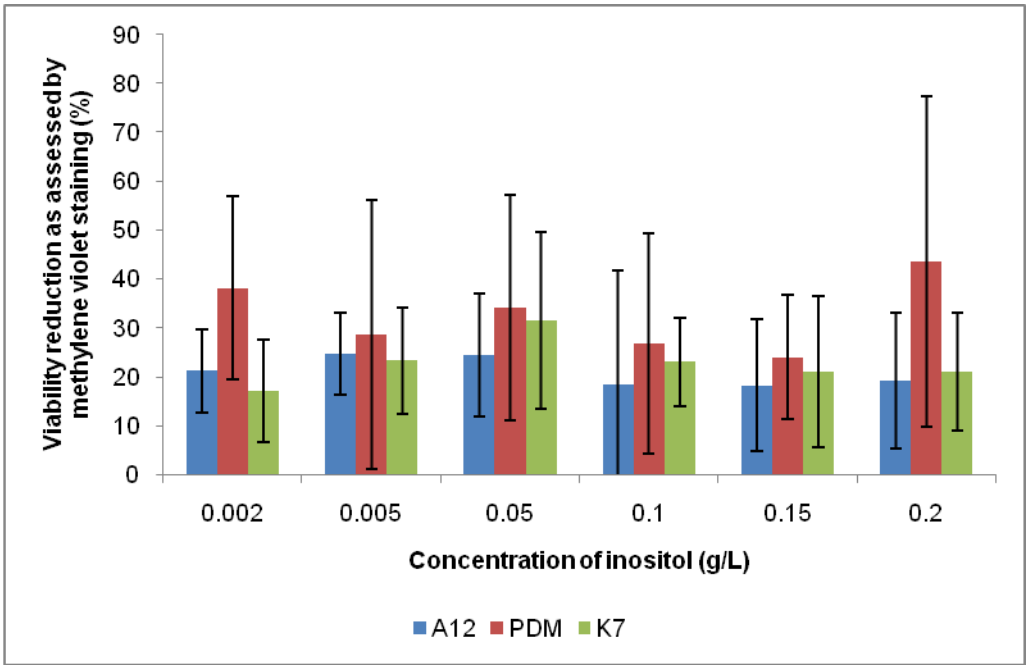


Figure 6.5 Viability reduction of yeast cells grown in inositol-supplemented or -unsupplemented media at 6 hours of culture. Viability reduction was determined by the methylene violet staining method after exposing yeast cells to 18% v/v ethanol. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviations.

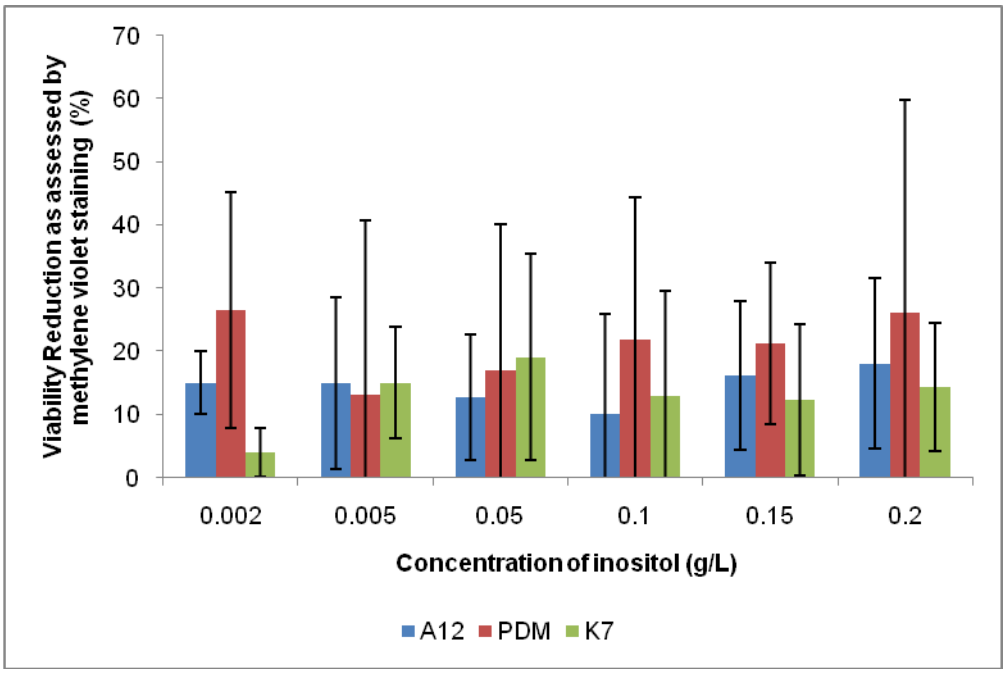


Figure 6.6 Viability reduction of yeast cells grown in inositol -supplemented and -unsupplemented media at 24 hours of culture. Viability reduction was determined by the methylene violet staining method after exposing yeast cells to 18% v/v ethanol. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviations.

6.3 Discussion

Generalized polarization data did not show any significant effects of inositol supplementation on membrane fluidity. Only slightly higher GP was detected and this was only for the PDM strain at 6 hours when supplemented with 0.05 g/L inositol, possibly indicating lower membrane fluidity compared to the other levels of supplementation. This result requires further study since a previous study (Chi, Kohlwein & Paltauf 1999) indicated that phospholipid composition was altered following inositol addition, which could lead to a changed plasma membrane fluidity. However, in the present study, in which fluidity was determined by a biophysical technique, any factors counterbalancing compositional changes would be taken into account as part of the measurement. Therefore, further study is required to investigate changes of phospholipid composition due to inositol supplementation and its relationship to membrane fluidity.

There were no significant differences in GP between yeast grown in inositol-supplemented and –unsupplemented media observed at 24 hours of culture. However, GP was increased significantly ($p < 0.05$) compared to 6 hours of culture, indicating significantly lower membrane fluidity. Similar results were also observed in Chapters 4 and 5. As in previous experiments, decreasing membrane fluidity was accompanied by increasing ethanol tolerance for all strains, independent of the level of inositol supplementation. This result supports previous study (Swan & Watson 1999) which indicated that ethanol tolerance is related to lower plasma membrane fluidity.

As in previous chapters (4 and 5) and other studies, respiro-fermentative cells, as expected, were more sensitive to ethanol.

No significant difference in viability reduction, either as measured by TPC or methylene violet staining, was detected between yeast grown in inositol-supplemented and -unsupplemented media. High variability between replicates of

viability measurements were again observed for TPC and methylene violet staining. Therefore, exploration of other methods for ethanol tolerance determination is required to achieve better results.

6.4 Conclusions

The present study could not confirm the results of previous studies which indicated that inositol supplementation and accumulation improved ethanol tolerance of yeast cells. Even though not significant, 0.05 g/L inositol might slightly change the membrane fluidity of the PDM strain. Further study is required to test this result. As in previous chapters of this thesis, PDM was found to be the yeast strain most susceptible to ethanol compared to A12 and K7.

CHAPTER SEVEN: GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 Discussion

Three different yeast strains were used in the present study, *Saccharomyces cerevisiae* strains A12, PDM and K7. The results of this study revealed that these three strains, besides having slightly different growth parameters, had different intrinsic membrane fluidity and sensitivity to high ethanol concentrations. The present study also indicated a slightly different finding compared to previous published studies regarding the relationship between membrane fluidity and ethanol tolerance.

Growth parameter analysis of these three yeast strains indicated that K7 has a longer respiro-fermentative growth phase but higher total and viable cell numbers during the respiratory phase of the fermentation process. The cell viability of K7 was maintained at above 90% throughout the fermentation. These features make K7 the best of the strains studied in terms of potential for industrial high sugar fermentation, which requires cell survival throughout the entire fermentation process.

Generalized polarization of the three yeast strains used in this experiment showed that PDM has the highest membrane fluidity, followed by K7 and A12 has the lowest. Previous studies indicated that yeast cells with higher membrane fluidity are more tolerant to high ethanol concentrations (Alexandre, Berlot & Charpentier 1994; Alexandre, Rousseaux & Charpentier 1994; Kajiwara *et al.* 2000). However, our results indicate the opposite. In respiro-fermentative cultures, PDM, which has the highest membrane fluidity, was also found to be the most susceptible to ethanol, with no significant difference between A12 and K7. Therefore, our results lead us to report that, unlike some previous studies

(Alexandre, Berlot & Charpentier 1994; Alexandre, Rousseaux & Charpentier 1994; Kajiwara *et al.* 2000; You, Rosenfield & Knipple 2003) but similar to other studies (Learmonth 2011; Learmonth & Gratton 2002), higher ethanol tolerance is related to lower intrinsic membrane fluidity.

Previous studies used different approaches for determining membrane fluidity. Determination of the unsaturation index by measuring the fatty acid composition of the yeast plasma membrane has been widely used for indirect estimation of membrane fluidity (Kajiwara *et al.* 2000; Rodrıguez-Vargas *et al.* 2007; Swan & Watson 1999; You, Rosenfield & Knipple 2003). These inferences do not take into account a large number of factors that may modulate membrane fluidity (Butcher 2008; Learmonth 2011). Direct measurement by measuring anisotropy of DPH was also used to determine the relationship between membrane fluidity and ethanol tolerance (Alexandre, Rousseaux & Charpentier 1994). All studies generally reported that ethanol tolerance is related to higher membrane fluidity. To the best of our knowledge, apart from studies in this laboratory (Butcher 2008; Learmonth 2011; Learmonth & Gratton 2002), only one publication by Swan & Watson (1999) reported that yeast cells with higher membrane fluidity have lower ethanol tolerance, and these authors estimated the membrane fluidity by reporting the unsaturation index.

Our approach to membrane fluidity measurement was different in that we used generalized polarization of laurdan. We also used two different methods for ethanol tolerance determination; total plate count and methylene violet staining methods. TPC is well known and widely used as a standard method for determining yeast viability after exposing yeast cells to a particular stress factor (Chi, Kohlwein & Paltauf 1999; Swan & Watson 1999). The methylene violet staining method is known as an established method for discriminating viable and nonviable cells (Smart *et al.* 1999).

The present study indicates that even though TPC and methylene violet staining do not give results that have strong correlation, they tend to have different applications for ethanol tolerance testing. The TPC method was useful in discriminating differences in yeast viability at different time points, i.e. different yeast growth phases, but failed to discriminate differences in yeast viability of different yeast strains at the same time point. On the other hand, the methylene violet staining method was useful in discriminating the differences in viability of different yeast strains at the same time point, but failed to discriminate differences in the viability of the same strain at different time points. Therefore, we suggest that these two methods can be used for different purposes, depending on what is required. However, further studies are required to work out why these techniques did not, as expected, provide similar results. One major difference between the techniques is that methylene violet staining may identify live cells that have lost the ability to divide or grow and would therefore not be detected by plate counts.

The aim of the present study was to improve ethanol tolerance by supplementing growth media with L-proline and/or inositol, based on published reports that indicated protective effects against various stress factors. L-proline has been reported to protect cells against freezing (Sekine *et al.* 2007; Takagi *et al.* 2000; Terao, Nakamori & Takagi 2003) and high ethanol concentrations (Takagi *et al.* 2005). However, most of the published studies discussed the effect of intracellular L-proline content on improving stress tolerance of the yeast cell. Intracellular L-proline content was increased by means of genetic engineering of L-proline metabolic pathways, either by disrupting L-proline degrading enzymes (Takagi *et al.* 2000) or enhancing L-proline formation (Morita, Nakamori & Takagi 2003; Takagi *et al.* 2005; Terao, Nakamori & Takagi 2003).

