GENERATION AND CHARACTERISATION OF ETHANOL-TOLERANT *Saccharomyces cerevisiae* MUTANTS

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Doctor of Philosophy

GENERATION AND CHARACTERISATION OF ETHANOL-TOLERANT *Saccharomyces cerevisiae* MUTANTS

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

"I, Dragana Stanley, declare that the PhD thesis entitled 'Generation and Characterisation of Ethanol-Tolerant *Saccharomyces cerevisiae* Mutants' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work."

15/08/2008

Signature Date

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SUMMARY

Recent increases in the price of crude oil are providing impetus to develop alternative sources of energy, including biofuels. Ethanologenic microorganisms such as *Saccharomyces cerevisiae* are widely used by fermentation industries, and have been used for many years to produce BioEthanol. However the productivity of this microorganism is limited, in part because of its intolerance to the ethanol that it generates. As ethanol levels increase during fermentation, yeast metabolic rate and viability decrease which, in turn, compromises ethanol yield. Improving ethanol tolerance of yeast should, therefore, lead to increased fermentation productivity. To date, investigations on ethanol tolerance in yeast have mostly focused on analysis of defective (ethanol-sensitive) mutants. This study presents work describing the generation and characterisation of ethanol-tolerant mutants using an adaptive evolution approach on mutagenised and non-mutagenised populations of *S. cerevisiae* W303-1A.

Mutants CM1 (chemically mutagenised) and SM1 (spontaneously evolved) show significant increases in ethanol tolerance manifested in increased specific growth rates and reduced lag periods in non-lethal ethanol concentrations, and higher survival rates in medium with lethal ethanol concentrations. Both mutants also have higher threshold ethanol concentrations (*i.e.* the highest ethanol concentration at which a culture can recover and resume growth) than the wild-type strain. Both mutants showed considerable differences in the concentrations of a number of metabolites involved in carbohydrate metabolism. They consistently produced more glycerol than the wild-type parent in the presence and absence of ethanol, which might suggest higher efficiency in maintaining redox-balance. The mutants also produced more acetate than wild type, with SM1 producing substantially more than either CM1 or the wild-type. In contrast to glycerol, differences in acetate content were detected only in the presence of ethanol, suggesting that acetate production by SM1 could be a direct response to ethanol stress.

Both mutants utilised glucose at a higher rate than the wild-type in the presence of ethanol and an initial glucose concentration of 20 g l^{-1}. When the glucose concentration was increased to 100 g l^{-1}, SM1 had the highest rate of glucose utilisation with this strain showing little difference in its glucose utilisation rate in the presence or absence

of ethanol, suggesting that it was not severely affected by the ethanol concentration (6.5% v/v) employed in the experiment.

Microarray experiments, comparing the parent with two ethanol-tolerant mutants in the presence and absence of non-lethal ethanol concentrations, investigated transcriptional differences between the strains to identify functional categories of genes involved in increased ethanol tolerance. The results clearly suggest that the mutants are not nearly as affected by 6.5% (v/v) ethanol as wild-type W303-1A. The mutants had approximately 3 times higher expression levels of transcription and translation related genes than the wild-type, and showed a less pronounced up-regulation of genes associated with glycolysis, especially *TDH,* the product of which, glyceraldehyde-3-phosphate dehydrogenase, uses NAD^+ as a cofactor. Both mutants showed a higher expression level of a number of mitochondrial genes, especially genes whose products are involved in mitochondrial respiration, and also a number of categories involved in maintaining redox-balance. Overall, redox stress appears to be lower in both mutants during ethanol stress, possibly due to the effect of their higher glycerol production rates on the NAD^+/NADH ratio; this may be why glycolytic activity was higher in the mutants although this would need to be confirmed. The mutants also displayed increased expression of genes associated with fatty acid, sterol, vitamin, acetate and arginine metabolism and also genes related to the synthesis of cell wall and cell membrane components.

PUBLICATIONS AND PRESENTATIONS

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LIST OF ABBREVIATIONS

Organizations:

AWRI	Australian Wine Research Institute
CUB	Carlton & United Breweries
WEHI	Walter and Eliza Hall Institution
AGRF	Australian Genome Research Facility

Chemicals and Units:

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
AR1	N-terminal activation domain
AR2	C-terminal activation domain
ARE	AP-1 responsive element
ATP	Adenosine triphosphate
AZC	Azetidine-2-carboxilic acid
ATPase	Adenosine triphosphatase
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
cDNA	Complementary DNA
CoA	Coenzyme A
CO_2	Carbon dioxide
CRD	Cysteine rich domain
Cy3	Cyanine dye 3
Cy5	Cyanine dye 5
DBD	DNA binding domain
DEPC	Diethyl pyrocarbonate
DHAP	Dihydroxyacetone phosphate
dATP	Deoxyadenosine 5'-triphosphate

dCTP	Deoxycytidine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
DTT	Dithiothreitol
dTTP	Deoxythymidine 5'-triphosphate
dUTP	Deoxyuridine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methane sulphonate
ESG	Ethanol significant genes
ESR	Environmental stress response
G3P	Glycerol 3 phosphate
GO	Gene ontology
HPLC	High pressure liquid chromatography
HSE	Heat shock element
HSF	Heat shock factor
Hsp	Heat shock protein
ISG	Interaction significant genes
IVA	Isovaleric acid
MIPS	Munich Information Centre for Protein Sequences
mRNA	Messenger RNA
MW	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
OD	Optical density
ORFs	Open reading frames
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKA	Protein kinase A

RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SGD	*Saccharomyces* genome database
SGR	Specific growth rate
SSC	Sodium chloride-sodium citrate
SSG	Strain significant genes
SS-DNA	Salmon sperm DNA
STRE	stress response element
STRP	stress-related proteins
TCA	tricarboxylic acid cycle
TRIS	Tris-(hydroxymethy)-aminomethane
UAC	Upstream activating sequence
UV	Ultra violet
v/v	Volume per volume
YAPRP	AP-1 related proteins
YEASTRACT	Yeast Search for Transcriptional Regulators and Consensus Tracking
YEPD	Yeast extract, peptone and D-glucose
YRE	Yap1-Responsive Elements

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Ethanol production by fermentation has a long history, initially being used to produce alcoholic beverages in which microbial inocula were unknowingly transferred between batches or initiated from remnants in previously used fermentation vessels. In fact, yeast has been used for the fermentation of alcoholic beverages and the leavening of dough for several thousands of years. Following alcoholic fermentation, spent brewer's yeast was traditionally utilized as a dough inoculum. The role of microorganisms in fermentation was first identified by Louis Pasteur in 1876 and the first pure yeast culture to be used in alcoholic beverage production was isolated by Emil Christian Hansen in 1883.

Ethanologenic microorganisms, such as yeast, and in particular *Saccharomyces cerevisiae*, perform a crucial role in the production of alcoholic beverages and industrial ethanol by converting sugars into ethanol. Although the fermentation process has been a commercial success, increasing competition within the fermentation industries, particularly in the alcoholic beverages sector, has applied increasing pressure on the need to reduce fermentation costs. One way of achieving this is to improve microbial performance which may result in increased fermentation productivity and ethanol yield. Microbial performance during brewing is currently limited by the inhibitory effects of the various stresses associated with the fermentation process on yeast vitality. The principal stresses encountered by yeast during fermentation are high ethanol concentrations, temperature stress and limited nutrient availability, although other stresses may also play a role. In particular, the exposure of yeast to stressful ethanol levels decreases their metabolic rate, induces changes in the catabolic pathways used and leads to a decline in yeast vitality. Such effects have a substantial impact on fermentation productivity by increasing growth/fermentation lag periods, reducing final ethanol yields, escalating fermentation turnover periods and limiting the life span of repitched (recycled) yeast. The maturation stage of the brewing process is particularly affected by ethanol stress (Mansure *et al.*, 1994). Acceleration of this step in particular

would be of great economic importance to the brewing industry (Suihko *et al.*, 1990). Minimizing the impact of ethanol toxicity on yeast is of fundamental importance for improving brewing productivity and ethanol yield.

From a renewable energy perspective, there is considerable interest in the use of ethanol as a replacement for fossil fuels. Bioethanol can make a significant contribution toward securing the long term supply of renewable transport fuels as well as the containment of greenhouse gas emissions, provide employment and new markets for the local agricultural industry, and reduce security concerns over national energy supplies. The cost of BioEthanol production however currently limits the economic competiveness of ethanol as a fuel, although the recent surge in the price of crude oil is swinging the balance in favour of ethanol. The current trend in BioEthanol production is to move away from using food-based substrates such as starches and sugar juice by developing processes that use lignocellulose-based substrates; these second generation BioEthanol processes are operationally complex and costly. BioEthanol would be more economically competitive as a fuel if ethanol yields and productivity could be improved by genetically modifying, or otherwise, ethanologenic microorganisms such that they are less sensitive to the inhibitory effects of ethanol. Although ethanol yields from the fermentation of lignocellulose hydrolysates are currently limited by low substrate concentrations, the ability to improve ethanol-tolerance would still be of substantial benefit to genetically modified microorganisms that catabolise both hexose and pentose sugars, but are not normally ethanologenic and are therefore ethanol sensitive, such as *E. coli* KO11 (de Carvalho Lima *et al.*, 2002; Yomano *et al.*, 1998). Even for yeast-based lignocellulose hydrolysate fermentations, improving the ethanol-tolerance of *S. cerevisiae* can potentially improve fermentation performance when advances in technology improve sugar yields from cellulosic biomass, as seems likely.

The objective of this project was to better understand the physiological and molecular processes that occur in *S. cerevisiae* during ethanol stress and, in particular, how the cell acclimatises to such stress. This was achieved by creating ethanol-tolerant mutants of *S. cerevisiae* using evolutionary engineering approaches. The ethanol-tolerant isolates were physiologically and metabolically characterised in the presence and absence of various ethanol concentrations. Gene expression analysis was then conducted to identify transcriptional differences between the mutants and parent strain in the absence

and presence of non-lethal ethanol stress, the purpose being to identify genes that are potentially responsible for the increase in ethanol-tolerance of the mutants. The close genetic relatedness of the mutants and the parent strain, but their differences in ethanol stress tolerance, provided an opportunity to identify areas of cell function that have a role in cell adaptation to, and protection from, the inhibitory effects of ethanol stress. The following literature review summarises the current state of knowledge in areas relevant to the project described in this thesis. Discussed in particular are aspects of the general stress response and ethanol stress response of *S. cerevisiae*, and the metabolic and evolutionary engineering of yeast.

1.2 GENERAL STRESS RESPONSE OF *Saccharomyces cerevisiae*

1.2.1 Introduction

Microorganisms encounter a number of stresses during fermentation, which subsequently affects fermentation productivity. These stresses include osmotic pressure, ethanol stress, nutrient limitation, changing viscosity and CO_2 concentration, rapid changes in temperature and pH, exposure to toxins or DNA damaging chemicals, and, in the case of industrial fermentations, high hydrostatic pressure in large fermentation vessels. These stresses generally lead to reduced growth and metabolic activity, and are known to trigger the expression of stress protective genes, resulting in cell-based changes aimed at ensuring cell survival and recovery (see reviews by Attfield 1997; Mager and Ferreira 1993).