Furthermore, there are no reports of extracellular L-proline supplementation affecting yeast cell survival after exposure to various stress factors. It has been shown that the yeast *S. cerevisiae* has the ability to assimilate L-proline from the culture medium, more so under aerobic conditions (Horak & Kotyk 1986). There were also some indications that excessive amounts of L-proline may have lethal effects on yeast (Takagi 2008). Therefore, the present study aimed to investigate the effect of extracellular L-proline supplementation and also to determine the optimum concentration of L-proline to have a positive effect on ethanol tolerance.

L-proline effects on maintaining membrane stability have been reported previously (Rudolph & Crowe 1985; Rudolph, Crowe & Crowe 1986; Takagi 2008). Those studies indicated that L-proline may affect the physical properties of the membrane. However, in this study GP measurement of yeasts grown in L-proline-supplemented and -unsupplemented media did not indicate any general change in membrane fluidity. Only one significant difference was detected, at 0.5 g/L L-proline supplementation of the PDM strain which had a higher GP value than other levels of supplementation. This indicates that for this strain L-proline supplementation at 0.5 g/L may lower membrane fluidity, and according to previous results, lower membrane fluidity should lead to better ethanol tolerance. However, viability reduction determination failed to show any effect of L-proline supplementation on ethanol tolerance.

In the present study, L-proline supplementation did not have any significant effect on improving yeast tolerance to high ethanol concentrations. Further investigation is required to confirm whether L-proline supplementation does change the plasma membrane fluidity and affect the ethanol tolerance property of the yeast cell.

Inositol was the second supplement used in the present experiments. Previous reports indicated that inositol supplementation may change plasma

membrane phospholipid composition (Chi, Kohlwein & Paltauf 1999) and improve the ethanol tolerance of the yeast cells (Chi, Kohlwein & Paltauf 1999; Furukawa *et al.* 2004; Krause *et al.* 2007). However, excessive inositol was found to have negative effects on cell growth and ethanol production (Ji *et al.* 2008). Therefore, in the present study one aim was to investigate the effect of different levels of inositol supplementation on yeast tolerance to ethanol and to determine the optimum level of inositol supplementation.

We found that inositol supplementation did not induce significant differences in membrane fluidity in the yeast strains studied. However, our experiment did not determine phospholipid or fatty acid composition, and while we can confirm that inositol supplementation did not affect the measured fluidity, we cannot fully determine relationships between inositol supplementation, phospholipid content and membrane fluidity. Further study to investigate this aspect is required to fully understand yeast adaptation mechanisms, especially in relation to inositol supplementation.

Ethanol tolerance of yeast cells was reportedly improved when the yeast were grown in inositol-supplemented media (Chi, Kohlwein & Paltauf 1999; Krause *et al.* 2007). Our results indicate that none of the inositol supplementation levels enhanced ethanol tolerance of the yeast cells. No significant differences were detected between yeast grown in inositol-supplemented and -unsupplemented media. Therefore, our results could not confirm the results of previous published studies that reported that inositol has a positive effect on enhancing ethanol the tolerance of yeast cells.

One aim of the present study was that once effective concentrations of L-proline and inositol were determined, then the effect of co-supplementation would be investigated. However, given that no consistent effects were seen for either supplement, co-supplementation experiments were not warranted. Also, due to

the lack of discernable effects of supplementation and also due to time constraints it was not possible to scale back to industrial sugar concentrations.

The yeasts studied were selected as “non-traditional” alternative strains with potential for industrial bioethanol fermentation. The overall results indicated that of the strains studied, the wine yeast (PDM) was the most susceptible when exposed to high ethanol concentration either in media with no supplementation or media additionally supplemented with L-proline or inositol. The susceptibility of this strain was most likely associated with the higher membrane fluidity compared to the other strains. Furthermore, of the strains studied, the sake strain (K7) appeared to be the most promising, mainly due to its maintenance of higher viability throughout the fermentation.

7.2 Conclusions and Contributions of This Study

The data acquired in this study led us to conclude some important points. Compared to A12 and K7, PDM tended to have the highest membrane fluidity and was the most susceptible to ethanol. Therefore, we conclude, unlike some previous published studies, that higher ethanol tolerance is related to lower intrinsic membrane fluidity.

This conclusion is also supported by comparison of membrane fluidity and ethanol tolerance data from respiro-fermentative (6 hour of culture) and respiratory (24 hour of culture) cells which indicated that respiratory yeasts had lower membrane fluidity and higher ethanol tolerance. Correlation analysis also showed that correlation between the viability measured by total plate count and methylene violet staining was poor, and warrants further study. However, individual interpretation of TPC and methylene violet staining indicate that these two methods can be applied for different purposes. The TPC method seemed better for discriminating viability of different growth phases, while methylene violet

staining seemed better for discriminating cell viability of different yeast strains at a particular time point.

Although not statistically significant, supplementation with 0.5 g/L L-proline seems to have a positive effect on changing membrane fluidity. Further study to test this result is required. As for the inositol supplementation, the present study could not confirm the results of previous published work which indicated that inositol supplementation has a positive effect on enhancing ethanol tolerance of the yeast cell.

Among the three strains used in the present study, K7 showed better performance compared to the other two strains. Although the ethanol tolerance of K7 was slightly lower than that of A12, this strain showed the best cell viability throughout the fermentation, which is an important property for fermentation of media with high sugar concentration. Therefore, K7 is recommended for further exploration.

7.3 Future Directions

Enhancing ethanol tolerance of yeasts is very important in order to achieve better ethanol productivity for bioethanol production. Higher ethanol production will lead to lower costs for ethanol separation, and eventually this will improve the economy of bioethanol production. Fundamental aspects of ethanol tolerance also need to be further explored in order to understand the underlying mechanisms, with a view to manipulation of these mechanisms to and enhance ethanol productivity. Therefore, from the present study, there are some potential approaches for further research:

1. *Investigation of the relationships between membrane fluidity, plasma membrane composition, and yeast cell susceptibility to various stress factors.*

Although it is widely accepted that high membrane fluidity is related to high unsaturation index, direct measurement of membrane fluidity may reveal exceptions to this rule as described by Alexandre, Rousseaux & Charpentier (1994), Butcher (2008) and Learmonth (2011). Therefore, all three parameters need to be measured when investigating relationships between membrane fluidity and ethanol tolerance. The present study also found that ethanol tolerance is related to lower membrane fluidity, a finding different to that of some other previous studies. Therefore, full elucidation of plasma membrane phospholipid and fatty acid composition is required to fully understand the relationships between plasma membrane composition, membrane fluidity and yeast tolerance to various stress factors.

2. *Supplementation with potential stress protectants and/or genetic modification of yeast metabolic pathways to improve accumulation of stress protectants and the effect on plasma membrane composition and membrane fluidity*

Many published studies have used genetic engineering to improve yeast tolerance to various stress factors. Supplementation with various stress protectants such as unsaturated fatty acids, inositol, metal ions etc. are also widely reported to improve tolerance to stress. However, only few of these studies investigated the effect of the various treatments on membrane fluidity, although the plasma membrane is the first cellular barrier to various stress factors. Therefore, investigation of the effect of the various treatments on stress tolerance and including plasma membrane fluidity measurements are essential to fully understand yeast tolerance to the various stress factors.

In our experiments, we failed to observe any change in ethanol tolerance following L-proline or inositol supplementation. However, our study did not assess potential changes in plasma membrane composition due to supplementation. Therefore, further studies are planned to investigate changes in plasma membrane composition and fluidity after supplementation and/or genetic modification. A further aim is to correlate these parameters to ethanol tolerance data, to further elucidate the stress tolerance mechanisms.

3. Fermentation of high sugar concentration medium

High sugar concentrations are preferred in industrial processes, since more ethanol can be produced. However, when high sugar concentrations are used, yeast strains with high survival rate throughout the fermentation are required in order to get the sugars fully converted. The present study found that K7 maintained high cell viability throughout the fermentation, and therefore this strain has potential for fermentation of high sugar concentration media. However, when high sugar concentrations are used, the yeast cells are exposed to high osmolarity stress. Therefore, further study to investigate the osmotolerance of K7 is required.

4. Investigation of viability after exposure to high ethanol concentrations

An interesting observation made during the present study was that when cells of respiro-fermentative phase were exposed to high ethanol concentrations, methylene violet staining indicated that not all cells were dead. However, total plate counts did not show any colonies, indicating the cells were nonviable or had lost the ability to divide or grow. Further study is required to investigate whether the viable cells detected by methylene violet staining may maintain their viability and are still able to ferment sugars. If the cells can still ferment sugar, but cannot grow, there might be potential for increasing ethanol productivity, since the sugar will not be taken up by the yeast cells and used for growth.

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Apppendix 1.1 Recipes for media

Yeast Extract Peptone Medium (YEP)

- 0.5% Yeast extract (w/v)
- 0.5% Bacteriological peptone (w/v)
- 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

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

- 6.7 g Bacto yeast nitrogen base
- 20 g D-glucose
- 100 mL Milli-Q water

Filter sterilize using a 0.22 µm membrane filter into a sterile Schott bottle. Store at 4°C until required.

Appendix 1.2 Formulae for proline and inositol supplementation

Proline Supplementation

Media	Final Proline Concentration (g/L)	YNB2G* (mL)	MiliQ Water (mL)	Proline Stock** (mL)	Final Volume (mL)
A	0	5	45	0	50
B	0.1	5	44.9	0.1	50
C	0.5	5	44.5	0.5	50
D	1.0	5	44	1	50
E	2.0	5	43	2	50
F	3.0	5	42	3	50

*Yeast nitrogen base medium with 2% glucose

**Proline stock = 50 g/L = 0.5 g / 10 mL

Inositol Supplementation

Media	Final Inositol Concentration (g/L)	YNB2G[#] (mL)	MiliQ Water (mL)	Inositol Stock[##] (mL)	Final Volume (mL)
G	0	5	45	0	50
H	0.005	5	44.95	0.05	50
I	0.05	5	44.2	0.8	50
J	0.1	5	43.365	1.635	50
K	0.15	5	42.535	2.465	50
L	0.2	5	41.7	3.3	50

[#] Yeast nitrogen base medium with 2% glucose

[##]Inositol Stock = 3 mg/mL = 30 mg/10 mL = 0.030 g / 10 mL

Inositol concentration in YNB = 0.002 g/L

For 100 mL media:

Inositol from YNB (g)	Additional Inositol (g)	Total Inositol (g)	Total Volume (mL)	Final Concentration	Volume of Inositol stock added (mL)
0.0002	0.0003	0.0005	100	0.005 g / 1000 mL	0.1
0.0002	0.0048	0.0050	100	0.05 g / 1000 mL	1.6
0.0002	0.0098	0.0100	100	0.10 g / 1000 mL	3.27
0.0002	0.0148	0.0150	100	0.15 g / 1000 mL	4.93
0.0002	0.0198	0.0200	100	0.20 g / 1000 mL	6.6

Appendix 2.1 Raw data for batch culture growth of different yeast strains. Cultures were grown under aerobic condition in YNB media with 16% glucose at 30°C and 180 opm.