A large number of studies have been conducted in the last few decades on the effect of various stressors, particularly temperature stress, on yeast physiology, biochemistry and cell structure. Some of the early work on yeast stress tolerance, although relatively simple in design, was of fundamental importance for developing our understanding of the nature of the yeast stress response. In particular, two important experimental approaches used to evaluate cellular stress response are pre-treatment and cross-protection. Pre-treatment refers to the exposure of a cell to a non-lethal stress, followed immediately by exposure to the same stress at previously lethal levels. For example, it was shown that the pre-treatment of *S. cerevisiae* with a mild temperature stress increases thermotolerance such that cells are better able to survive subsequent exposure

to previously lethal temperatures (Plesset *et al.,* 1982). This effect was also found to be dose-dependent with higher pre-treatment temperatures, within a sub-lethal range, conferring higher subsequent induced thermotolerance (Coote *et al.,* 1991).

Cross-protection refers to the pre-treatment of cells using a particular non-lethal stress, followed by exposure to a different stressor, usually at a previously lethal dose. In most cases, it was observed that pre-treatment equips the cell with the necessary defences to better withstand the subsequent stress condition, despite different stressors being used for the pre-treatment and lethal treatment steps. Cross-protection however is not always effective at improving stress tolerance, with some stressor combinations providing no protection to the cell when exposed to the second stressor. For example, when *S. cerevisiae* was pre-treated with heat shock, the cells acquired higher oxidative stress (H_2O_2) tolerance, however, pre-exposure to oxidative stress failed to induce thermotolerance (Collinson and Dawes 1992). Other studies have observed that heat pre-treatment can fortify yeast cells against subsequent ethanol stress (Watson and Cavicchioli 1983) and oxidative stress (Lewis *et al.,* 1995), whilst pre-treatment using osmotic stress (Varela *et al.,* 1992), ethanol stress, sorbic acid exposure, low pH (Coote *et al.,* 1991; Plesset *et al.,* 1982) or nutrient limitation (Piper 1993), leads to induced thermotolerance.

The above studies provide considerable insight on how yeast respond to a stressful environment. The ability to pre-treat yeast indicates the existence of molecular mechanisms that are responsible for a stress response which, upon activation by a non-lethal stress, can boost cellular defences and protect the cell from a normally lethal stress level (Ruis and Schuller 1995). Furthermore, the existence of cross-protection suggests that there are some cell functions that are commonly affected by many stressors and that there exists a general stress response in yeast that is activated regardless of the type of stressor involved. In yeast as well as in other eukaryotes, it has been found that the activation of stress protective genes is initially determined and controlled by specific stress response elements, located in gene promoter regions of the genome.

1.2.2. Yeast stress elements

Yeast cells are continuously exposed to environmental changes that may be unfavourable for cell vitality and survival. The efficiency with which they respond to these environmental stresses can be the difference between life and death. The first line of defence, immediate or early stress response, does not involve the transcription of stress-combating proteins, rather the modulation of existing proteins and activation of stress-signalling pathways. These, in turn, initiate later transcriptional responses. There are at least three groups of stress-induced proteins in yeast, heat shock proteins (HSPs), stress-related proteins (STRPs) and AP-1 related proteins (YAPRPs). These are regulated by their upstream transcriptional elements HSE, STRE and ARE respectively and they govern the stress response in cells ranging from yeast to mammals (Ruis and Schuller 1995; Shinohara *et al.,* 2002).

1.2.2.1 Heat stress element (HSE)

Heat stress elements (HSE), which are probably the most documented and certainly most conserved stress elements in eukaryotes, bind to transcriptional activators known as heat shock factor (HSF), triggering a cellular response to a number of chemical and physical stresses in addition to heat shock. The HSE/HSF interaction is conserved across yeast, plants and humans, however the number and importance of HSF genes can vary (Eastmond and Nelson 2006). Some organisms, including *Arabidopsis thaliana,* have 21 different HSF genes, mammals have 3 and *Drosophila melanogaster* and *S. cerevisiae* have a single HSF. Furthermore, the role of the HSF can vary significantly in different organisms (Eastmond and Nelson 2006; Hahn *et al.,* 2004).

In higher eukaryotes HSF binds to a HSE immediately upon heat induction, whereas in yeast HSF interacts with HSE even during normal conditions, albeit with low transcriptional activity, and mediates transcription of a number of constitutive genes (Sorger and Pelham 1988). This view has recently been challenged by results showing that Hsf1 is not constitutively bound to HSEs upstream of *SSA4* or *HSP30* until there is a temperature upshift. Hsf1 does bind with *SSA1* and *HSP104* at normal temperatures but only at a very low level (Li *et al.,* 2006). Genes dependent on Hsf1 for transcriptional activation are involved in protein folding, degradation and trafficking,

detoxification, carbohydrate metabolism, energy metabolism, cell wall organisation, cell signalling, small molecule transport, transcription and maintenance of cell integrity (Eastmond and Nelson 2006; Hahn *et al.*, 2004).

In yeast *HSF1* is an essential gene and *hsf1* null mutants are defective in a number of cell processes such as maintenance of cell wall integrity, protein transport and cell cycle progression (Imazu and Sakurai 2005; Zarzov *et al.*, 1997). A recessive *mas3* mutation in *HSF1* was found to cause temperature sensitive defects in mitochondrial protein import and cell cycle progression (Smith and Yaffe 1991).

Hsf1 binds to highly conserved HSE sequences (Figure 1.1) consisting of three tandem inverted repeats of nGAAn, known as "perfect HSE" (Perisic *et al.*, 1989). There are also "gap type HSEs" that have two consecutive nGAAn units followed by a 5 bp gap before the last nGAAn repeat (Santoro *et al.*, 1998). Recently a new Hsf1 binding sequence was discovered and named "step type HSE", comprising of three non-inverted nTTCn repeats separated by 5 bp blocks (Yamamoto *et al.*, 2005). The discovery of this novel HSE sequence has enriched the list of genes that potentially can be regulated by HSF. There are however genes that are reported to be regulated by Hsf1, but contain no known HSE (Yamamoto *et al.*, 2005) or contain non-conventional HSE with a gap of 11 bp between the first two repeats (Tachibana *et al.*, 2002). This, combined with the fact that some deviations in known HSE sequences are possible, suggests that there are more HSE sequences to be discovered and that this motif is far more abundant in the yeast genome than previously thought (Santoro *et al.*, 1998).

S. cerevisiae HSF1 contains four major distinct functional domains. On the N terminal and C terminal ends there are activation domains, each with a different role. The N-terminal activation domain (AR1) is responsible for the stress response (Nieto-Sotelo *et al.*, 1990) and the C-terminal activation domain (AR2) is responsible for activity under conditions of continuing stress and for cell cycle progression during stress (Morano *et al.*, 1999; Nieto-Sotelo *et al.*, 1990). The core of *HSF1* consists of a winged-helix-turn-helix DNA binding domain (DBD) that negatively regulates Hsf1 transcriptional activity (Bulman *et al.*, 2001; Littlefield and Nelson 1999) and a trimerisation domain (triple stranded alpha helical coiled coil) that facilitates the formation of Hsf1 trimers (Peteranderl and Nelson 1992). In addition to the four major domains, there is the

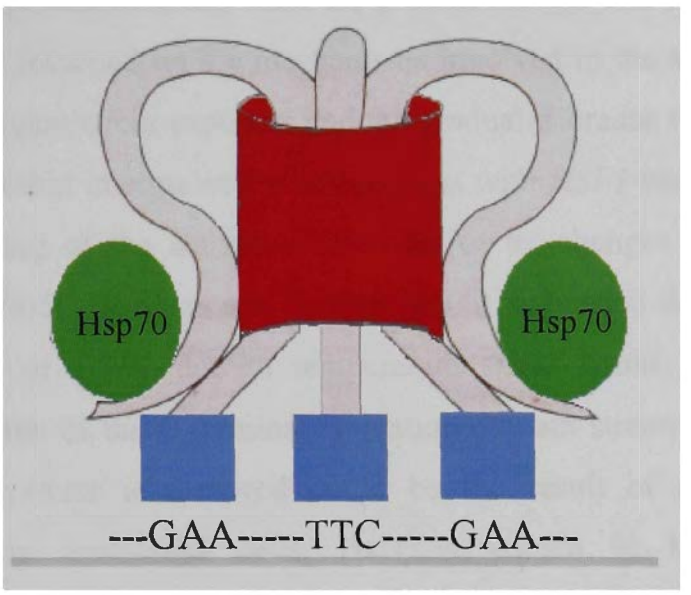

Figure 1.1: Heat shock factor binding to HSE repeated motifs. Under non-stress conditions Hsf binds to HSE as a trimer and is kept inactivated through interaction with Hsp70. Stress exposure initiates the release of Hsp70 and allows the protein to assume its active form (adapted from Mager and Ferreira 1993).

negative regulatory domain Conserved Element 2 (CE2) (Jakobsen and Pelham 1991) and the C-terminal Modulator (CTM) that alleviates CE2 repression (Sakurai and Fukasawa 2001).

DBDs on each subunit of an Hsf1 trimer, recognise one of the nGAAn inverted repeats in the HSE sequence and constitutively bind the protein (Sorger and Nelson 1989). A number of studies have focussed on the mechanisms involved in the sudden change in transcriptional activity upon stress exposure and the gradual decrease when the stress is removed. It is believed that intermolecular interactions with *HSF1* cause de-repression either through unmasking of the activation domains or by changes in conformation (Bulman and Nelson 2005). Bulman and Nelson (2005) suggested that the change in transcriptional activity of *HSF1* due to temperature stress could be regulated by trehalose, by modification of the C terminal activation domain structure in Hsf1. The loss of activity when stress is removed could be the result of post-translational modifications since, in unstressed cells, Hsf1 is known to be constitutively phosphorylated (Burnie *et al.,* 2006). The mechanisms behind the regulation of HSF are however still to be confirmed.

1.2.2.2 Stress response element (STRE)

The Stress Response Element (STRE) has been studied for nearly 30 years. This element with consensus core sequence AGGGG or CTTTT, binds specifically to transcription factors Msn2p and Msn4p (Martinez-Pastor *et al.,* 1996) and is able to mediate transcription induced by a number of different stresses.

Kobayashi and McEntee (1990) found that gene *DDR2* was induced greater than 20-fold by DNA damage or heat stress in the cell. By deleting the upstream promoter sequence of *DDR2*, the authors identified a 50 bp element that confers induction of this gene during heat shock. They also found however that Hsf1 (a known stress induced transcriptional regulator described above) could not bind to this oligonucleotide sequence *in vitro*. It was suggested that there exists a second, HSF-independent, stress response transcriptional element. In a subsequent study, the same authors found that the above oligonucleotide was capable of causing heat shock induction of the *CYC1*-lac gene when placed upstream of its reporter (Kobayashi and McEntee 1990). It was

found that the sequence CCCCT was essential for this induction and that their initial 50 bp oligonucleotide contained two such sequences. The insertion of two CCCCT sequences upstream of *CYC1*-lac enabled heat shock induction of this gene; mutations in this CCCCT element abolished induction of the reporter by heat shock. It was also found that the CCCCT sequence binds to a single 140 kDa polypeptide (later revised to 97 kDa) that is distinct from the HSF protein.