OD_{600nm}

Strain / Time (hours)	PDM	A12	A14
0	0.668	0.436	0.426
4	1.456	1.098	1.266
8	3.474	3.156	3.230
24	5.080	4.572	4.272
30	5.858	4.664	5.060
48	5.832	4.630	4.928
72	5.842	4.564	4.742
96	6.098	4.420	4.818
120	6.438	4.772	4.954
144	6.218	4.746	4.824
168	5.930	4.138	4.868

Total Cell ($\times 10^6$ cell/mL)

Strain / Time (hours)	PDM	A12	A14
0	8.190	3.680	6.540
4	37.700	27.100	43.500
8	84.700	53.300	57.200
24	100.800	58.800	72.000
30	97.900	69.000	95.300
48	86.100	55.500	63.700
72	103.800	72.500	74.800
96	129.900	45.900	99.400
120	117.400	60.200	106.600
144	99.300	66.100	90.600
168	107.000	68.200	81.600

Viable Cell (× 10^6 cell/mL)

Strain / Time (hours)	PDM	A12	A14
0	6.640	3.275	5.930
4	34.800	25.700	42.400
8	73.000	47.900	53.500
24	66.800	46.600	62.400
30	60.900	44.600	86.800
48	22.900	10.600	51.700
72	8.500	1.900	32.000
96	6.600	0.600	18.800
120	3.900	0.700	11.000
144	0.600	0.100	2.100
168	0.400	0.100	1.400

Cell Viability (%)

Strain / Time (hours)	PDM	A12	A14
0	81.13	87.90	90.63
4	92.24	94.90	97.52
8	86.30	89.96	93.42
24	66.36	79.46	86.66
30	61.86	64.64	91.16
48	26.68	18.94	81.06
72	8.14	2.74	42.86
96	5.22	1.22	18.84
120	2.46	0.80	10.28
144	0.60	0.14	2.30
168	0.36	0.16	1.70

Sugar Concentration (% w/v)

Strain / Time (hours)	PDM	A12	A14
0	14.28	15.93	16.01
4	16.04	14.66	14.30
8	13.92	13.24	11.92
24	10.43	10.79	10.79
30	9.18	10.44	10.09
48	7.97	9.85	8.94
72	6.54	9.86	6.23
96	6.52	9.52	5.37
120	6.76	9.61	4.92
144	5.13	9.91	5.21
168	6.90	7.74	5.98

Appendix 2.2 Raw data for batch culture growth of A12 strains in different media. Cultures were grown under aerobic condition in various media with 16% glucose at 30°C and 180 opm.

OD_{600nm}

Strain / Time (hours)	YEP16S	YEP16G	YNB16G	YNBAS2G	YNB2G	YNBAS16S
0	0.326	0.392	0.450	0.322	0.390	0.436
4	0.998	1.076	1.422	1.524	1.524	1.098
8	4.188	3.514	3.392	3.768	3.408	3.156
24	6.188	4.972	4.774	3.952	3.276	4.572
30	6.730	6.210	4.954	4.006	3.290	4.664
48	6.900	9.354	4.968	4.148	3.550	4.630
72	7.186	11.062	4.762	3.890	3.756	4.564
96	7.542	12.104	5.050	4.044	3.642	4.420
120	8.644	12.166	5.052	3.692	3.394	4.772
144	9.728	13.280	5.192	3.842	3.514	4.746
168	10.202	14.208	5.102	4.080	3.618	4.138

Total Cell ($\times 10^6$ cell/mL)

Strain / Time (hours)	YEP16S	YEP16G	YNB16G	YNBAS2G	YNB2G	YNBAS16S
0	6.53	0.41	8.17	1.12	13.34	2.27
4	18.56	3.74	24.19	2.91	49.70	7.51
8	94.90	9.02	69.70	4.96	79.00	12.60
24	68.70	8.82	64.50	4.51	78.40	5.79
30	78.50	6.71	79.90	9.93	68.10	6.88
48	90.70	5.53	146.60	12.86	104.10	3.75
72	73.10	3.99	126.20	10.07	87.90	5.65
96	88.40	3.19	145.10	17.22	80.40	10.45
120	108.20	4.72	142.00	3.00	89.50	4.92
144	106.20	3.63	200.00	16.96	87.90	3.60
168	122.80	10.80	182.40	25.95	88.10	7.82

Viable Cell (× 10^6 cell/mL)

Strain / Time (hours)	YEP16S	YEP16G	YNB16G	YNBAS2G	YNB2G	YNBAS16S
0	5.63	0.28	6.06	1.13	10.69	1.81
4	17.25	3.54	21.57	3.07	44.80	7.23
8	93.90	8.93	66.60	4.87	66.60	10.25
24	38.50	9.66	27.50	1.77	49.40	2.25
30	35.40	2.97	32.80	7.43	35.80	4.54
48	15.10	1.64	42.40	7.93	23.40	2.48
72	6.40	2.10	1.61	0.25	3.60	1.14
96	6.00	1.58	7.80	1.44	0.40	0.55
120	26.40	5.16	10.00	2.92	0.00	0.00
144	15.40	3.97	12.00	6.67	0.30	0.27
168	7.60	3.51	9.60	3.65	0.10	0.22

Cell Viability (%)

Strain / Time (hours)	YEP16S	YEP16G	YNB16G	YNBAS2G	YNB2G	YNBAS16S
0	86.36	3.13	73.82	5.46	80.10	0.87
4	92.94	3.88	89.00	3.20	90.02	1.43
8	98.92	0.40	95.72	1.69	84.40	2.51
24	55.40	6.72	42.66	1.32	63.16	3.25
30	45.14	2.04	39.88	5.60	52.46	1.41
48	16.66	1.32	28.74	3.68	22.48	2.44
72	8.84	3.15	12.74	1.72	4.18	1.55
96	6.78	1.84	5.36	0.77	0.46	0.64
120	24.34	4.18	7.04	2.07	0.00	0.00
144	14.48	3.75	5.88	2.98	0.36	0.33
168	6.08	2.75	5.12	1.29	0.12	0.27

Sugar Concentration (% w/v)

Strain / Time (hours)	YEP16S	YEP16G	YNBAS16G	YNBAS16S	YNB16S	YNBAS2G	YNB2G
0	14.13	17.29	17.11	15.93	15.40	1.21	1.23
4	13.18	16.72	16.82	14.66	12.37	0.91	0.94
8	12.40	14.33	14.84	13.24	12.21	0.05	0.17
24	9.25	11.86	11.89	10.79	10.02	0.01	0.01
30	7.99	10.19	11.24	10.44	11.21	0.01	0.01
48	6.38	6.54	9.82	9.85	9.54	0.01	0.01
72	6.53	3.20	9.05	9.86	10.17	0.01	0.01
96	5.56	2.16	9.03	9.52	10.07	0.01	0.01
120	4.37	1.80	9.70	9.61	ND*	0.01	0.02
144	1.62	0.84	9.70	9.91	ND*	0.02	0.02
168	0.52	0.28	10.16	7.74	ND*	0.02	0.02

*ND: Not Determined

Legend:

YEP16S = Yeast extract peptone with 16% sucrose
YEP16G = Yeast extract peptone with 16% glucose
YNB16G = Yeast Nitrogen Base with 16% glucose
YNBAS2G = Yeast nitrogen base with 2% glucose and 1% additional ammonium sulphate
YNB2G = Yeast nitrogen base with 2% glucose
YNBAS16S = Yeast nitrogen base with 16% sucrose and 1% additional ammonium sulphate
YNBAS16G = Yeast nitrogen base with 16% glucose and 1% additional ammonium sulphate
YNB16S = Yeast nitrogen base with 16% glucose

Appendix 2.3 Raw data for batch culture growth. Cultures were grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four independent experiments.

$OD_{600\ nm}$

Strain / Time (hours)	A12	PDM	K7
0	0.120 ± 0.033	0.107 ± 0.017	0.107 ± 0.010
6	1.031 ± 0.345	0.677 ± 0.197	0.490 ± 0.095
12	3.266 ± 0.073	3.378 ± 0.437	2.881 ± 0.345
18	3.305 ± 0.057	3.826 ± 0.234	4.344 ± 0.450
24	3.315 ± 0.076	3.924 ± 0.079	4.587 ± 0.150
30	3.365 ± 0.187	3.954 ± 0.150	4.551 ± 0.311

Total Cell (× 10^6 cell/mL)

Strain / Time (hours)	A12	PDM	K7
0	1.178 ± 0.186	1.355 ± 0.243	1.005 ± 0.060
6	17.768 ± 7.379	12.875 ± 2.843	8.351 ± 1.555
12	50.998 ± 20.429	68.750 ± 8.471	44.100 ± 3.265
18	63.600 ± 27.710	79.425 ± 11.796	55.650 ± 13.728
24	71.450 ± 43.376	77.050 ± 7.728	60.500 ± 16.140
30	70.175 ± 29.774	81.100 ± 13.851	70.575 ± 14.842

Viable Cell (× 10^6 cell/mL)

Strain / Time (hours)	A12	PDM	K7
0	0.908 ± 0.219	1.048 ± 0.174	0.920 ± 0.076
6	17.155 ± 7.194	12.328 ± 2.270	8.021 ± 1.472
12	39.861 ± 18.510	55.875 ± 8.168	41.700 ± 3.661
18	49.400 ± 24.352	63.200 ± 11.650	52.325 ± 13.575
24	55.275 ± 37.508	58.275 ± 4.652	56.550 ± 15.435
30	52.925 ± 23.386	62.325 ± 13.271	64.825 ± 14.533

Cell Viability (%)

Strain / Time (hours)	A12	PDM	K7
0	76.47 ± 9.43	77.50 ± 6.47	91.26 ± 2.22
6	96.30 ± 1.33	95.72 ± 0.41	96.15 ± 1.51
12	76.91 ± 4.61	81.06 ± 3.33	94.49 ± 4.05
18	76.65 ± 4.90	79.25 ± 3.00	93.82 ± 2.77
24	75.43 ± 6.83	75.71 ± 2.93	93.36 ± 2.00
30	75.11 ± 4.95	76.54 ± 3.31	91.61 ± 2.61

Budding Rate (%)

Strain / Time (hours)	A12	PDM	K7
0	44.67 ± 19.60	36.68 ± 6.79	22.60 ± 4.90
6	26.26 ± 3.22	22.07 ± 4.14	19.71 ± 1.80
12	30.71 ± 3.07	34.51 ± 7.44	25.84 ± 3.52
18	32.32 ± 5.72	35.72 ± 4.09	19.49 ± 8.11
24	31.50 ± 5.08	37.94 ± 5.33	21.91 ± 8.73
30	33.36 ± 4.26	32.50 ± 3.07	25.16 ±10.12

Glucose (% w/v)

Strain / Time (hours)	A12	PDM	K7
0	1.47 ± 0.39	1.50 ± 0.34	1.51 ± 0.41
6	1.02 ± 0.33	1.20 ± 0.32	1.56 ± 0.27
12	0.12 ± 0.07	0.32 ± 0.14	0.58 ± 0.14
18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
30	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Ethanol (% v/v)