Bellazi *et al.,* (1991) demonstrated that a 58 bp upstream region of *CTT1* (an oxidative stress response gene) in yeast was able to mediate negative cAMP transcriptional control during nitrogen starvation. It was also found that this oligo could induce a heat stress response, that it comprises two subunits and that it is not related to a HSF (Wieser *et al.,* 1991). These authors subsequently demonstrated that the upstream activating sequence (UAC) was in this case also STRE and that it was able to mediate stress response caused by heat, nutrient starvation, oxidative and osmotic stress (Marchler *et al.,* 1993). It was also suggested that STRE, more than HSE, is important for induced thermotolerance. Subsequent work extended the list of STRE-controlled genes and it was found that Msn2 and Msn4 bind specifically to STRE-containing oligonucleotides (Martinez-Pastor *et al.,* 1996; Ruis and Schuller 1995). The authors suggested that Msn2 and Msn4 are likely to be STRE-controlling transcription factors. Schmitt and McEntee (1996) screened a yeast genomic DNA library to isolate clones of STRE binding proteins, finding only Msn2. The authors found that STRE binding activity was abolished by *MSN2* disruption and increased by *MSN2* over-expression, suggesting that Msn2 is a transcription factor that activates STRE genes. It was also found that Msn4 can partially replace Msn2 in transcriptional activation resulting from stress and identified yet another transcription factor, distinct and independent from both Msn2 and Msn4, that binds to the STRE motif; it was also predicted to be a zinc-finger protein. Estruch and Carlson (1993) found that single deletions of either *MSN2* or *MSN4* genes did not result in a significant change in phenotype, however, double knockout mutants (*ie* both *MSN2* and *MSN4*) were hypersensitive to range of stressful conditions. Over-expression of these genes in yeast caused reduced sensitivity to starvation and heat stress.

Treger *et al.* (1998) conducted a computerised pattern search of the entire yeast genome for a minimum of two upstream STRE motifs. The authors confined the search to only

300 bp upstream from the TATA sequence and allowed up to 200 bp distance between two STRE, although subsequent studies showed that, for a number of documented STRE-regulated genes, this distance can be much higher (Nevitt *et al.*, 2004). The results revealed 186 putative STRE-containing genes. In a further analysis using a series of multiple regulatory mutants, the authors selected 8 STRE-containing genes and demonstrated that three of them required Msn2/Msn4 for activation. Some of the HSPs were found to also have a STRE motif and were either predominantly regulated by HSF (*HSP26*) or by Msn2/STRE (*HSP12*). For other genes (*HSP78* and *HSP104*) each pathway was independently capable of activating transcription during stress, however, they also had residual transcription in a triple knockout mutant (inactivated STRE and HSE) indicating that additional transcriptional activators were involved in their stress regulation. This was supported by results where the inactivation of both pathways had relatively little effect on stress-related transcription of *HSP82*. It was further shown that the individual contributions of Msn2 and Msn4 are different for specific genes and under different stress conditions (see review by Estruch 2000).

The role of protein kinase A (PKA) in regulating Msn2-mediated transcriptional activation has been well documented (Durchschlag *et al.*, 2004; Gorner *et al.*, 1998; Smith *et al.*, 1998; Thevelein and de Winde 1999). Low PKA activity has been shown to coincide with increased Msn2-dependant transcription, while high PKA activity has the opposite effect (Jones *et al.*, 2003). PKA activity influences Msn2-induced transcription associated with nuclear import, export and DNA binding (Gorner *et al.*, 1998). Both Msn2 and Msn4 contain a nuclear localisation signal adjacent to their zinc-finger area which is inhibited by PKA phosphorylation, but can be activated by proteinphosphatase 1 (PP1) dephosphorylation (De Wever *et al.*, 2005; Gorner *et al.*, 1998). Durchschlag *et al.*, (2004) demonstrated that when PKA activity remains constantly low, such as when glucose is depleted or during prolonged stress, there is an increase in nuclear localisation of Msn2. The authors argued that stress conditions cause prolonged nuclear localisation of Msn2 to reduce its cytoplasmic levels, suggesting that growth may occur if it was possible to increase the degradation of nuclear Msn2 under these conditions.

Although both Msn2 and Msn4 relocalise from cytoplasm to nucleus in response to stress, it has been shown that a portion of these transcription factors continue to shuttle

in and out of nucleus with periodicity of few minutes (Jacquet *et al.*, 2003). This shuttling was shown to be independent of their cellular protein levels. Unlike Msn4, Msn2 was shown to oscillate only in presence of PKA. Nuclear export of Msn2 has been shown to be regulated by exportin Msn5 and both export and import, as discussed above, were shown to be regulated by PKA. Msn2 was also shown to be positively regulated by Psr1/Whi2 phosphatase complex and negatively regulated by Snf1 (Kaida *et al.*, 2002). Its nuclear degradation was shown to be Ssn3 (alias Srb10)-dependent (Bose *et al.*, 2005).

1.2.2.3 AP1 Responsive Element (ARE)

The AP-1 responsive element (ARE) is another well-studied stress response element. ARE binds to the AP-1 group of transcription factors, one of which, Yap1, has been shown to activate transcription in response to oxidative stress (Dumond *et al.*, 2000; Folch-Mallol *et al.*, 2004; He and Fassler 2005). *S. cerevisiae yap1* deletion mutants show reduced activity of enzymes associated with oxygen detoxification (Schnell *et al.*, 1992). Yap1 is also involved in the mediation of a number of yeast responses to toxic chemicals that alter the redox status of the cell (Wu *et al.*, 1993) and the cadmium stress response (Wemmie *et al.*, 1994).

Yap1 was first identified by its ability to bind to an SV40-derived AP-1 recognition site (ARE element TGACTAA). Later, more Yap1-Responsive Elements (YRE) sequences were identified; TTACTAA and TGACTCA (Fernandes *et al.*, 1997); TKACAAA (Nguyen *et al.*, 2001) and TNACAAA (He and Fassler 2005). Yap1 can also bind to upstream regions of genes that do not contain any of the above sequences, suggesting that there could be more YRE yet to be discovered (He and Fassler 2005).

Yap1 is located in the cytoplasm in non-stressed cells but resides in the nucleus under stress conditions. A nuclear export factor, Crm1, was shown to regulate this relocalisation (Yan *et al.*, 1998). Repression of *CRM1* expression results in Yap1 relocalisation to the nucleus and increased transcription of Yap1-regulated genes (Kuge *et al.*, 1997). A protein is recognized as belonging to the AP-1 protein family according to similarity in its bZIP protein domain, however, a number of proteins from this group, including Yap1, share another amino acid motif that is rich in cysteine residues and is

called the Cysteine Rich Domain (CRD). Localisation of Yap1 in the nucleus requires formation of a disulphide bond between the C and N terminal of the CRD. Dually disulphide bonded, Yap1 forms a bi-functional protein domain that controls both nuclear localisation and activation of Yap1-dependant transcription (Gulshan *et al.*, 2005).

A number of stress-induced genes that have multiple upstream stress response elements (HSE, STRE and ARE) have been shown to require more than one type of stress element for successful stress-induced transcription. For example, a gene from the Yap family, *YAP4*, is induced by several different stressors and is regulated by both YRE and STRE upstream sequences. This gene, which contains one YRE, four STRE and multiple HSE elements in its upstream region, was shown to be transcriptionally dependent on YRE and its most proximal STRE sequences. Deletion of these two stress elements lead to complete abrogation of both *YAP4* mRNA and protein (Nevitt *et al.*, 2004). Similar dependence on more than one stress element is observed in the regulation of *FLR1* (a gene encoding a multiple drug transporter). The promoter of this gene has three YRE sequences, each of them functional and necessary for optimal transcription (Nguyen *et al.*, 2001).

Yap1, Hog1 and Skn7 regulate transcription associated with the oxidative stress response (Ikner and Shiozaki 2005). It was demonstrated that among the genes regulated by Yap1 in response to peroxide stress (32 genes), some also require the presence of Skn7 (15 genes), however, only Yap1 was necessary for the cadmium stress response (Lee *et al.*, 1999). Skn7 has also been shown to interact with Hsf1 *in vivo* and as required for induction of heat shock proteins under oxidative stress (Raitt *et al.*, 2000).

A second member of the Yap family, Yap2, is highly homologous with Yap1 and known to be a mediator of responses to a range of stressors. Cohen *et al.*, (2002) showed that microarray analysis can distinguish between these two highly homologous genes, which are 88% identical in their DNA binding domains, and demonstrated that these proteins activate non-overlapping sets of genes in response to various stresses. Yap1 activates genes involved in response to the detoxification of reactive oxygen species, whereas Yap2 controls a set of genes important for stabilizing proteins during

stress. They also demonstrated that the binding sites are different for the two genes. Miyahara *et al.*, (1996) used *YAP1* and *YAP2* double deletion mutants to confirm their role as mediators of heat shock-induced transcriptional activation of multidrug resistance ABC transporters in yeast, namely *PDR5* and *SNQ2*.

There are other proteins in yeast able to bind to the AP-1 optimal site. So far, 14 proteins have been identified that are able to bind to the AP-1 site including Ccn4, Yap1, Yap2, Met28, Sko1 and Hac1. Further sequence analysis revealed more proteins from the Yap family including Yap3-Yap8, which have sequence similarities to Yap1 and Yap2. More on these extended AP-1 family members and their role in stress can be found in a review by Rodrigues-Pousada *et al.*, (2004).

Yeast stress elements initiate the transcription of many genes in *S. cerevisiae* upon exposure to various stressors. Their interaction with transcription factors and subsequent activation of stress response genes is complex and not fully understood. One of the more important roles of yeast stress elements is the regulation of genes that encode heat shock proteins, which have vital roles in protecting the cell from the damaging effects of stress exposure. The roles of heat shock proteins in the stress response of *S. cerevisiae* are discussed in the following sections.

1.2.3 The role of heat shock proteins in cellular stress response

Heat shock proteins (HSPs) are among the most conserved of proteins. They are present in all organisms from bacteria to humans (Lindquist and Craig 1988), which is indicative of their importance in cell survival. The role of HSPs is very important in cellular stress response, with HSPs being rapidly produced in response to all cellular stressors investigated so far (Feder *et al.*, 1995; Holbrook and Udelsman 1994; Lindquist 1993; Lindquist and Craig 1988). HSPs are classified according to their molecular weight into three groups: high molecular weight HSPs of about 104 or 83 kDa, medium-sized HSPs of around 70 kDa and small HSPs with molecular weights between 15 and 30 kDa (Khlebodarova 2002).

1.2.3.1 Hsp104

Yeast are known to express a specific chaperone gene *HSP104*, which is crucial for protection from heat and ethanol stress, which induce this gene up to 1000-fold (Sanchez and Lindquist 1990; Sanchez *et al.*, 1992). *HSP104* is not found in mammals however its expression in mammalian cells enhances chaperone capacity and reduces heat shock-induced loss of viability (Mosser *et al.*, 2004).

HSP104 does not appear to have a role in the stress response of yeast subjected to sub-lethal heat shock temperatures (Sanchez and Lindquist 1990), however, mild heat pre-treatment of a *hsp104*Δ mutant and its wild-type strain resulted in acquired thermotolerance to lethal temperatures in wild-type cells, but not in the *hsp104*Δ mutant. It was found that *S. cerevisiae* cells with deleted *HSP104* grew at the same rate as the wild-type at 25 and 37°C and died at the same rate at 50°C. If, however, they were pre-treated with a mild heat stress of 37°C, the differences became more obvious. Pre-treatment of wild-type and *hsp104*Δ cells prior to 10 minutes treatment at 50°C led to a 1000-fold increase in wild-type viability compared to the deletion mutant, indicating that Hsp104 has a role in induced thermotolerance. During mild heat shock pre-treatment, acquisition of thermotolerance was dependent on the levels of accumulated Hsp104 (Kawai *et al.*, 1999). Further work by Parsell *et al.*, (1994) found that the wild-type, with a functional *HSP104*, rapidly removed aggregated heat-damaged proteins whereas this did not occur in the *hsp104*Δ mutant. Although it was generally accepted that Hsp104 has a role associated with the protection and repair of heat denatured proteins (Hanninen *et al.*, 1999; Kawai *et al.*, 1999; Sanchez *et al.*, 1993; Vogel *et al.*, 1995), there was considerable controversy over its suggested role in disaggregating or removing accumulated protein agglomerates. Glover and Lindquist (1998) further investigated this role for Hsp104 and found that it contributes to aggregated protein rescue by protein refolding, a role shared with Hsp70 and Hsp40.

Hsp104 is reported to be necessary for the survival of yeast at lethal temperatures (Kawai *et al.*, 1999; Winkler *et al.*, 1991) and it has been suggested that it has a stress protective role in synergy with trehalose (see Section 1.2.4.1). Elliott *et al.*, (1996) compared the heat resistance of stationary phase *tps2*Δ and *hsp104*Δ single mutants and found increased sensitivity to heat, however, double null mutations had a synergistic

effect with almost a complete loss of stationary phase heat resistance; this synergy was confirmed in *tps1Δhsp104Δ* double mutants. Hsp104 was also found to contribute to an increase in both the accumulation and degradation of trehalose during heat shock (Iwahashi *et al.,* 1998). Among other roles, Hsp104 is required for reassembly of heat-damaged small nuclear ribonucleoproteins (Bracken and Bond 1999) and it has a role in the propagation of yeast prion-like factor (PSI^+) (Chernoff *et al.,* 1995; Lindquist *et al.,* 1995).

1.2.3.2 Hsp70

The HSP family of middle molecular weight, Hsp70, are highly conserved. Their main role during stress is to act as protein chaperones, binding to newly translated proteins to assist in their correct folding and to prevent aggregation (Piper *et al.,* 1997). They are also involved in disassembling aggregates of misfolded proteins, translocating proteins to the endoplasmic reticulum and mitochondria, and possibly being involved in the regulation of expression of other HSPs, as presented in Figure 1.1, (reviewd in Becker and Craig 1994; Bukau and Horwich 1998). The Hsp70 family includes nine cytosolic chaperones, Ssa1-4, Ssb1, Ssb2, Sse1, Sse2, Ssz1, three mitochondrial chaperones, Ssc1, Ssq1, Ecm10 (Voos and Rottgers 2002) and two ER-located chaperones, Kar2 and Lhs1 (Baxter *et al.,* 1996). The expression of many Hsp70 genes is increased during heat stress and their functions are often overlapping and complementary (Weitzel and Li 1993; Werner-Washburne and Craig 1989; Werner-Washburne *et al.,* 1987). Members of the Hsp70 family however do not function in isolation. Indeed, during heat stress response and recovery, Hsp70 proteins work as a part of a chaperone team with Hsp104 and other HSPs as described in the following section.

1.2.3.3 Low Molecular Weight HSPs

A number of HSPs with low molecular weights have also been reported. These include HSPs 12, 26, 30 and 42, which are produced in response to ethanol stress (Alexandre *et al.,* 2001; Chandler *et al.,* 2004). One of these, *HSP12*, is induced by many different stressors and the monitoring of its expression has been recommended for determining the stress status of industrial yeast cultures (Karreman and Lindsey 2005). When yeast cells are subjected to heat shock, small HSPs, such as Hsp26 and Hsp42, assemble to

form large co-complexes with proteins prone to aggregation (Haslbeck *et al.*, 2004; Haslbeck *et al.*, 1999; Susek and Lindquist 1989; Wotton *et al.*, 1996). It has been suggested that these sHSPs assist denaturated proteins in refolding by holding them in a repair-competent state, following which reactivation is carried out by Hsp104/Hsp70/Hsp40 (Haslbeck and Buchner 2002; Parsell *et al.*, 1994). Hsp26 has also been shown to facilitate disaggregation of aggregated proteins after heat shock (Cashikar *et al.*, 2005).

Most stressors that activate HSPs are proteotoxic, *i.e.* they increase the number of unfolded and/or missfolded or aggregated proteins. This suggests that HSPs may be activated as a response to an increased level of damaged proteins, rather than a stress signalling mechanism *per se*. Trotter *et al.*, (2002) investigated the effect of misfolded protein accumulation on gene expression in yeast cells. The authors used a proline analogue, azetidine-2-carboxilic acid (AZC), which incorporates into proteins to cause the misfolding. They found that chemically-induced misfolding activated HSP expression, leading to the repression of ribosomal proteins in a HSF-dependent manner, failing to induce the stress response regulon (STRE). The authors also confirmed that AZC treatment, as with heat shock treatment, does not activate the endoplasmic reticulum Unfolded Protein Response, however, it strongly and robustly causes HSF-dependant expression changes, induces some genes (*HSP12*) that partially depend on Msn2/4 and causes G1 cell cycle arrest. It was also demonstrated that ethanol treatment, known to increase protein misfolding, mimics AZC treatment by causing high expression of HSP genes and weak expression of STRE genes (Schuler *et al.*, 1994). Both ethanol and AZC stress resulted in unchanged *ACT1* expression and in ribosomal protein repression. Although AZC treatment and heat shock effects partially overlap, AZC treatment fails to activate cell integrity pathways and accumulation of glycogen and trehalose as reported for heat shock (Trotter *et al.*, 2002), however, there is sufficient evidence to suggest that misfolded protein accumulation could be a mediator and/or sensor of cellular stress and stimulate at least part of the stress response.

1.2.4 Stress-protective roles of trehalose and glycerol

Proteins are not the only molecules that have a stress protection role in *S. cerevisiae*. Often referred to as "chemical chaperones", osmolytes (named for their role in maintaining osmotic pressure in the cell) are small organic molecules that have been shown to stabilise the native conformation of cellular proteins under a range of stresses in a similar fashion to protein-based chaperones (Crowe *et al.*, 1998; Wang and White 1999; Yancey *et al.*, 1982). Osmolytes comprise mostly sugars, polyols, methylamines and free amino acids or their derivatives. Among the osmolytes, trehalose and glycerol have received most attention for their role in cellular stress protection.

1.2.4.1 Trehalose

Trehalose attracted interest in its stress protection role when it became evident that almost all the so-called "resurrection" plants and microorganisms, *ie.* organisms able to survive and recover from almost complete desiccation, contained large amounts of trehalose (see review by Crowe and Crowe 1992). Further investigation demonstrated that trehalose accumulates in cells during a range of stresses such as high temperature (Eleutherio *et al.*, 1995; Hottiger *et al.*, 1989), freezing (Kim *et al.*, 1996), starvation (Lillie and Pringle 1980), dehydration and desiccation (Eleutherio *et al.*, 1993), osmotic shock (Hounsa *et al.*, 1998), ethanol stress (Attfield 1987; Hounsa *et al.*, 1998; Kim *et al.*, 1996; Soto *et al.*, 1999), salt stress (Carvalheiro *et al.*, 1999) and exposure to copper or hydrogen peroxide (Attfield 1987; Hounsa *et al.*, 1998; Kim *et al.*, 1996; Soto *et al.*, 1999).

Trehalose has been shown to protect enzymes upon heat exposure. For example, yeast pyrophosphatase activity is preserved at 50°C in the presence of trehalose (Sola-Penna and Meyer-Fernades 1994). The authors showed that the half-life for thermal inactivation of the cytosolic enzyme pyrophosphatase at 50°C is increased 13-fold in the presence of 1.5M trehalose. Furthermore, after 10 minutes exposure to 50°C, pyrophosphatase activity was completely lost in the control (with no added trehalose), whereas the presence of 2M trehalose preserved 95% of enzyme activity. Other carbohydrates could not reproduce this effect. Trehalose also protects the activity of yeast glucose-6-phosphate dehydrogenase and phosphoglucose-isomerase against

thermal inactivation (Hottiger *et al.*, 1994). Other proteins protected by trehalose include trypsin during freeze-thaw stress (Lopez-Diez and Bone 2004), alcohol dehydrogenase during heat stress (Miroliaei *et al.*, 2007) and plasma membrane H^+-ATPase from *Kluyveromyces lactis* (Sampedro *et al.*, 2001).

The protective role of trehalose on membrane proteins is well documented (Crowe *et al.*, 1988; Crowe, J.H.Crowe, L.M. *et al.*, 1984; Crowe, J.H.Whittam, M.A. *et al.*, 1984; Crowe, L.M. *et al.*, 1984). Water removal leads to detrimental phase transitions in biological membranes in any stressed cell, which can lead to a loss of membrane integrity. Trehalose is capable of preventing phase transitions by inhibiting fusion between lipids and maintaining the lipid bilayer in a fluid state under severe drying conditions (Crowe *et al.*, 1988). Using Fourier transform infrared spectroscopy, Leslie *et al.*, (1994) measured the transition temperature of phospholipids in *S. cerevisiae* and compared the effect of trehalose on the properties of dehydrated plasma membranes in intact *S. cerevisiae* with dried plasma membranes. The presence of trehalose lowered the phase transition in yeast from around 60°C to around 40°C, thus preventing the destruction of membrane integrity by phase transition in yeast rehydrated between 40°C and 60°C. Mansure *et al.*, (1994) studied the effect of 10% (v/v) ethanol on plasma membrane integrity in the presence and absence of trehalose. They found that cell viability increased in close correlation with trehalose concentration. Trehalose reversed the leakage of electrolytes from ethanol-damaged cells and, regardless of whether it was internal, added externally, or on both sides of the membrane, trehalose inhibited ethanol-induced leakage of liposomes.

Studies on mutants with gene deletions that affect trehalose metabolism have however been inconclusive regarding the role of trehalose in the yeast stress response. Although a *TPS1* null yeast mutant (such mutant is unable to synthesise trehalose) resulted in cells with extremely reduced thermotolerance, it was also shown that this mutant was impaired in the synthesis of HSPs, confusing the role of trehalose and loss of Hsp production with the observed loss in thermotolerance (De Virgilio *et al.*, 1994; Hazell *et al.*, 1995). Nwaka *et al.*, (1994) found that yeast mutants deficient in trehalose-hydrolysing neutral trehalase accumulated extremely high trehalose concentrations, but displayed low thermotolerance. They noted that when glucose was added to thermotolerant yeast cells during stationary phase, their trehalose concentration

decreases while thermotolerance remains high. These authors also observed that a *ubc4Δubc5Δ* mutant, which can synthesise HSPs constitutively, had high thermotolerance, but their trehalose levels were low compared to the wild-type strain. Although the authors did not reject a role for trehalose as a thermoprotectant, they argued that under certain conditions HSPs can assume such a role. In a subsequent review, Singer and Lindquist (1998) noted that trehalose levels in the neutral trehalase *nth1Δ* mutant used by Nwaka *et al.*, (1994) would have been excessive and could possibly have interfered with other cell functions, including chaperone activity.