Strain / Time (hours)	A12	PDM	K7
0	0.06 ± 0.13	0.13 ± 0.15	-0.03 ± 0.06
6	0.24 ± 0.17	0.18 ± 0.19	0.04 ± 0.13
12	0.52 ± 0.24	0.59 ± 0.08	0.45 ± 0.22
18	0.65 ± 0.14	0.75 ± 0.24	0.64 ± 0.22
24	0.53 ± 0.08	0.58 ± 0.22	0.60 ± 0.16
30	0.39 ± 0.11	0.45 ± 0.23	0.52 ± 0.23

Appendix 2.4 Raw data for batch culture growth of strain A12 supplemented with proline. Cultures were grown u
medium with 2% glucose at 30°C and 180 opm. Data represents means of two independent experiments. Media
Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2 g/L Proline; F = YNB + 3 g/L Proline

OD_{600nm}

Media / Time (hours)	A	B	C	D	E	F
0	0.122 ± 0.054	0.121 ± 0.044	0.117 ± 0.041	0.103 ± 0.001	0.110 ± 0.028	0.106 ± 0.01
6	1.061 ± 0.576	0.971 ± 0.400	1.055 ± 0.706	1.294 ± 0.025	1.396 ± 0.223	1.340 ± 0.12
12	3.244 ± 0.107	3.381 ± 0.361	3.648 ± 0.294	3.561 ± 0.420	4.039 ± 0.307	4.257 ± 0.28
18	3.299 ± 0.081	3.501 ± 0.380	3.547 ± 0.100	3.636 ± 0.322	4.148 ± 0.204	4.059 ± 0.64
24	3.334 ± 0.102	3.454 ± 0.240	3.401 ± 0.083	3.481 ± 0.298	4.116 ± 0.102	3.983 ± 0.00
30	3.436 ± 0.272	3.504 ± 0.283	3.581 ± 0.018	3.447 ± 0.247	4.208 ± 0.003	4.156 ± 0.20

Total Cell (× 10^6 Cell/mL)

Media / Time (hours)	A	B	C	D	E
0	1.203 ± 0.053	1.315 ± 0.049	1.248 ± 0.004	1.005 ± 0.071	1.233 ± 0.039
6	18.592 ± 12.671	19.376 ± 7.852	22.952 ± 10.805	24.168 ± 7.116	30.512 ± 11.155

Viable Cell (× 10^6 Cell/mL)

Media / Time (hours)	A	B	C	D	E
0	0.843 ± 0.117	0.923 ± 0.152	0.900 ± 0.042	0.778 ± 0.110	0.923 ± 0.110
6	17.880 ± 12.366	18.824 ± 7.863	22.392 ± 10.510	23.462 ± 6.842	29.658 ± 10.807
12	51.650 ± 21.142	60.650 ± 16.334	53.450 ± 26.092	71.400 ± 46.952	83.550 ± 40.234
18	61.850 ± 34.012	50.350 ± 16.193	48.900 ± 19.799	71.650 ± 40.800	85.700 ± 40.447
24	76.950 ± 48.295	54.950 ± 18.314	59.800 ± 16.122	63.600 ± 22.345	90.200 ± 13.294
30	68.500 ± 25.456	72.300 ± 22.627	65.300 ± 18.385	60.500 ± 19.799	83.350 ± 21.850

Cell Viability (%)

Media / Time (hours)	A	B	C	D	E
0	69.804 ± 6.650	69.941 ± 8.991	72.680 ± 2.629	76.707 ± 6.767	74.369 ± 6.765
6	95.569 ± 1.426	96.882 ± 1.310	97.508 ± 0.099	97.179 ± 0.105	97.273 ± 0.105
12	80.439 ± 3.399	81.148 ± 0.508	79.006 ± 1.020	82.067 ± 2.404	87.064 ± 2.404

Budding Rate (%)

Media / Time (hours)	A	B	C	D	E
0	47.811 ± 21.048	63.622 ± 0.064	57.610 ± 7.499	37.771 ± 6.571	59.095 ± 3.277
6	23.956 ± 0.189	26.600 ± 2.760	26.378 ± 1.419	27.866 ± 0.802	28.929 ± 1.456
12	33.261 ± 0.864	29.877 ± 3.185	26.688 ± 0.134	21.671 ± 3.738	21.811 ± 1.018
18	27.494 ± 1.365	28.764 ± 0.671	25.978 ± 3.313	21.339 ± 6.368	25.339 ± 5.777
24	27.256 ± 2.223	26.971 ± 1.253	28.973 ± 0.071	22.056 ± 4.609	22.081 ± 1.960
30	29.936 ± 0.780	29.816 ± 1.022	23.587 ± 3.071	24.561 ± 6.246	21.165 ± 0.797

Glucose (% w/v)

Media / Time (hours)	A	B	C	D	E
0	1.29 ± 0.47	1.00 ± 0.42	1.10 ± 0.76	0.86 ± 0.56	1.33 ± 0.66
6	0.91 ± 0.39	0.77 ± 0.37	0.87 ± 0.50	0.66 ± 0.45	0.74 ± 0.60
12	0.07 ± 0.04	0.07 ± 0.07	0.06 ± 0.08	0.00 ± 0.00	0.00 ± 0.00
18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Ethanol (% v/v)

Media / Time (hours)	A	B	C	D	E
0	0.08 ± 0.18	0.84 ± 0.44	0.20 ± 0.05	0.03 ± 0.00	0.00 ± 0.01
6	0.21 ± 0.26	0.34 ± 0.26	0.09 ± 0.15	0.10 ± 0.11	0.13 ± 0.11
12	0.47 ± 0.35	0.58 ± 0.17	0.59 ± 0.13	0.49 ± 0.15	0.80 ± 0.23
18	0.65 ± 0.15	0.80 ± 0.15	0.83 ± 0.06	0.98 ± 0.06	0.71 ± 0.17
24	0.48 ± 0.05	0.43 ± 0.08	0.34 ± 0.21	0.28 ± 0.13	0.43 ± 0.04
30	0.33 ± 0.13	0.43 ± 0.13	0.60 ± 0.07	0.58 ± 012	0.41 ± 0.33

Proline (g/L)

Media / Time (hours)	A	B	C	D	E
0	0.034 ± 0.000	0.072 ± 0.011	0.306 ± 0.183	0.474 ± 0.283	1.430 ± 0.611
6	0.034 ± 0.000	0.089 ± 0.045	0.319 ± 0.199	0.512 ± 0.334	1.195 ± 0.777
12	0.034 ± 0.000	0.095 ± 0.035	0.382 ± 0.089	0.700 ± 0.116	1.736 ± 0.111

Appendix 2.5 Raw data for batch culture growth of strain PDM supplemented with proline. Cultures were grown u
medium with 2% glucose at 30°C and 180 opm. Data represents means of two independent experiments. Media
Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2 g/L Proline; F = YNB + 3 g/L Proline

OD_{600nm}

Media / Time (hours)	A	B	C	D	E	F
0	0.113 ± 0.027	0.145 ± 0.030	0.118 ± 0.031	0.124 ± 0.011	0.113 ± 0.030	0.121 ± 0.06
6	0.771 ± 0.245	0.797 ± 0.259	0.707 ± 0.165	0.971 ± 0.386	1.055 ± 0.412	0.928 ± 0.46
12	3.753 ± 0.092	3.986 ± 0.133	3.957 ± 0.004	4.506 ± 0.255	4.753 ± 0.486	5.088 ± 0.15
18	3.928 ± 0.263	4.089 ± 0.174	4.394 ± 0.218	4.703 ± 0.236	4.945 ± 0.058	5.055 ± 0.19
24	3.954 ± 0.093	4.028 ± 0.246	4.406 ± 0.025	4.358 ± 0.404	4.864 ± 0.351	4.908 ± 0.12
30	4.027 ± 0.106	4.207 ± 0.024	4.412 ± 0.096	4.726 ± 0.173	5.019 ± 0.188	5.090 ± 0.13

Total Cell (× 10^6 Cell/mL)

Media / Time (hours)	A	B	C	D	E
0	1.538 ± 0.209	1.443 ± 0.067	1.608 ± 0.187	1.440 ± 0.191	1.505 ± 0.212
6	14.413 ± 3.164	14.696 ± 5.555	13.997 ± 0.811	20.704 ± 9.322	22.392 ± 11.732

Viable Cell (× 10^6 Cell/mL)

Media / Time (hours)	A	B	C	D	E
0	1.198 ± 0.011	1.023 ± 0.081	1.180 ± 0.085	1.110 ± 0.007	1.220 ± 0.134
6	13.832 ± 2.976	13.992 ± 5.374	13.605 ± 0.777	17.400 ± 5.306	18.944 ± 7.377
12	54.050 ± 13.081	66.900 ± 9.334	72.300 ± 23.617	74.400 ± 23.759	90.900 ± 0.707
18	68.750 ± 10.960	72.200 ± 0.990	79.850 ± 11.950	72.300 ± 17.112	80.750 ± 17.041
24	59.750 ± 2.051	65.500 ± 3.394	82.700 ± 17.112	88.400 ± 2.828	95.400 ± 20.506
30	70.050 ± 16.758	58.900 ± 4.667	63.100 ± 7.354	76.000 ± 9.617	86.750 ± 32.456

Cell Viability (%)

Media / Time (hours)	A	B	C	D	E
0	78.52 ± 10.09	70.99 ± 9.26	74.31 ± 13.99	77.90 ± 9.60	81.30 ± 2.62
6	96.02 ± 0.37	95.08 ± 0.53	97.16 ± 0.21	96.60 ± 0.64	96.96 ± 0.64
12	81.19 ± 5.34	85.45 ± 1.04	87.17 ± 2.54	86.84 ± 4.42	89.38 ± 4.42
18	80.50 ± 2.65	80.52 ± 1.65	81.31 ± 0.88	83.58 ± 4.64	86.02 ± 4.64
24	76.66 ± 4.58	79.63 ± 2.85	81.33 ± 3.20	81.17 ± 4.36	84.93 ± 4.36

Budding Rate (%)

Media / Time (hours)	A	B	C	D	E
0	35.79 ± 11.62	46.32 ± 16.31	47.27 ± 12.08	34.78 ± 4.09	38.29 ± 5.14
6	20.94 ± 2.85	23.59 ± 7.96	21.49 ± 5.93	26.86 ± 1.59	24.57 ± 1.26
12	36.42 ± 10.66	34.91 ± 9.74	35.25 ± 10.21	33.24 ± 3.60	31.93 ± 4.17
18	38.41 ± 3.52	35.64 ± 2.57	35.28 ± 0.68	33.58 ± 1.86	28.19 ± 2.03
24	36.62 ± 3.21	34.69 ± 3.24	31.72 ± 0.48	30.23 ± 8.49	27.33 ± 9.30
30	34.82 ± 0.50	29.37 ± 3.06	29.59 ± 0.06	32.47 ± 2.88	26.82 ± 2.37

Glucose (% w/v)

Media / Time (hours)	A	B	C	D	E
0	1.67 ± 0.28	1.51 ± 0.24	1.63 ± 0.07	1.53 ± 0.15	1.55 ± 0.09
6	1.26 ± 0.22	1.31 ± 0.03	1.29 ± 0.06	1.14 ± 0.20	1.13 ± 0.15
12	0.24 ± 0.11	0.21 ± 0.14	0.26 ± 0.05	0.08 ± 0.12	0.08 ± 0.11
18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Ethanol (% v/v)

Media / Time (hours)	A	B	C	D	E
0	0.16 ± 0.08	0.54 ± 0.37	0.22 ± 0.02	0.15 ± 0.02	0.21 ± 0.07
6	0.17 ± 0.13	0.32 ± 0.00	0.15 ± 0.17	0.35 ± 0.35	0.26 ± 0.12
12	0.61 ± 0.04	0.58 ± 0.08	0.72 ± 0.07	0.79 ± 0.01	0.71 ± 0.14
18	0.84 ± 0.19	0.80 ± 0.19	0.92 ± 0.28	0.67 ± 0.13	0.73 ± 0.26
24	0.60 ± 0.32	0.57 ± 0.03	0.51 ± 0.17	0.56 ± 0.26	0.62 ± 0.41
30	0.46 ± 0.49	0.55 ± 0.38	0.61 ± 0.38	0.49 ± 0.32	0.46 ± 0.26

Proline (g/L)

Media / Time (hours)	A	B	C	D	E
0	0.034 ± 0.000	0.131 ± 0.034	0.425 ± 0.023	0.790 ± 0.059	1.558 ± 0.046
6	0.034 ± 0.000	0.141 ± 0.042	0.425 ± 0.047	0.727 ± 0.105	1.415 ± 0.102
12	0.034 ± 0.000	0.102 ± 0.023	0.333 ± 0.025	0.733 ± 0.040	1.380 ± 0.087
18	0.034 ± 0.000	0.098 ± 0.032	0.317 ± 0.007	0.588 ± 0.042	1.294 ± 0.074

Appendix 2.6 Raw data for batch culture growth of strain K7 supplemented with proline. Cultures were grown u
medium with 2% glucose at 30°C and 180 opm. Data represents means of two independent experiments. Media
Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2 g/L Proline; F = YNB + 3 g/L Proline

OD_{600nm}

Media / Time (hours)	A	B	C	D	E	F
0	0.108 ± 0.017	0.094 ± 0.017	0.106 ± 0.006	0.105 ± 0.007	0.113 ± 0.007	0.103 ± 0.00
6	0.458 ± 0.150	0.463 ± 0.117	0.537 ± 0.081	0.747 ± 0.180	0.710 ± 0.192	0.745 ± 0.18
12	3.022 ± 0.501	3.226 ± 0.368	3.336 ± 0.300	3.267 ± 0.049	3.616 ± 0.130	4.437 ± 0.70
18	4.058 ± 0.526	4.088 ± 0.308	4.502 ± 0.221	4.863 ± 0.324	5.170 ± 0.628	5.500 ± 0.88
24	4.517 ± 0.182	4.152 ± 0.201	5.015 ± 0.856	5.202 ± 0.724	5.666 ± 0.973	5.114 ± 0.41
30	4.301 ± 0.095	4.672 ± 0.911	5.054 ± 1.355	5.503 ± 1.184	5.379 ± 0.921	6.389 ± 1.94

Total Cell (× 10^6 Cell/mL)

Media / Time (hours)	A	B	C	D	E
0	1.033 ± 0.074	1.185 ± 0.424	1.030 ± 0.276	1.050 ± 0.269	1.345 ± 0.417
6	7.795 ± 2.123	8.760 ± 1.392	10.552 ± 6.490	14.205 ± 6.490	12.341 ± 6.351

Viable Cell (× 10^6 Cell/mL)

Media / Time (hours)	A	B	C	D	E
0	0.960 ± 0.092	1.120 ± 0.375	0.983 ± 0.244	0.990 ± 0.255	1.168 ± 0.265
6	7.499 ± 2.112	8.120 ± 1.633	9.872 ± 0.226	12.480 ± 4.480	10.925 ± 4.718
12	40.600 ± 0.283	44.650 ± 2.785	47.450 ± 14.071	53.150 ± 2.051	59.750 ± 14.071
18	59.200 ± 18.102	55.500 ± 14.708	61.800 ± 21.496	72.700 ± 1.980	73.100 ± 16.263
24	63.800 ± 22.203	63.900 ± 18.809	53.200 ± 12.445	76.850 ± 11.526	72.450 ± 0.495
30	74.200 ± 14.849	64.650 ± 17.041	62.600 ± 13.567	81.100 ± 32.668	76.600 ± 31.369

Cell Viability (%)

Media / Time (hours)	A	B	C	D	E
0	92.62 ± 2.61	95.02 ± 2.35	96.13 ± 1.77	94.49 ± 0.64	87.86 ± 7.19
6	96.11 ± 1.04	92.38 ± 3.86	93.79 ± 4.07	97.14 ± 0.36	97.79 ± 1.48
12	96.52 ± 1.85	96.34 ± 1.95	97.03 ± 0.11	97.89 ± 1.07	96.73 ± 0.06
18	94.96 ± 0.20	96.37 ± 0.33	96.51 ± 0.17	96.36 ± 0.70	97.13 ± 0.80

Budding Rate (%)

Media / Time (hours)	A	B	C	D	E
0	18.71 ± 0.43	23.62 ± 1.49	23.34 ± 5.16	21.34 ± 9.93	20.89 ± 2.68
6	19.82 ± 3.09	19.77 ± 3.49	23.67 ± 8.59	20.26 ± 4.18	20.43 ± 4.25
12	25.31 ± 3.89	22.22 ± 12.64	20.97 ± 12.58	18.53 ± 8.31	19.37 ± 13.19
18	23.06 ± 7.18	21.03 ± 14.81	17.01 ± 10.85	13.71 ± 9.46	15.37 ± 12.76
24	24.37 ± 12.95	25.54 ± 23.55	21.96 ± 20.82	20.03 ± 14.57	20.01 ± 14.55
30	28.14 ± 15.66	23.55 ± 17.97	23.87 ± 16.34	20.59 ± 15.87	18.12 ± 15.70

Glucose (% w/v)

Media / Time (hours)	A	B	C	D	E
0	1.37 ± 0.50	1.32 ± 0.50	1.33 ± 0.09	1.50 ± 0.17	1.66 ± 0.15
6	1.35 ± 0.20	1.14 ± 0.40	1.24 ± 0.28	1.30 ± 0.16	1.38 ± 0.13
12	0.46 ± 0.02	0.42 ± 0.01	0.39 ± 0.00	0.36 ± 0.02	0.34 ± 0.04
18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Ethanol (% v/v)

Media / Time (hours)	A	B	C	D	E
0	0.02 ± 0.05	0.07 ± 0.06	0.19 ± 0.14	0.10 ± 0.04	0.09 ± 0.01
6	0.01 ± 0.14	0.30 ± 0.35	0.02 ± 0.00	0.05 ± 0.03	0.04 ± 0.06
12	0.53 ± 0.30	0.39 ± 0.10	0.41 ± 0.08	0.51 ± 0.18	0.77 ± 0.51
18	0.55 ± 0.08	0.56 ± 0.05	0.77 ± 0.27	0.55 ± 0.01	0.60 ± 0.05
24	0.50 ± 0.17	0.48 ± 0.02	0.45 ± 0.03	0.46 ± 0.00	0.43 ± 0.01
30	0.54 ± 0.20	0.68 ± 0.41	0.88 ± 0.75	0.58 ± 0.58	0.70 ± 0.50

Proline (g/L)

Media / Time (hours)	A	B	C	D	E
0	0.034 ± 0.000	0.100 ± 0.017	0.361 ± 0.022	0.782 ± 0.095	1.693 ± 0.149
6	0.034 ± 0.000	0.094 ± 0.014	0.379 ± 0.086	0.797 ± 0.072	1.645 ± 0.090
12	0.034 ± 0.000	0.097 ± 0.001	0.378 ± 0.071	0.808 ± 0.128	1.604 ± 0.127
18	0.034 ± 0.000	0.100 ± 0.016	0.365 ± 0.035	0.725 ± 0.021	1.605 ± 0.070
24	0.034 ± 0.000	0.096 ± 0.010	0.339 ± 0.027	0.692 ± 0.018	1.246 ± 0.063
30	0.034 ± 0.000	0.094 ± 0.004	0.325 ± 0.045	0.554 ± 0.228	1.217 ± 0.074

Appendix 2.7 Raw data for batch culture growth of strain A12 supplemented with inositol. Cultures were grown
medium with 2% glucose at 30°C and 180 opm. Data represents means of two independent experiments. Me
inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g/L inositol; K = YNB + 0.15
inositol

OD_{600nm}

Media / Time (hours)	G	H	I	J	K	L
0	0.118 ± 0.017	0.112 ± 0.006	0.100 ± 0.000	0.114 ± 0.008	0.122 ± 0.000	0.121 ± 0.00
6	1.001 ± 0.146	0.987 ± 0.095	0.993 ± 0.038	1.297 ± 0.089	1.268 ± 0.102	1.273 ± 0.09
12	3.288 ± 0.048	3.118 ± 0.105	2.995 ± 0.281	3.304 ± 0.175	3.276 ± 0.127	3.211 ± 0.25
18	3.311 ± 0.055	3.187 ± 0.038	3.240 ± 0.079	3.361 ± 0.038	3.239 ± 0.081	3.360 ± 0.04
24	3.295 ± 0.075	3.199 ± 0.049	3.150 ± 0.096	3.320 ± 0.059	3.249 ± 0.013	3.317 ± 0.02
30	3.293 ± 0.103	3.295 ± 0.095	3.258 ± 0.079	3.338 ± 0.040	3.351 ± 0.123	3.210 ± 0.02

Total Cell (× 10^6 Cell/mL)

Media / Time (hours)	G	H	I	J	K
0	1.153 ± 0.315	1.108 ± 0.194	1.135 ± 0.035	1.235 ± 0.297	0.933 ± 0.230
6	16.944 ± 0.294	14.888 ± 0.826	17.160 ± 2.794	24.736 ± 4.390	25.312 ± 2.534

Viable Cell (× 10^6 Cell/mL)

Media / Time (hours)	G	H	I	J	K
0	0.973 ± 0.336	0.938 ± 0.293	0.903 ± 0.145	1.033 ± 0.343	0.775 ± 0.247
6	16.429 ± 0.494	14.517 ± 0.830	16.744 ± 2.749	23.776 ± 4.028	24.032 ± 2.082
12	28.072 ± 4.989	26.228 ± 2.789	29.378 ± 0.455	32.400 ± 1.273	31.950 ± 1.202
18	36.950 ± 1.485	30.200 ± 1.556	35.200 ± 13.435	34.450 ± 5.020	32.850 ± 9.122
24	33.600 ± 2.970	35.950 ± 4.738	34.950 ± 6.152	35.850 ± 3.606	31.450 ± 3.465
30	37.350 ± 4.738	38.900 ± 11.783	38.050 ± 5.445	32.800 ± 4.950	31.900 ± 1.131

Cell Viability (%)

Media / Time (hours)	G	H	I	J	K
0	83.14 ± 6.78	83.82 ± 11.53	78.98 ± 10.33	83.22 ± 6.77	82.67 ± 5.83
6	97.04 ± 1.05	97.56 ± 0.08	97.55 ± 0.15	96.20 ± 0.77	95.11 ± 1.48
12	73.37 ± 1.53	73.79 ± 1.87	74.28 ± 1.23	73.47 ± 2.67	75.21 ± 5.51
18	72.86 ± 3.35	72.38 ± 5.15	73.58 ± 3.31	73.30 ± 0.43	76.21 ± 6.78
24	69.63 ± 2.43	71.84 ± 1.52	67.90 ± 1.08	74.86 ± 2.90	72.32 ± 2.37