Trehalose has a number of properties that could distinguish it from other disaccharides with respect to protein protection. Trehalose is a non-reducing disaccharide comprised of two glucose molecules linked by an α1-1 bond. As a non-reducing sugar, trehalose does not participate in Mail-Larad reactions which are known to severely affect proteins. Trehalose is also one of the most heat stable sugars that does not caramelise in its native state and it has a relatively larger hydrated volume than other common carbohydrates (Sola-Penna and Meyer-Fernandes 1998; Sussich *et al.*, 2001). There are currently two hypotheses on what determines the protective abilities of trehalose. The first, also known as glassy-state hypothesis, proposes that trehalose provides a glassy protective environment around proteins and other cell structures. Trehalose forms an amber-like environment that captures and immobilises proteins, reducing movement, degradation and denaturation (Franks *et al.*, 1991; Green and Angell 1989; Levine and Slade 1992). Second, the water-replacement hypothesis, proposes that the hydroxy groups in trehalose are able to replace water by the formation of hydrogen bonds with protein to maintain secondary structures (Allison *et al.*, 1999; Crowe *et al.*, 1998). These two hypotheses are not mutually exclusive with the consensus being that both mechanisms are needed for stress-protection by trehalose (Crowe *et al.*, 1998). Recently, trehalose was also proven to directly eliminate reactive oxygen species, H_2O_2 and O_2^-, during heat stress in wheat (Luo *et al.*, 2008), suggesting another stress protective mechanism for trehalose.

1.2.4.2 Glycerol

Another osmolyte, glycerol, is also well known for its stress protective role. Glycerol production in yeast has been reported to increase with heat shock treatment (Kajiwara,

Y. *et al.*, 2000), salt stress (Carvalheiro *et al.*, 1999) and during osmotic stress (Krantz *et al.*, 2004; Mager and Siderius 2002; Mager and Varela 1993; Modig *et al.*, 2007; Wojda *et al.*, 2003). Yeast cells respond to osmotic stress by increasing the production and accumulation of glycerol to counterbalance the external osmotic pressure (Mager and Varela 1993). It has been shown that most of the special protein protective properties of trehalose can be replaced by glycerol (Sola-Penna and Meyer-Fernandes 1998), but this does not apply for all proteins (Faber-Barata and Sola-Penna 2005). Glycerol has been associated with thermal protection of a number of proteins in yeast, with both trehalose and glycerol able to significantly protect *S. cerevisiae* pyrophosphatase against inactivation at temperatures up to 95°C, and to allow renaturation of inactivated enzymes when the temperature was lowered (Henle *et al.*, 1982; Zancan and Sola-Penna 2005). The role of glycerol as a stress protectant and regulator of cellular redox balance is discussed further in Section 1.4.4.

1.3. THE ETHANOL STRESS RESPONSE OF *S. cerevisiae*

1.3.1 Effects of ethanol on *S. cerevisiae*

1.3.1.1 Overview

Although a major metabolic product of fermentation, ethanol in sufficiently high concentrations is a potent chemical stressor for yeast cells. Ethanol acts as a non-competitive inhibitor of yeast growth at relatively low concentrations, inhibiting cell division and causing loss in viability, decreasing cell volume and increasing thermal death. Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock-like proteins, lowering the rate of RNA and protein accumulation, enhancing the frequency of petite mutations, redirecting metabolism, denaturing intracellular proteins and glycolytic enzymes and reducing their activity. The main sites for ethanol effects in yeast are cellular membranes, hydrophobic and hydrophilic proteins and the endoplasmic reticulum (Walker 1998). For both ethanol stress and heat shock, vacuole morphology is altered from segregated structures to a single, large organelle (Meaden *et al.*, 1999). Membrane structure and function appear to be a predominant target of ethanol. Exposure of yeast to ethanol

results in increased membrane fluidity and consequential decrease in membrane integrity. The main effects of ethanol on the yeast cell are summarised in Table 1.1.

1.3.1.2 Effect of ethanol on the yeast plasma membrane

The plasma membrane is recognized as a prime target of ethanol toxicity in yeast (see reviews by D'Amore *et al.*, 1990; Rose 1993). Exposure of the yeast phospholipid bilayer to ethanol weakens the water-lattice structure, decreases the strength of fatty acid interactions, promotes leakage of small ions and reduces plasma membrane integrity (Sajbidor and Grego 1992). Ethanol locates into hydrophobic regions of the membrane, increasing polarity in that part of the membrane and the capacity to solubilise polar molecules (Ingram 1986). This influences protein transport across the membrane, increases proton influx and intracellular acidification (Alexandre *et al.*, 1998; Ogawa *et al.*, 2000; Walker 1998) and decreases nutrient uptake (Pascual *et al.*, 1988). Ethanol exposure is also known to stimulate H^+-ATPase activity (Monteiro and Sa-Correia 1998; Rosa and Sa-Correia 1991).

The influence of ethanol on membrane composition is well documented. The fact that ethanol affects growth rate and solute accumulation in a manner dependent on membrane lipid composition was noted by Thomas and Rose (Thomas and Rose 1979), and further investigated by Beaven *et al.*, (1982). These authors observed a dose-dependent increase in the proportion of mono-unsaturated fatty acids, especially oleic acid, at the expense of saturated fatty acyl residues. This was later confirmed by a number of studies with ethanol causing an increase in C18:1 fatty acyl residues (oleic acid) and a decrease in either C16:1 at 15% (v/v) ethanol (Sajbidor and Grego 1992) or in C16:0 at 10% (v/v) ethanol (Alexandre *et al.*, 1994b). Gupta *et al.*, (1994) observed an increase in lipid peroxidation in ethanol-treated cells, accompanied by a decreased level of total lipids, phospholipids and free sterols. The authors also noted that the glycolipid to phospholipid membrane ratio and diphosphatidylglycerol content increased during ethanol stress. Under anaerobic growth conditions, yeast cells depend on an extracellular supply of unsaturated fatty acids since the yeast desaturase enzyme requires oxygen (Walker 1998). Under anaerobic conditions, unsaturated fatty acid- and sterol-supplemented cultures show an increase in ethanol-tolerance when grown in

Table 1.1: Effects of ethanol on yeast cell physiology

Physiological function	Ethanol influence
Cell viability and growth:	
	• General inhibition of growth, cell division and cell viability
	• Decrease in cell volume
	• Induction of morphological transactions
	• Enhancement of thermal death
Intermediary metabolism and macromolecular biosynthesis:	
	• Denaturation of intracellular proteins and glycolytic enzymes
	• Lowered rate of RNA and protein accumulation
	• Reduction in V_{max} of main glycolytic enzymes
	• Enhancement of petite mutations
	• Induction of heat shock-like proteins
	• Elevated levels of cellular trehalose
	• Increase in oxygen free radicals
	• Induced synthesis of cytochrome P450
Membrane structure and function:	
	• Decrease in membrane saturated and increase in unsaturated fatty acids
	• Acceleration of sterol biosynthesis
	• Induced lipolysis of cellular phospholipids
	• Increased phospholipid biosynthesis
	• Increased ionic permeability
	• Inhibition of nutrient uptake
	• Inhibition of H^+-ATPase and dissipation of proton-motive force
	• Uncoupling of electrogenic processes by promoting passive re-entry of protons and consequential lowering of cytoplasmic pH
	• Hyperpolarisation of plasma membrane

medium containing more unsaturated fatty acids at the expense of saturated fatty acids (Thomas *et al.,* 1978). You *et al.,* (2003) compared the effects of different unsaturated fatty acids on ethanol toxicity and tolerance. Unsaturated fatty acids in yeast comprise, almost exclusively, palmitoleic (C16:1) and oleic acid (C18:1), both owing their synthesis to the OLE1 gene, a membrane-located desaturase. Using an *ole1* mutant and supplementing the medium with monounsaturated fatty acids, the authors found an unsaturated fatty acid composition, especially high in oleic acid content, to be the most effective in overcoming the inhibitory effects of ethanol (You *et al.*, 2003). Kajiwara *et al.,* (2000) overexpressed the *OLE1* gene to compare strain fermentation efficiency and ethanol productivity and found that the unsaturated fatty acid content, although increased in the recombinant strain, was not sufficient to significantly improve ethanol production despite its known influence on ethanol-tolerance.

Membrane sterol content is also affected by ethanol. It has been established that sterol composition affects yeast growth and the plasma membrane fatty acid composition (Buttke *et al.,* 1980). Ethanol-stressed yeast cells reduce their total sterol content and increase the proportion of unsaturated membrane sterols, especially ergosterol (Alexandre *et al.,* 1994a; Castillo Agudo 1992; Larue *et al.,* 1980; Walker-Caprioglio *et al.,* 1990). This agrees with the finding that supplementation of media with ergosterol makes yeast cells more ethanol tolerant than supplementation with other sterols (Thomas *et al.,* 1978). The 5-7 unsaturated sterols are also found to increase ethanol-tolerance and reduce the ethanol-related cell death rate (Novotny *et al.*, 1992a; Novotny *et al.,* 1992b).

Changes in the plasma membrane lipid composition have been correlated with an increase in membrane fluidity (Alexandre *et al.,* 1994a). An increase in membrane fluidity of *S. cerevisiae* as been observed to occur upon exposure to ethanol (Mishra and Prasad 1989; Thomas and Rose 1979). Jones and Greenfield (1987) observed a correlation between an ethanol-associated increase in membrane fluidity and increased membrane permeability, but only during step increases in ethanol concentrations; when the cells were gradually exposed to ethanol, membranes became both less fluid and less permeable, suggesting that gradual exposure provided sufficient time for the cells to correct the fluidity changes in the membrane caused by the ethanol. Aguilera *et al.*, (2006) speculated that the increased ethanol-tolerance of the yeast strains investigated in

their study could be attributed to the ability of their ethanol-tolerant strains to decrease membrane fluidity, offsetting the effects of membrane fluidisation caused by ethanol thus helping to maintain membrane functionality and ATPase activity.

Ethanol is also known to stimulate ATPase activity by disrupting the integrity of the cell membrane and causing increased proton influx into the cell (Alexandre *et al.*, 1994a; Cartwright *et al.*, 1987). ATPase is a major regulator of the membrane proton gradient and an increase in ATPase activity due to ethanol stress is thought to offset increased proton influx (Cartwright *et al.*, 1987; Leao and Van Uden 1984); the proton gradient drives nutrient uptake across the membrane and maintains intracellular pH homeostasis (Monteiro and Sa-Correia 1998). ATPase activity is known to be regulated by plasma membrane lipid composition, with ATPase activity increasing with an increase in the proportion of long chain fatty acids (Johannson *et al.*, 1981) and decreasing with a decrease in ergosterol content (Arami *et al.*, 1997). Most ethanol-tolerant strains are found to have higher ATPase activity (Aguilera *et al.*, 2006). The activity of other membrane-bound proteins are also known to be affected by ethanol stress, especially membrane transport systems for glucose (Leão and Van Uden 1982), maltose (Loureiro-Dias and Peinado 1982), ammonium (Leão and Van Uden 1983) and, under high ethanol concentrations, amino acids (Leão and van Uden 1984).