Budding Rate (%)

Media / Time (hours)	G	H	I	J	K
0	41.54 ± 25.88	37.60 ± 16.40	31.06 ± 17.30	31.48 ± 13.04	32.48 ± 17.6
6	28.57 ± 3.13	26.72 ± 0.61	23.98 ± 2.85	25.03 ± 0.42	27.47 ± 0.39
12	28.17 ± 1.28	29.68 ± 0.57	27.93 ± 0.65	33.12 ± 1.48	30.53 ± 1.99
18	37.14 ± 1.80	28.49 ± 4.85	32.28 ± 11.75	22.02 ± 0.02	30.73 ± 0.96
24	35.75 ± 0.54	34.21 ± 5.06	26.18 ± 0.79	30.37 ± 4.60	29.86 ± 1.17
30	36.78 ± 2.66	26.51 ± 1.81	25.26 ± 4.54	24.93 ± 0.16	25.96 ± 1.66

Glucose (% w/v)

Media / Time (hours)	G	H	I	J	K
0	1.65 ± 0.31	1.26 ± 0.74	1.15 ± 0.58	1.48 ± 0.47	1.22 ± 0.51
6	1.14 ± 0.35	1.50 ± 0.01	1.09 ± 0.52	1.02 ± 0.39	1.21 ± 0.19

Ethanol (% v/v)

Media / Time (hours)	G	H	I	J	K
0	0.04 ± 0.14	0.08 ± 0.07	0.27 ± 0.31	0.13 ± 0.01	0.20 ± 0.10
6	0.27 ± 0.13	0.22 ± 0.09	0.22 ± 0.14	0.21 ± 0.15	0.25 ± 0.26
12	0.58 ± 0.22	0.60 ± 0.20	0.62 ± 0.27	0.64 ± 0.20	0.66 ± 0.28
18	0.64 ± 0.18	0.67 ± 0.17	0.70 ± 0.11	0.71 ± 0.08	0.70 ± 0.08
24	0.58 ± 0.08	0.58 ± 0.05	0.59 ± 0.04	0.75 ± 0.25	0.55 ± 0.02
30	0.45 ± 0.08	0.46 ± 0.08	0.48 ± 0.15	0.49 ± 0.16	0.55 ± 0.21

Inositol (g/L)

Media / Time (hours)	G	H	I	J	K
0	0.002 ± 0.000	0.036 ± 0.045	0.092 ± 0.097	0.117 ± 0.085	0.158 ± 0.144
6	0.023 ± 0.030	0.024 ± 0.032	0.071 ± 0.061	0.095 ± 0.070	0.142 ± 0.074

Appendix 2.8 Raw data for batch culture growth of strain PDM supplemented with inositol. Cultures were grown
medium with 2% glucose at 30°C and 180 opm. Data represents means of two independent experiments. Me
inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g/L inositol; K = YNB + 0.15
inositol

OD_{600nm}

Media / Time (hours)	G	H	I	J	K	L
0	0.101 ± 0.001	0.103 ± 0.007	0.102 ± 0.003	0.093 ± 0.033	0.103 ± 0.033	0.106 ± 0.01
6	0.583 ± 0.146	0.607 ± 0.109	0.654 ± 0.059	0.793 ± 0.021	0.830 ± 0.079	0.835 ± 0.10
12	3.002 ± 0.034	3.266 ± 0.127	3.338 ± 0.322	3.453 ± 0.199	3.557 ± 0.066	3.486 ± 0.03
18	3.723 ± 0.231	3.919 ± 0.134	3.809 ± 0.072	3.707 ± 0.001	3.723 ± 0.027	3.751 ± 0.03
24	3.893 ± 0.081	3.696 ± 0.387	3.645 ± 0.066	3.877 ± 0.024	3.835 ± 0.185	3.846 ± 0.13
30	3.881 ± 0.188	3.956 ± 0.124	3.873 ± 0.134	3.912 ± 0.116	3.870 ± 0.144	3.973 ± 0.07

Total Cell (× 10^6 Cell/mL)

Media / Time (hours)	G	H	I	J	K
0	1.173 ± 0.032	1.438 ± 0.392	1.625 ± 0.629	1.295 ± 0.092	1.495 ± 0.071
6	11.336 ± 2.184	12.936 ± 3.111	14.552 ± 0.916	18.445 ± 1.082	16.976 ± 1.652

Viable Cell (× 10^6 Cell/mL)

Media / Time (hours)	G	H	I	J	K
0	0.898 ± 0.032	1.090 ± 0.318	1.218 ± 0.491	0.983 ± 0.074	1.103 ± 0.025
6	10.824 ± 2.070	12.344 ± 3.066	13.896 ± 0.803	17.624 ± 1.052	16.288 ± 1.539
12	57.700 ± 3.960	48.600 ± 1.273	52.250 ± 3.889	53.800 ± 2.546	49.200 ± 5.798
18	57.650 ± 12.799	57.150 ± 5.869	49.150 ± 2.192	44.350 ± 0.495	45.800 ± 2.263
24	56.800 ± 7.212	61.800 ± 0.566	44.050 ± 0.778	55.500 ± 12.162	46.350 ± 0.778
30	54.600 ± 2.970	64.750 ± 9.263	51.700 ± 4.808	62.600 ± 0.141	64.650 ± 11.384

Cell Viability (%)

Media / Time (hours)	G	H	I	J	K
0	76.48 ± 4.41	75.83 ± 1.09	74.00 ± 0.40	75.71 ± 0.53	74.31 ± 2.49
6	95.42 ± 0.10	95.32 ± 0.78	95.66 ± 0.26	95.53 ± 0.21	95.98 ± 0.32
12	80.93 ± 2.14	77.56 ± 4.17	79.35 ± 1.77	78.52 ± 1.12	76.52 ± 3.23
18	78.00 ± 3.70	75.03 ± 1.66	75.90 ± 0.54	73.65 ± 2.96	74.47 ± 0.09

Budding Rate (%)

Media / Time (hours)	G	H	I	J	K
0	37.57 ± 0.22	49.33 ± 7.97	52.41 ± 3.91	43.08 ± 6.75	48.52 ± 5.62
6	23.20 ± 6.19	25.58 ± 2.55	25.01 ± 6.02	22.62 ± 2.31	23.65 ± 0.30
12	32.61 ± 6.14	24.99 ± 4.67	29.67 ± 0.75	28.68 ± 2.25	28.95 ± 3.59
18	33.02 ± 2.96	30.27 ± 3.75	25.86 ± 3.02	31.73 ± 2.67	37.46 ± 12.49
24	39.25 ± 8.24	31.26 ± 2.70	31.16 ± 4.32	28.61 ± 1.26	27.65 ± 2.49
30	30.17 ± 2.52	22.58 ± 1.70	24.45 ± 0.76	23.47 ± 3.53	23.05 ± 3.21

Glucose (% w/v)

Media / Time (hours)	G	H	I	J	K
0	1.32 ± 0.38	1.51 ± 0.01	1.48 ± 0.60	1.89 ± 0.47	1.75 ± 0.33
6	1.14 ± 0.49	1.29 ± 0.23	1.29 ± 0.21	1.08 ± 0.36	1.28 ± 0.22
12	0.41 ± 0.15	0.32 ± 0.11	0.33 ± 0.09	0.23 ± 0.06	0.21 ± 0.06

Ethanol (% v/v)

Media / Time (hours)	G	H	I	J	K
0	0.11 ± 0.23	0.08 ± 0.18	0.01 ± 0.09	0.17 ± 0.33	0.09 ± 0.20
6	0.19 ± 0.30	0.14 ± 0.21	0.09 ± 0.14	0.23 ± 0.33	0.09 ± 0.11
12	0.56 ± 0.13	0.54 ± 0.19	0.58 ± 0.17	0.65 ± 0.34	0.57 ± 0.23
18	0.65 ± 0.32	0.70 ± 0.32	0.64 ± 0.34	0.82 ± 0.49	0.63 ± 0.28
24	0.55 ± 0.21	0.63 ± 0.29	0.51 ± 0.09	0.54 ± 0.17	0.54 ± 0.18
30	0.44 ± 0.14	0.50 ± 0.21	0.49 ± 0.23	0.70 ± 0.43	0.71 ± 0.44

Inositol (g/L)

Media / Time (hours)	G	H	I	J	K
0	0.007 ± 0.007	0.006 ± 0.006	0.051 ± 0.027	0.126 ± 0.004	0.124 ± 0.009
6	0.006 ± 0.006	0.007 ± 0.008	0.044 ± 0.006	0.063 ± 0.028	0.103 ± 0.034
12	0.002 ± 0.000	0.002 ± 0.000	0.037 ± 0.010	0.068 ± 0.029	0.097 ± 0.032
18	0.002 ± 0.000	0.002 ± 0.000	0.035 ± 0.014	0.073 ± 0.024	0.110 ± 0.042
24	0.002 ± 0.000	0.002 ± 0.000	0.035 ± 0.005	0.065 ± 0.011	0.106 ± 0.032

Appendix 2.9 Raw data for batch culture growth of strain K7 supplemented with inositol. Cultures were grown u
medium with 2% glucose at 30°C and 180 opm. Data represents means of two independent experiments. Me
inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g/L inositol; K = YNB + 0.15
inositol

OD_{600nm}

Media / Time (hours)	G	H	I	J	K	L
0	0.105 ± 0.001	0.100 ± 0.014	0.099 ± 0.013	0.104 ± 0.011	0.120 ± 0.011	0.108 ± 0.00
6	0.522 ± 0.017	0.466 ± 0.085	0.461 ± 0.134	0.570 ± 0.025	0.625 ± 0.007	0.575 ± 0.08
12	2.740 ± 0.164	2.711 ± 0.098	2.868 ± 0.088	2.952 ± 0.458	3.038 ± 0.141	3.150 ± 0.15
18	4.630 ± 0.068	4.160 ± 0.492	4.700 ± 0.311	4.634 ± 0.042	4.648 ± 0.566	4.623 ± 0.00
24	4.656 ± 0.122	4.593 ± 0.055	4.602 ± 0.359	4.945 ± 0.086	4.971 ± 0.256	4.922 ± 0.17
30	4.800 ± 0.181	4.902 ± 0.410	4.908 ± 0.105	5.133 ± 0.075	5.055 ± 0.225	4.862 ± 0.00

Total Cell (× 10^6 Cell/mL)

Media / Time (hours)	G	H	I	J	K
0	0.978 ± 0.046	1.145 ± 0.141	1.243 ± 0.279	1.065 ± 0.014	1.145 ± 0.021

Viable Cell (× 10^6 Cell/mL)

Media / Time (hours)	G	H	I	J	K
0	0.880 ± 0.049	1.043 ± 0.166	1.133 ± 0.265	0.998 ± 0.018	1.073 ± 0.046
6	8.544 ± 0.973	9.072 ± 1.018	8.415 ± 2.331	11.128 ± 0.735	11.413 ± 1.569
12	42.800 ± 5.940	42.450 ± 3.748	43.500 ± 3.394	45.000 ± 13.294	35.550 ± 15.203
18	45.450 ± 6.010	51.250 ± 1.768	46.700 ± 4.808	45.750 ± 3.465	49.600 ± 10.182
24	49.300 ± 3.394	47.400 ± 8.344	49.050 ± 0.071	61.050 ± 7.707	55.500 ± 1.273
30	55.450 ± 7.849	49.550 ± 4.455	57.950 ± 6.859	59.700 ± 2.970	57.650 ± 4.172

Cell Viability (%)

Media / Time (hours)	G	H	I	J	K
0	89.89 ± 0.75	91.00 ± 3.00	91.21 ± 0.98	93.49 ± 2.80	93.66 ± 2.28
6	96.19 ± 2.40	97.46 ± 0.59	97.50 ± 0.26	93.37 ± 2.35	95.33 ± 2.50
12	92.45 ± 5.40	95.51 ± 1.94	94.17 ± 4.11	95.32 ± 2.68	95.78 ± 1.10

Budding Rate (%)

Media / Time (hours)	G	H	I	J	K
0	26.49 ± 3.34	34.44 ± 8.54	25.42 ± 1.39	31.42 ± 10.30	38.49 ± 9.81
6	19.60 ± 0.33	18.62 ± 5.19	19.71 ± 0.91	19.70 ± 1.51	21.00 ± 4.29
12	26.37 ± 4.56	29.53 ± 9.76	26.60 ± 7.23	26.41 ± 1.37	24.99 ± 7.55
18	15.91 ± 9.74	19.25 ± 9.19	21.06 ± 8.16	21.92 ± 5.13	19.15 ± 10.33
24	19.44 ± 6.07	20.50 ± 11.30	16.61 ± 11.02	19.13 ± 10.49	20.29 ± 13.77
30	22.19 ± 5.19	24.16 ± 7.25	21.70 ± 9.06	23.56 ± 13.45	23.27 ± 10.66

Glucose (% w/v)

Media / Time (hours)	G	H	I	J	K
0	1.65 ± 0.43	1.80 ± 0.03	1.45 ± 0.13	1.65 ± 0.22	1.49 ± 0.54
6	1.77 ± 0.04	1.53 ± 0.33	1.49 ± 0.31	1.70 ± 0.13	1.69 ± 0.04
12	0.69 ± 0.09	0.57 ± 0.10	0.58 ± 0.15	0.54 ± 0.01	0.62 ± 0.08

Ethanol (% v/v)

Media / Time (hours)	G	H	I	J	K
0	-0.08 ± 0.01	0.18 ± 0.36	0.18 ± 0.33	-0.01 ± 0.10	0.03 ± 0.14
6	0.07 ± 0.16	0.07 ± 0.14	0.05 ± 0.11	0.08 ± 0.14	0.09 ± 0.16
12	0.37 ± 0.19	0.41 ± 0.23	0.42 ± 0.20	0.48 ± 0.32	0.46 ± 0.32
18	0.72 ± 0.33	0.72 ± 0.27	0.71 ± 0.28	0.76 ± 0.35	0.63 ± 0.24
24	0.69 ± 0.09	0.68 ± 0.15	0.64 ± 0.18	0.62 ± 0.21	0.64 ± 0.19
30	0.50 ± 0.34	0.56 ± 0.25	0.54 ± 0.30	0.57 ± 0.31	0.66 ± 0.25

Inositol (g/L)

Media / Time (hours)	G	H	I	J	K
0	0.008 ± 0.009	0.007 ± 0.007	0.027 ± 0.012	0.091 ± 0.011	0.105 ± 0.053
6	0.004 ± 0.003	0.005 ± 0.004	0.057 ± 0.016	0.055 ± 0.075	0.078 ± 0.107
12	0.002 ± 0.000	0.002 ± 0.000	0.045 ± 0.025	0.077 ± 0.044	0.111 ± 0.053

Appendix 2.10 Raw data for generalized polarization. Cultures were grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four independent experiments.

Strains / Time (hours)	A12	PDM	K7
6	0.3613 ± 0.0148	0.2563 ± 0.0302	0.3065 ± 0.0094
24	0.5213 ± 0.0260	0.4812 ± 0.0494	0.4914 ± 0.0565

Appendix 2.11 Raw data for viability reduction measured by total plate count. Cultures were grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four and six independent experiments at 6 and 24 hours, respectively.

Strains / Time (hours)	A12	PDM	K7
6	96.39 ± 6.02	99.65 ± 0.50	99.92 ± 0.06
24	46.05 ± 22.29	68.15 ± 16.13	49.22 ± 19.94

Appendix 2.12 Raw data for viability reduction measured by methylene violet staining. Cultures were grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of eight independent experiments

Strains / Time (hours)	A12	PDM	K7
6	23.25 ± 12.81	38.28 ± 14.34	21.05 ± 9.04
24	17.04 ± 9.74	31.95 ± 16.01	10.70 ± 11.00

Appendix 2.13 Raw data for generalized polarization of strain A12 supplemented with proline. Cultures were gr
YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four independent experiments. M
g/L Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2 g/L Proline; F = YNB + 3 g/L Proline

Media / Time (hours)	A	B	C	D	E	
6	0.3587 ± 0.0186	0.3691 ± 0.0304	0.3810 ± 0.0128	0.3728 ± 0.0388	0.3255 ± 0.0303	0.
24	0.5222 ± 0.0343	0.4972 ± 0.0288	0.5039 ± 0.0065	0.5018 ± 0.0272	0.4718 ± 0.0305	0.

Appendix 2.14 Raw data for viability reduction measured by total plate count of strain A12 supplemented with
under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means
experiments at 6 and 24 hours, respectively. Media: A = YNB; B = YNB + 0.1 g/L Proline; C = YNB + 0.5 g/L Proli
= YNB + 2 g/L Proline; F = YNB + 3 g/L Proline

Media / Time (hours)	A	B	C	D	E	
6	99.27 ± 0.62	99.43 ± 0.54	99.44 ± 0.50	99.83 ± 0.04	99.82 ± 0.05	
24	59.98 ± 11.21	62.43 ± 9.44	51.04 ± 18.72	54.74 ± 15.28	60.40 ± 24.78	

Appendix 2.15 Raw data for viability reduction measured by methylene violet staining of strain A12 supplemen
grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of
Media: A = YNB; B = YNB + 0.1 g/L Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2
Proline

Media / Time (hours)	A	B	C	D	E	
6	25.25 ± 17.29	35.10 ± 18.21	34.49 ± 16.08	29.69 ± 8.78	37.84 ± 21.74	
24	19.00 ± 13.67	21.78 ± 12.90	19.81 ± 12.33	22.17 ± 12.93	14.48 ± 11.20	

Appendix 2.16 Raw data for generalized polarization of strain PDM supplemented with proline. Cultures were gr
YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four independent experiments. M
g/L Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2 g/L Proline; F = YNB + 3 g/L Proline

Media / Time (hours)	A	B	C	D	E	
6	0.2533 ± 0.0102	0.2977 ± 0.0321	0.3419 ± 0.1010	0.2484 ± 0.0560	0.2089 ± 0.0435	0.
24	0.4943 ± 0.0636	0.4841 ± 0.0064	0.4960 ± 0.0222	0.4687 ± 0.0731	0.4324 ± 0.0203	0.

Appendix 2.17 Raw data for viability reduction measured by total plate count of strain PDM supplemented with under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means experiments at 6 and 24 hours, respectively. Media: A = YNB; B = YNB + 0.1 g/L Proline; C = YNB + 0.5 g/L Proli = YNB + 2 g/L Proline; F = YNB + 3 g/L Proline

Media / Time (hours)	A	B	C	D	E	
6	99.88 ± 0.05	99.98 ± 0.02	99.95 ± 0.07	99.98 ± 0.03	99.99 ± 0.01	
24	76.32 ± 19.57	71.25 ± 30.80	79.40 ± 8.00	68.25 ± 14.21	61.49 ± 19.15	

Appendix 2.18 Raw data for viability reduction measured by methylene violet staining of strain PDM supplemen grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of Media: A = YNB; B = YNB + 0.1 g/L Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2 Proline

Media / Time (hours)	A	B	C	D	E	
6	38.38 ± 11.39	53.69 ± 24.13	41.85 ± 19.41	26.87 ± 17.78	27.69 ± 16.70	
24	37.34 ± 16.19	24.13 ± 13.65	35.86 ± 19.81	23.34 ± 9.87	17.98 ± 9.66	

Appendix 2.19 Raw data for generalized polarization of strain K7 supplemented with proline. Cultures were gr
YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four independent experiments. M
g/L Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2 g/L Proline; F = YNB + 3 g/L Proline

Media / Time (hours)	A	B	C	D	E	
6	0.3088 ± 0.0110	0.3366 ± 0.0280	0.3366 ± 0.0311	0.3042 ± 0.0366	0.3082 ± 0.0488	0.
24	0.4926 ± 0.0770	0.5081 ± 0.0108	0.5316 ± 0.0165	0.4829 ± 0.0519	0.4785 ± 0.0460	0.

Appendix 2.20 Raw data for viability reduction measured by total plate count of strain K7 supplemented with proli
aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of two and three
and 24 hours, respectively. Media: A = YNB; B = YNB + 0.1 g/L Proline; C = YNB + 0.5 g/L Proline; D = YNB +
Proline; F = YNB + 3 g/L Proline

Media / Time (hours)	A	B	C	D	E	
6	99.97 ± 0.03	99.94 ± 0.05	99.98 ± 0.00	99.51 ± 0.51	98.91 ± 1.35	
24	57.64 ± 19.62	54.60 ± 22.80	66.73 ± 21.35	45.98 ± 6.58	67.01 ± 15.49	

Appendix 2.21 Raw data for viability reduction measured by methylene violet staining of strain K7 supplement
grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of
Media: A = YNB; B = YNB + 0.1 g/L Proline; C = YNB + 0.5 g/L Proline; D = YNB + 1 g/L Proline; E = YNB + 2
Proline

Media / Time (hours)	A	B	C	D	E	
6	25.06 ± 6.13	18.77 ± 5.13	16.40 ± 10.80	14.57 ± 8.24	19.02 ± 7.85	
24	17.34 ± 12.25	13.86 ± 15.04	16.50 ± 15.36	9.27 ± 11.15	5.14 ± 2.31	

Appendix 2.22 Raw data for generalized polarization of strain A12 supplemented with inositol. Cultures were gr
YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four independent experiments. M
inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g/L inositol; K = YNB + 0.15
inositol

Media / Time (hours)	G	H	I	J	K	
6	0.3640 ± 0.0122	0.3436 ± 0.0358	0.3803 ± 0.0296	0.3689 ± 0.0327	0.3523 ± 0.0545	0.
24	0.5204 ± 0.0199	0.4887 ± 0.0113	0.4899 ± 0.0296	0.4763 ± 0.0055	0.4825 ± 0.0364	0.