1.3.1.3 The effect of ethanol on organelles

Mitochondria appear to have a significant role in ethanol-tolerance, as evident by the fact that petite (respiration deficient) mutants have reduced survival rates relative to their grande parent strain in presence of ethanol (Aguilera and Benitez 1985). Costa *et al.*, (1993) showed that mitochondrial, and not cytoplasmic, superoxide dismutase is involved in ethanol stress resistance, with an MnSOD (mitochondrial superoxide dismutase)-deficient strain showing increased ethanol sensitivity, which was not the case for a deficient cytoplasmic SOD mutant (Costa *et al.*, 1997). The authors presumed that MnSOD is inactivating reactive oxygen species in mitochondria during respiratory growth on ethanol.

A number of studies using knockout strains to examine ethanol sensitivity have reported a higher ethanol sensitivity in strains containing mitochondrial gene deletions, with

Fujita *et al.*, (2006) reporting 5, Kubota *et al.*, (2004) 14 and van Voorst *et al.*, (2006) 4 genes associated with ethanol-tolerance *i.e.* a total of 21 different genes across three separate studies.

Ethanol is known to affect vacuole function. Lucero *et al.*, (1997) observed that vacuole degradation of a maltose transporter in yeast is inhibited by ethanol. These authors suggest that this was due to compromised endocytosis resulting from ethanol-induced membrane alterations. Although it was found that passive proton influx does not occur across the vacuole membrane, as is observed for the cellular plasma membrane, it has been demonstrated that vacuole morphology alters to form a single large organelle in response to ethanol stress (Loureiro-Dias and Santos 1990; Meaden *et al.*, 1999). Matsuura and Takagi (2005) recently demonstrated that the stress protective role of proline depends on vacuole function during heat and ethanol stress.

1.3.1.4 Other ethanol stress protectants

There are a number of ethanol stress protectants that, when added to the medium, improve yeast survival rate and tolerance to ethanol stress. Known to be involved in growth, cell division and enzyme activity, magnesium ions also stabilize the membrane bilayer and are involved in ethanol stress relief (Walker 1994). Magnesium ions decrease the permeability of the plasma membrane to protons and anions by interacting with membrane phospholipids (Petrov and Okorokov 1990). Magnesium ions have been shown to protect yeast cells against 10% (v/v) ethanol stress and either addition to the growth medium or preconditioning with magnesium significantly increases yeast survival under non-lethal and lethal ethanol concentrations (Birch and Walker 2000; Dasari *et al.*, 1990; Hu *et al.*, 2003b). Similarly, calcium ions increase ethanol-tolerance when added extracellularly. In one study, the exposure of yeast to 20% (v/v) ethanol for 9 hours resulted in the total loss of viable cells in control cultures, however, cultures supplemented with Ca^{2+} (1.64 mM) retained 50% viability (Hu *et al.*, 2003a). The authors showed this calcium-induced ethanol-tolerance to be related to its ability to decrease plasma membrane permeability under ethanol stress.

The protective role of trehalose in yeast subjected to various stresses includes ethanol stress (see Section 1.2.4.1). D'Amore *et al.*, (1991) observed that trehalose

accumulation increased yeast viability when exposed to 5% (v/v) ethanol. Mansure *et al.*, (1994) also demonstrated a positive correlation between yeast viability and trehalose concentration in the presence of 10% (v/v) ethanol, and attributed this to the ability of trehalose to reverse the effect of ethanol-induced leakage of electrolytes across the plasma membrane. Contrary to the above, Alexandre *et al.*, (1998) claimed there was no correlation between cell viability and trehalose concentration during ethanol stress. In this regard, the role of trehalose in the ethanol-tolerance of yeast cells remains controversial.

1.3.2 The response of yeast to ethanol stress

1.3.2.1 Overview

Yeast have built-in protective mechanisms to counteract the toxic effects of a range of stresses, but the effectiveness of these mechanisms is limited in native yeast. Studies on the yeast stress response have mostly been confined to the effects of heat treatment although stress responses have been observed following exposure to ethanol and other stressors such as oxidative stress and low nutrient conditions. Importantly, the stress-response affords the cell some protection against a range of stresses and is therefore significant to fermentation industries because it enables yeast to survive in what would otherwise be lethal conditions.

The stress response of yeast, which is a property of all living organisms, is a transient reprogramming of cellular activities to ensure survival during stress, to protect essential cell components and to enable a rapid resumption of 'normal' cellular activities during the recovery period. The response of yeast to environmental stress is complex, involving various aspects of cell sensing, signal transduction, transcriptional and posttranscriptional control, protein-targeting to organelles, accumulation of protectants, and increased activity of repair functions. The efficiency of these processes in a given yeast strain determines its robustness and, to a large extent, whether it is able to perform well in industrial processes.

1.3.2.2 Transcriptional response to ethanol stress

Above a critical threshold level, ethanol stress and heat shock induce heat shock-like proteins (HSP), otherwise known as stress response proteins (see Section 1.2.3). These are evolutionarily conserved proteins, originally characterized on the basis of their strong induction by heat shock. HSPs induced in *S. cerevisiae* by ethanol stress appear, on the whole, to be identical to those induced by heat shock (Walker 1998). Both heat and ethanol stress in yeast leads to the acquisition of thermotolerance and ethanol-tolerance.

Yeast cells exposed to ethanol synthesise a range of Heat Shock Proteins (HSP) (Table 1.2), including Hsp104, Hsp26, Hsp30, Hsp70, Hsp82, and Hsp12, but only Hsp104 and Hsp12 have been shown to physiologically influence yeast tolerance to ethanol. Hsp104 acts as a remodelling agent in the disaggregation of denaturated proteins (Glover and Lindquist 1998), whereas Hsp12 is a membrane-associated protein that can protect liposomal membrane integrity against desiccation and ethanol (Sales *et al.*, 2000).

In addition to the work on HSPs and their influence on ethanol-tolerance, there have been a number of studies that take a more generic look at the effect of ethanol on the transcriptome. These studies on the transcriptional response of *S. cerevisiae* to ethanol are discussed in the following sections according to two different experimental approaches, gene expression studies and genome-wide screens.

(a) Global gene expression and ethanol stress in *S. cerevisiae*

There are a number of gene expression studies on the effects of ethanol on yeast transcription. Ogawa *et al.*, (2000) compared gene expression in a parent sake yeast and an ethanol-tolerant mutant, thereof the objective being to investigate the mechanisms of ethanol-tolerance acquired by the mutant. It was found that several genes (*CTT1*, *GPD1*, *SPI1*, *HSP12* and *HOR7*) were highly expressed only in the mutant in the absence of ethanol stress, with their level of expression increasing following exposure to ethanol stress; these genes having already been identified as ethanol-stress response genes by other workers. The authors also found that catalase, glycerol and trehalose

Table 1.2: Genes reported as overexpressed in *S. cerevisiae* during ethanol stress in at least two of the following studies; Fujita *et al.*, (2004), Chandler *et al.*, (2004), Alexandre *et al.*, (2001) and Ogawa *et al.*, (2000). It should be noted that Fujita *et al.*, (2004) reported 271 genes with higher expression levels during ethanol stress, but only 42 were presented in their paper. The other 229 genes could not be included in this table since the website containing supplementary material with the full gene lists was not accessible.

Gene	Description	Reference
HSP12, 26, 30, 78, 104	Heat shock proteins	(Fujita *et al.*, 2004, Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
HSP82	Heat shock protein	(Fujita *et al.*, 2004, Alexandre *et al.*, 2001)
HSP42	Heat shock protein	(Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
CTT1	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide	(Fujita *et al.*, 2004, Chandler *et al.*, 2004, Alexandre *et al.*, 2001, Ogawa *et al.*, 2000)
DDR2	Multi-stress response protein	(Fujita *et al.*, 2004, Chandler *et al.*, 2004)
SSA4	Member of the heat shock protein 70 (HSP70) family	(Fujita *et al.*, 2004, Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
YRO2	Putative protein of unknown function	(Fujita *et al.*, 2004, Chandler *et al.*, 2004)
TDH1	Glyceraldehyde-3-phosphate dehydrogenase	(Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
TSL1	Large subunit of trehalose 6-phosphate synthase	(Chandler *et al.*, 2004, Alexandre *et al.*, 2001)

Gene	Description	Reference
TPS1	Synthase subunit of trehalose-6-phosphate synthase	(Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
ALD4	Mitochondrial aldehyde dehydrogenase	(Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
GLK1	Glucokinase, catalyzes the phosphorylation of glucose at C6 in the first irreversible step of glucose metabolism	(Fujita *et al.*, 2004, Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
YGP1	Cell wall-related secretory glycoprotein	(Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
HOR7	Protein of unknown function; induced under hyperosmotic stress	(Fujita *et al.*, 2004, Chandler *et al.*, 2004, Alexandre *et al.*, 2001, Ogawa *et al.*, 2000)
PYC1	Pyruvate carboxylase isoform	(Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
DAK1	Dihydroxyacetone kinase, required for detoxification of dihydroxyacetone (DHA); involved in stress adaptation	(Fujita *et al.*, 2004, Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
YER053C YDR516C YBR139W	Proteins of unknown function	(Chandler *et al.*, 2004, Alexandre *et al.*, 2001)
HXK1	Hexokinase isoenzyme 1, a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism	(Fujita *et al.*, 2004, Chandler *et al.*, 2004, Alexandre *et al.*, 2001)

Gene	Description	Reference
PGK1	3-phosphoglycerate kinase, key enzyme in glycolysis and gluconeogenesis	(Fujita *et al.*, 2004, Chandler *et al.*, 2004)
SPI1	GPI-anchored cell wall protein involved in weak acid resistance	(Chandler *et al.*, 2004, Ogawa *et al.*, 2000)
CYC7	Cytochrome c isoform 2, expressed under hypoxic conditions	(Fujita *et al.*, 2004, Ogawa *et al.*, 2000)

accumulated to a greater extent in the mutant compared to the parent, and that the mutant exhibited resistance to other stressors such as heat, high osmolarity and oxidative stress. The authors concluded that the mutant had multiple stress tolerance because of it elevated expression of stress response genes, resulting in the accumulation of stress-protective substances. This study reported the higher expression of only a few genes in the mutant compared to the parent, which can be attributed to a number of factors. Gene filters were used to measure global transcription differences between the parent and mutant strain only when grown in the absence of stress, suggesting that the reported stress response genes were constitutively expressed by the mutant; ethanol-stress conditions were used only to confirm the increased expression of these genes under stress conditions using Northern analysis. Also, as noted by the authors, the study would most likely have reported more mutant-specific ethanol responsive genes if methods other than visual observation had been used to analyse the gene filters. Although the concept was good, the outcomes of this study were limited by poor experimental design and low sensitivity of the visual approach used to analyse the gene filters.

A number of studies have directly compared stressed and non-stressed *S. cerevisiae* cells during short term non-lethal exposure to ethanol (Alexandre *et al.*, 2001; Chandler *et al.*, 2004; Fujita *et al.*, 2004). Although these studies used different strains and ethanol concentrations, and there were differences in the reported expression of some

individual genes, the Gene Ontology (GO) categories that were enhanced or repressed by ethanol stress are very comparable. Alexandre *et al.*, (2001) reported enhanced expression of GO categories associated with cell energetics, stress response, protein destination and ionic homeostasis. They reported that out of the 300 genes comprising the Environmental Stress Response (ESR) family, 73 were up-regulated in the presence of 7% (v/v) ethanol stress. They observed increased expression levels of genes regulating both biosynthesis and degradation of trehalose, as well as the up-regulation of sugar kinases *HXK1* and *GLK1*.