Appendix 2.23 Raw data for viability reduction measured by total plate count of strain A12 supplemented with under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means experiments at 6 and 24 hours, respectively. Media: G = YNB (with 0.002 g/L inositol); H = YNB + 0.005 g/L inosito = YNB + 0.1 g/L inositol; K = YNB + 0.15 g/L inositol; L = YNB + 0.2 g/L inositol

Media / Time (hours)	G	H	I	J	K	
6	93.51 ± 8.67	86.86 ± 18.30	95.19 ± 6.66	93.92 ± 1.91	91.54 ± 0.10	
24	40.87 ± 24.73	46.96 ± 2.15	59.72 ± 0.64	43.28 ± 5.92	59.50 ± 0.46	

Appendix 2.24 Raw data for viability reduction measured by methylene violet staining of strain A12 supplemen grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of Media G = YNB (with 0.002 g/L inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g inositol; L = YNB + 0.2 g/L inositol

Media / Time (hours)	G	H	I	J	K	
6	21.24 ± 8.55	24.76 ± 8.36	24.40 ± 12.60	18.39 ± 23.42	18.28 ± 13.46	
24	15.08 ± 4.92	15.06 ± 13.60	12.80 ± 9.95	10.08 ± 15.77	16.23 ± 11.75	

Appendix 2.25 Raw data for generalized polarization of strain PDM supplemented with inositol. Cultures were gr
YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four independent experiments. M
inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g/L inositol; K = YNB + 0.15
inositol

Media / Time (hours)	G	H	I	J	K	
6	0.2593 ± 0.0447	0.2784 ± 0.0511	0.3649 ± 0.0960	0.2403 ± 0.0434	0.3005 ± 0.0850	0.
24	0.4681 ± 0.0345	0.4770 ± 0.0183	0.4507 ± 0.0371	0.4774 ± 0.0520	0.4546 ± 0.0292	0.

Appendix 2.26 Raw data for viability reduction measured by total plate count of strain PDM supplemented with
under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means
experiments at 6 and 24 hours, respectively. Media: G = YNB (with 0.002 g/L inositol); H = YNB + 0.005 g/L inosito
= YNB + 0.1 g/L inositol; K = YNB + 0.15 g/L inositol; L = YNB + 0.2 g/L inositol

Media / Time (hours)	G	H	I	J	K	
6	99.42 ± 0.73	91.47 ± 11.90	97.85 ± 3.04	93.54 ± 9.02	99.49 ± 0.67	
24	59.99 ± 8.21	68.97 ± 15.54	74.04 ± 16.62	60.83 ± 11.38	68.71 ± 7.12	

Appendix 2.27 Raw data for viability reduction measured by methylene violet staining of strain PDM supplemen grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of Media G = YNB (with 0.002 g/L inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g inositol; L = YNB + 0.2 g/L inositol

Media / Time (hours)	G	H	I	J	K	
6	38.18 ± 18.71	28.65 ± 27.49	34.23 ± 23.07	26.95 ± 22.52	24.04 ± 12.74	
24	26.56 ± 16.07	13.26 ± 9.05	17.11 ± 13.78	21.89 ± 17.16	21.33 ± 13.24	

Appendix 2.28 Raw data for generalized polarization of strain K7 supplemented with inositol. Cultures were gr YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of four independent experiments. M inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g/L inositol; K = YNB + 0.15 inositol

Media / Time (hours)	G	H	I	J	K	
6	0.3043 ± 0.0085	0.3066 ± 0.0299	0.3361 ± 0.0437	0.3121 ± 0.0247	0.3390 ± 0.0237	0.
24	0.4903 ± 0.0390	0.5038 ± 0.0264	0.5106 ± 0.0127	0.5084 ± 0.0159	0.5050 ± 0.0112	0.

Appendix 2.29 Raw data for viability reduction measured by total plate count of strain K7 supplemented with inos
aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of two and three
and 24 hours, respectively. Media: G = YNB (with 0.002 g/L inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05
inositol; K = YNB + 0.15 g/L inositol; L = YNB + 0.2 g/L inositol

Media / Time (hours)	G	H	I	J	K	
6	99.87 ± 0.05	99.88 ± 0.12	99.72 ± 0.16	95.51 ± 5.87	99.20 ± 0.87	
24	40.79 ± 19.91	4.93 ± 43.23	8.20 ± 34.96	38.04 ± 8.08	9.52 ± 26.96	

Appendix 2.30 Raw data for viability reduction measured by methylene violet staining of strain K7 supplement
grown under aerobic condition in YNB medium with 2% glucose at 30°C and 180 opm. Data represents means of
Media G = YNB (with 0.002 g/L inositol); H = YNB + 0.005 g/L inositol; I = YNB + 0.05 g/L inositol; J = YNB + 0.1 g
inositol; L = YNB + 0.2 g/L inositol

Media / Time (hours)	G	H	I	J	K	
6	17.05 ± 10.51	23.32 ± 10.77	31.48 ± 18.12	23.10 ± 9.03	21.15 ± 15.46	
24	4.06 ± 3.83	15.03 ± 8.79	19.14 ± 16.39	13.05 ± 16.51	12.26 ± 11.97	

Statistical Analysis

Raw data was initially compiled into Minitab 15® for Windows®. This software package was then used to perform one-way analysis of variance, which compared the variance of each parameter (i.e. GP, viability reduction by TPC, viability reduction by methylene violet staining, OD_{600nm}, total cell number, viable cell number, cell viability, budding rate, 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 differences between the data.

When significant differences detected, the test was followed by Fisher's LSD test to determine which strain did and did not differ significantly ($p<0.05$) from each other. Pairwise comparison was used to determine which value different significantly. When an interval upper and lower limit of Fisher's pairwise comparisons do not contain zero (0) value, the pairs are significantly different. Negative value of lower and upper limits indicate the value is significantly lower than the subtracted value, whereas positive value indicate higher compared to the subtracted value.

This appendix provides examples of output from the statistical analysis.

Appendix 3.1 One way between groups ANOVA for determination of significant variance between generalized polarization of strains A12, PDM and K7 at 6 hours culture, followed by Fisher's LSD test.

One-way ANOVA: GP6 versus Strains

```
Source   DF        SS        MS      F      P
Strains   2  0.044138  0.022069  54.32  0.000
Error    21  0.008531  0.000406
Total    23  0.052669

S = 0.02016   R-Sq = 83.80%   R-Sq(adj) = 82.26%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
A12    8  0.36131  0.01483                                (---*---)
K7     8  0.30653  0.00943                     (----*---)
PDM    8  0.25630  0.03016   (---*---)
                           -+---------+---------+---------+--------
                          0.245     0.280     0.315     0.350

Pooled StDev = 0.02016


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of Strains

Simultaneous confidence level = 88.16%


Strains = A12 subtracted from:

Strains     Lower    Center     Upper  -----+---------+---------+---------+----
K7       -0.07575  -0.05479  -0.03383            (---*---)
PDM      -0.12597  -0.10501  -0.08405  (---*---)
                                     -----+---------+---------+---------+----
                                       -0.100    -0.050    -0.000     0.050


Strains = K7 subtracted from:

Strains     Lower    Center     Upper  -----+---------+---------+---------+----
PDM      -0.07118  -0.05023  -0.02927              (---*---)
                                     -----+---------+---------+---------+----
                                       -0.100    -0.050    -0.000     0.050
```

Appendix 3.2 One way between groups ANOVA for determination of significant variance between generalized polarization of strains A12, PDM and K7 at 24 hours culture, followed by Fisher's LSD test.

One-way ANOVA: GP24 versus Strains

```
Source   DF       SS       MS     F      P
Strains   2  0.00695  0.00348  1.65  0.215
Error    21  0.04418  0.00210
Total    23  0.05113

S = 0.04586   R-Sq = 13.60%   R-Sq(adj) = 5.37%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
A12    8  0.52129  0.02602               (----------*----------)
K7     8  0.49144  0.05651       (----------*----------)
PDM    8  0.48115  0.04940    (----------*----------)
                           -+---------+---------+---------+--------
                          0.450     0.480     0.510     0.540

Pooled StDev = 0.04586


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of Strains

Simultaneous confidence level = 88.16%


Strains = A12 subtracted from:

Strains     Lower    Center    Upper   --+---------+---------+---------+-------
K7       -0.07754  -0.02985  0.01784      (-----------*----------)
PDM      -0.08783  -0.04014  0.00755   (-----------*-----------)
                                      --+---------+---------+---------+-------
                                    -0.080    -0.040     0.000     0.040


Strains = K7 subtracted from:

Strains     Lower    Center    Upper   --+---------+---------+---------+-------
PDM      -0.05798  -0.01029  0.03740           (----------*-----------)
                                      --+---------+---------+---------+-------
                                    -0.080    -0.040     0.000     0.040
```

Appendix 3.3 One way between groups ANOVA for determination of significant variance between generalized polarization of strains PDM with different proline supplementation at 6 hours culture, followed by Fisher's LSD test.

One-way ANOVA: GP6 versus [Pro]

```
Source  DF       SS       MS     F      P
[Pro]    5  0.04411  0.00882  2.96  0.040
Error   18  0.05367  0.00298
Total   23  0.09777

S = 0.05460   R-Sq = 45.11%   R-Sq(adj) = 29.86%

                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N     Mean    StDev  --------+---------+---------+---------+-
0.0    4  0.25333  0.01023        (-------*-------)
0.1    4  0.29770  0.03211              (--------*-------)
0.5    4  0.34188  0.10096                      (-------*-------)
1.0    4  0.24835  0.05602       (--------*--------)
2.0    4  0.20885  0.04349  (-------*--------)
3.0    4  0.24302  0.03913       (-------*-------)
                              --------+---------+---------+---------+-
                                     0.210     0.280     0.350     0.420

Pooled StDev = 0.05460


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons among Levels of [Pro]

Simultaneous confidence level = 67.02%


[Pro] = 0.0 subtracted from:

[Pro]     Lower    Center    Upper  --------+---------+---------+---------+-
0.1    -0.03674   0.04437  0.12549                 (------*-----)
0.5     0.00743   0.08855  0.16967                     (-----*------)
1.0    -0.08609  -0.00498  0.07614             (------*-----)
2.0    -0.12559  -0.04448  0.03664          (-----*------)
3.0    -0.09142  -0.01030  0.07082            (------*------)
                           --------+---------+---------+---------+-
                                -0.12      0.00      0.12      0.24


[Pro] = 0.1 subtracted from:

[Pro]     Lower    Center     Upper  --------+---------+---------+---------+-
0.5    -0.03694   0.04418   0.12529                 (------*-----)
1.0    -0.13047  -0.04935   0.03177          (------*------)
2.0    -0.16997  -0.08885  -0.00773       (------*-----)
3.0    -0.13579  -0.05467   0.02644          (-----*------)
                           --------+---------+---------+---------+-
                                -0.12      0.00      0.12      0.24
```

```
[Pro] = 0.5 subtracted from:

[Pro]     Lower    Center     Upper  --------+---------+---------+---------+-
1.0    -0.17464  -0.09353  -0.01241      (------*------)
2.0    -0.21414  -0.13303  -0.05191  (------*------)
3.0    -0.17997  -0.09885  -0.01773      (------*------)
                                     --------+---------+---------+---------+-
                                          -0.12      0.00      0.12      0.24


[Pro] = 1.0 subtracted from:

[Pro]     Lower    Center     Upper  --------+---------+---------+---------+-
2.0    -0.12062  -0.03950  0.04162           (------*-----)
3.0    -0.08644  -0.00532  0.07579              (------*-----)
                                     --------+---------+---------+---------+-
                                          -0.12      0.00      0.12      0.24


[Pro] = 2.0 subtracted from:

[Pro]     Lower    Center     Upper  --------+---------+---------+---------+-
3.0    -0.04694  0.03417  0.11529                 (------*------)
                                     --------+---------+---------+---------+-
                                          -0.12      0.00      0.12      0.24
```