Chandler *et al.*, (2004) extended the above study by investigating both the early (1 hour) and late stress response (3 hours) of *S. cerevisiae* cultures grown in the presence of 5% (v/v) ethanol. The authors observed significant increases in the expression of genes associated with the general stress response, energy utilisation, transport mechanisms, cell surface interactions and lipid metabolism. Unlike the work of Alexandre *et al.*, (2001), they reported increased expression in genes associated with hexose transport (*HXK6* and *HXK7*), the up-regulation of trehalose biosynthesis, but not degradation, genes (see Section 1.2.4.1) and an increase in the expression of many glycolysis and TCA cycle-associated genes, despite surplus glucose being present in the medium. The authors proposed that the induction of hexose transport and glycolysis genes, which normally increase expression under conditions of low glucose levels or starvation, is due to the cell entering a pseudo-starvation state when stressed by ethanol exposure. The reason for the cell entering a pseudo-starvation state during ethanol stress was not investigated.

Fujita *et al.*, (2004) investigated the effect of stress caused by ethanol and other straight chain alcohols, on gene expression levels in *S. cerevisiae*. They found that 271 genes were up-regulated by factor of 3 or more in 9% (v/v) ethanol stress. They reported increased gene expression during alcohol stress in GO categories associated with stress response, cell rescue defence and virulence, TCA cycle and glycolysis, energy and amino acid metabolism. The web-site containing supplementary material accompanying the publication, with complete lists of genes affected by alcohol stress in Fujita *et al.*, (2004) study, was not accessible at the time of writing this thesis.

Similar to Alexandre *et al.*, (2001), Fujita *et al.*, (2004) focussed on genes with increased expression levels. Chandler *et al.*, (2004) found that 274 genes had lowered expression levels during ethanol stress. These genes were mostly associated with protein synthesis, RNA synthesis and processing, amino acid metabolism and nucleotide metabolism, which supports other observations of genes with decreased expression during growth arrest (Gasch *et al.*, 2000). Chandler *et al.*, (2004) found that gene expression profiles of ethanol-stressed cells were quite different in the late ethanol stress response (after 3 hours of stress exposure). They found that the total number of highly expressed genes decreased from 100 (after 1 hour of stress exposure) to 14 genes, 7 of which were also induced during the early stress response; these genes were associated with energy utilisation, general stress response and vacuole functions. The number of genes with decreased expression levels changed from 274 (1 hour of ethanol stress) to 99 (3 hours of ethanol stress), most of these being associated with ribosomal function. Overall, the findings from the above gene expression studies had substantial overlap, with a high number of genes similarly induced by ethanol exposure. Table 1.2 summarizes some of the genes reported as ethanol stress-induced by at least two of the above three ethanol-stress studies.

Hirasawa *et al.*, (2007) conducted a similar ethanol-stress study to those described above but, instead of reporting up-regulated genes, they used two-dimensional clustering and identified a cluster of tryptophan-related genes that were induced by 5% (v/v) ethanol stress. The authors focused on the tryptophan cluster without further inspecting other ethanol stress-responsive genes. They found that tryptophan supplementation in cultures and over-expression of tryptophan biosynthesis genes increases ethanol-tolerance.

(b) Functional genomic screens of *S. cerevisiae* during ethanol stress

A number of studies on ethanol stress tolerance in yeast used mutant yeast strains to better understand the genetic basis of ethanol-tolerance. Takahashi *et al.*, (2001) created approximately 7000 transposon mutants and compared their growth in rich medium with and without 6% (v/v) ethanol. They initially found 260 clones that grew slower on ethanol and 5 clones that had no growth at all. Selecting the latter 5 mutants for sequencing analysis, they found that transposons were inserted into the coding

regions of the following functionally unrelated genes, *BEM2*, *PAT1*, *ROM2*, *VPS34* and *ADA2*. *BEM2* (alias *TSL1*) is involved in the control of cytoskeleton organization and cellular morphogenesis (Kim *et al.*, 1994) and is required for bud emergence (Wang and Bretscher 1995). *PAT1* is a topoisomerase II-associated, deadenylation-dependent, mRNA-decapping factor required for faithful chromosome transmission (Wang *et al.*, 1999; Wang *et al.*, 1996). *ROM2* expresses a GDP/GTP exchange protein (Gep) for Rho1 and Rho2 (Ozaki *et al.*, 1996). *VPS34* encodes an enzyme responsible for the synthesis of phosphatidylinositol 3-phosphate; this protein is required for the localization of a variety of vacuole proteins (Herman and Emr 1990), vacuole segregation (Schu *et al.*, 1993) and endocytosis (Strahl and Thorner 2007). *ADA2* is a transcriptional activator required for acetylation of histones (Sterner *et al.*, 2002). The authors confirmed their findings using knockouts of the above genes and concluded that they are important for growth during ethanol stress. They also acknowledged that the number of genes involved in ethanol-tolerance is probably much higher, but this would require the screening of at least 35,000 mutants to ensure coverage of the whole yeast genome using a transposon mutation approach.

Kubota *et al.*,(2004) investigated the ethanol sensitivity of 4,847 *S. cerevisiae* mutants containing single deletions in non-essential genes and found that 256 mutants had impaired growth in the presence of 11% (v/v) ethanol compared to the wild-type; 181 of these genes were also sensitive to 8% (v/v) ethanol. Genes that appeared to be important for ethanol-tolerance were associated with biosynthesis (43 genes), cell cycle (17), cytoskeleton (18), the mitochondrion (22), morphogenesis (14), nucleic acid binding (12), protease (4), protein transport/vacuole (45), signal transduction (4), transcription (25), transport (11) and of unknown function (41). In the same publication, and independent from the deletion library ethanol experiments, the authors demonstrated that the addition of ethanol causes cell-cycle delay and also that Swe1 (a negative regulator of mitosis) is related to the regulation of cell growth under ethanol stress. The increase in cell size observed during ethanol stress, resulting from cell-cycle delay, did not occur in the *Swe1Δ* mutant. The authors also found that Swe1 levels increased 10 minutes after ethanol exposure, but returned to normal (unstressed) levels within 20 minutes. This provided evidence of the very short term nature of the response of some genes to ethanol stress, which may in part explain the lack of correlation across

deletion studies and expression studies in this area. Samples for gene-expression analysis were taken 30 minutes (Alexandre *et al.*, 2001), 1 hour (Chandler *et al.*, 2004) and 2 hours (Fujita *et al.*, 2004) after stress exposure. This is supported by Chandler *et al.*, (2004) who found that late ethanol stress response genes (3 hours of stress) shared only 7% commonality with genes up-regulated after 1 hour of exposure in the same experiment. Also, Kubota *et al.*, (2004) did not find the same set of genes important for ethanol-tolerance in the presence of 8% (v/v) and 11% (v/v) ethanol, indicating that the severity of ethanol stress can also contribute to the results, noting that in all of these studies different strains and media were used.

Fujita *et al.*, (2006) performed a robotic-based screen of a *S. cerevisiae* single gene knockout library to identify genes required for growth in the presence of alcohols, including 10% (v/v) ethanol. They identified 137 mutants as being ethanol sensitive, reporting a number of vacuole function-related genes as being necessary for growth in the presence of all the alcohols inspected. Similarly, van Voorst *et al.*, (2006) identified 46 genes in *S. cerevisiae* associated with impaired growth at 6% (v/v) ethanol. They also noted that none of the 22 mitochondrial-associated genes found to be important for ethanol-tolerance at 11% (v/v) ethanol (as reported by Kubota *et al.*, 2004) were sensitive to 6% (v/v) ethanol, suggesting that the mechanism of ethanol sensitivity could be ethanol concentration-dependent. This may account, together with strain differences, for the lack of correlation between deletion library screenings. There are only 4 genes (*GIM5*, *VPS36*, *SMI1* and *GIM4*) commonly reported by all three yeast genome-wide screenings (Fujita *et al.*, 2006; Kubota *et al.*, 2004; van Voorst *et al.*, 2006). The correlation between these studies is schematically presented in Figure 1.2.

Alper *et al.*, (2006) used 'global transcription machinery engineering' to isolate ethanol-tolerant strains. In this method, the binding preferences of key global transcription factors are modified by a combination of mutagenesis and selection. The paper specifically describes the introduction of mutations in the TATA-binding protein gene *SPT15* using the polymerase chain reaction, followed by selection for ethanol-tolerant phenotypes using serial subculturing in 6% (v/v) ethanol. The best performing isolate displayed a prolonged exponential growth phase, more rapid and complete glucose utilisation and increased ethanol yield under a number of different conditions and glucose concentrations. The desired phenotype was shown to be due to three mutations

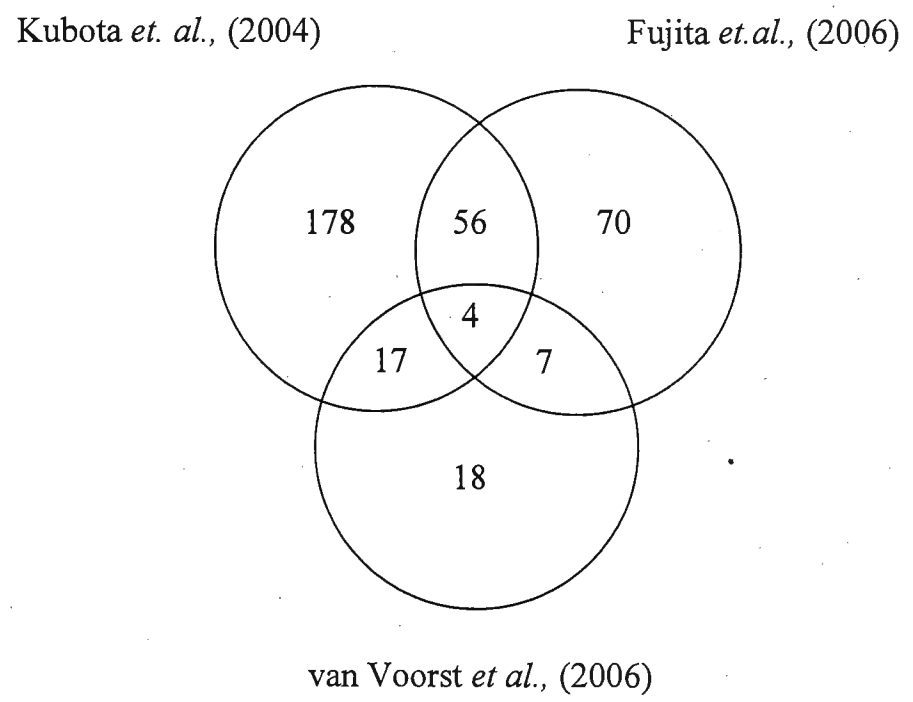

Figure 1.2: Venn-diagram comparison of the results from three independent deletion library screens of *S. cerevisiae* genes associated with ethanol-tolerance. The number of genes identified uniquely or commonly is shown.

in the *SPT15* gene that appear to alter the gene product's interaction with Spt3 – a subunit of the SAGA histone acetyltransferase that regulates a number of RNA polymerase II-dependent genes. Microarray analysis of an *spt15Δ* mutant demonstrated the overexpression of a number of Spt3-dependent genes with broad function. While overexpression of these genes individually did not produce the desired effect, many of the most highly overexpressed genes were essential for the Spt15-dependent tolerance, suggesting that each gene encodes a necessary component of a complex, interconnected network that supports the ethanol-tolerant phenotype. Such work demonstrates the complex nature of ethanol-tolerance in microorganisms and the challenges faced in attempting to increase ethanol-tolerance in strains using recombinant DNA techniques.

1.4 YEAST CENTRAL METABOLISM AND ETHANOL STRESS

1.4.1 Introduction

The fermentation of glucose to ethanol and carbon dioxide should be a redox neutral process, however, some products are removed from the cycle to form different by-products creating an excess of reducing cofactors. Biomass also contributes to the imbalance, and together with organic acid production, creates a surplus of NADH (Albers *et al.,* 1998). NADH is a major reducing cofactor and its oxidation is necessary to provide NAD^+, the driving force for oxidising metabolic reactions, so there is a need for the cell to maintain the NADH/NAD^+ balance. Figure 1.3 summarises the major processes drawing from, and contributing to, the NADH pool in the cell. Bakker *et al.,* (2001) discussed the various mechanisms for NADH reoxidation in yeast. At least five mechanisms for NADH reoxidation in *S. cerevisiae* were considered:

1. Alcoholic fermentation
2. Oxidation of intra-mitochondrial NADH via a mitochondrial 'internal' NADH dehydrogenase
3. Respiration of cytosolic NADH via external mitochondrial NADH dehydrogenases
4. Respiration of cytosolic NADH via the glycerol-3-phosphate shuttle
5. Glycerol production

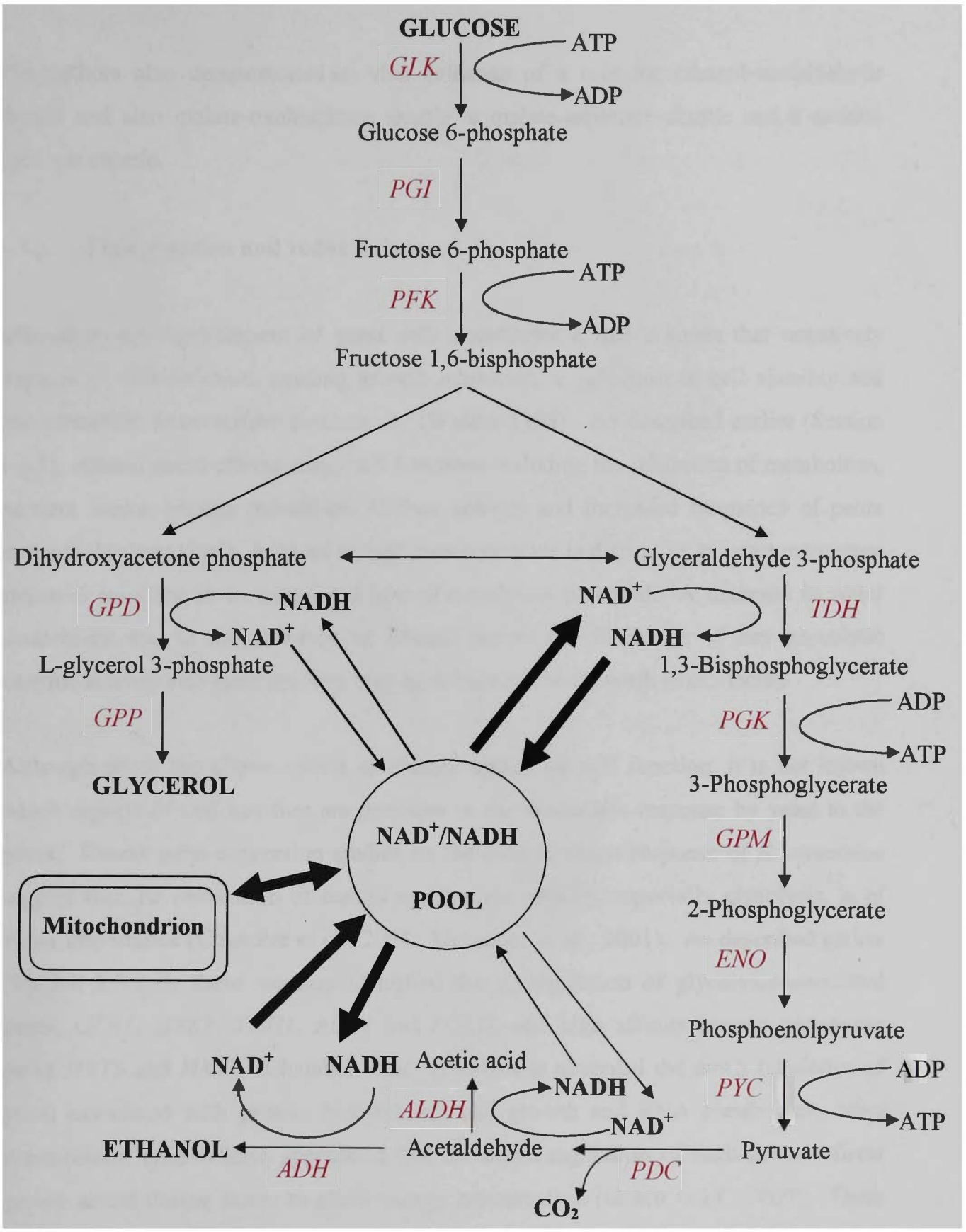

Figure 1.3: Central metabolism of *S. cerevisiae* illustrating the main contributions to the NAD^+/NADH redox equilibrium. Thick arrows represent major contributions to the NAD^+/NADH pool. The enzymes are marked in red: Glucokinase (*GLK*), Phosphoglucose isomerase (*PGI*), Phosphofructokinase (*PFK*), Aldolase (*ALD*), Triosephosphate isomerase (*TPI*), Glycerol-3-phosphate dehydrogenase (*GPD*), Glycerol-3-phosphatase (*GPP*), Triosephosphate dehydrogenase (*TDH*), Phosphoglycerate kinase (*PGK*), Phosphoglycerate mutase (*GPM*), Enolase (*ENO*), Pyruvate decarboxylase (*PDC*), Aldehyde dehydrogenase (*ALDH*), Alcohol dehydrogenase (*ADH*).

The authors also demonstrated *in vivo* evidence of a role for ethanol-acetaldehyde shuttle and also malate-oxaloacetate shuttle, a malate-aspartate shuttle and a malate-pyruvate shuttle.

1.4.2 Fermentation and redox balance

Ethanol in the environment of yeast cells constitutes a major stress that negatively impacts on cell function, causing growth inhibition, a reduction in cell viability and compromising fermentation productivity (Walker 1998). As described earlier (Section 1.3.1), ethanol stress affects many cell functions including the inhibition of metabolism, nutrient intake, plasma membrane ATPase activity and increased frequency of petite mutants (Walker 1998). Ethanol in high concentrations is disruptive to yeast membrane structure resulting in an associated loss of membrane potential. A decrease in water availability due to the presence of ethanol causes the inhibition of key glycolytic enzyme activity and these proteins may be denatured (Hallsworth *et al.*, 1998).

Although all of the above effects of ethanol impact on cell function, it is not known which aspects of cell function are priorities in the immediate response by yeast to the stress. Recent gene expression studies on the ethanol stress response of *S. cerevisiae* suggest that the restoration of central metabolism activity, especially glycolysis, is of major importance (Chandler *et al.*, 2004; Alexandre *et al.*, 2001). As described earlier (Section 1.3.2.2), these workers identified the up-regulation of glycolysis-associated genes, *GLK1*, *HXK1*, *TDH1*, *ALD4* and *PGM2*, and high affinity hexose transporter genes *HXT6* and *HXT7*. Chandler *et al.*, (2004) also observed the down regulation of genes associated with protein biosynthesis, cell growth and RNA metabolism; other stress-related studies have speculated that the down regulation of such genes reflects growth arrest during stress to allow energy conservation (Gasch *et al.*, 2000). These observations led Chandler *et al.*, (2004) to propose that when exposed to ethanol stress, yeast cells enter a pseudo-starvation state since the molecular response to ethanol stress was similar to that when cells are starved of glucose, except that in this case there was ample glucose in the medium but, because of the impact of ethanol, access for cellular catabolism was apparently limited.

The stimulatory effect of acetaldehyde on the ethanol stress response of *S. cerevisiae* appears to support the above 'pseudo-starvation' concept (Stanley *et al.,* 1997). Acetaldehyde is a metabolite in the fermentation pathway that is reduced to ethanol by alcohol dehydrogenase (Adh), which uses NADH as a cofactor, thereby regenerating cytosolic NAD^+ supply (Fig. 1.3). Although at high concentrations acetaldehyde is a known inhibitor of yeast, being regarded as more toxic to the cell than ethanol, the addition of a small amount of acetaldehyde to ethanol-stressed cultures can substantially reduce the lag period and increase the specific growth rate (Stanley *et al.,* 1993; Stanley *et al.,* 1997; Walker-Caprioglio and Parks 1987). Acetaldehyde concentrations between 0.05 and 0.5 g l^{-1} have been shown to stimulate the acclimatisation rate of yeast to ethanol shock (Stanley *et al.,* 1997). Reduction of the lag phase of ethanol-stressed yeast cultures was also observed in cultures stressed by other alcohols such as propanol and butanol (Barber *et al.,* 2002). Stanley *et al.,* (1997) suggested the added acetaldehyde replaces intracellular acetaldehyde that is lost due to the effect of ethanol stress on plasma membrane integrity. In earlier work, Stanley and Pamment (1993) demonstrated that acetaldehyde levels could be up to 10-fold higher intracellularly than outside the cell in the absence of ethanol stress, however, this concentration gradient could be lost during ethanol stress due to its impact on membrane fluidity. This loss of intracellular acetaldehyde probably leads to a redox imbalance since the Adh-catalysed reduction of acetaldehyde to ethanol is a means of regenerating cytoplasmic NAD^+. Limited availability in the cytoplasm can affect glycolytic flux by constraining glyceraldehyde-3-phosphate dehydrogenase activity, which requires NAD^+ as a cofactor (Fig. 1.3). It was speculated that added acetaldehyde could replace the lost intracellular acetaldehyde enabling more efficient NAD^+ regeneration (Stanley *et al.,* 1997).

The impact of acetaldehyde on intracellular redox balance is well documented. Betz and Becker (1975) reported that the addition of acetaldehyde (0.009 g l^{-1}) to an anaerobic yeast culture significantly reduced the NADH levels. They suggested that the added acetaldehyde was converted to ethanol, during which NADH is oxidised to NAD^+. Barber *et al.,* (2002) demonstrated that when propanal (a three carbon aldehyde) was used instead of acetaldehyde to stimulate the ethanol stress response of *S. cerevisae*, the stressed cultures had a reduced lag period similar to that when acetaldehyde was added, and an equivalent amount of propanol was produced relative to the amount of propanal lost; this supported the concept that the stimulatory effect may

be due to aldehyde reduction to alcohol, and therefore its role in maintaining the NAD^+/NADH redox balance. The stimulatory effect of acetaldehyde on the ethanol stress response of yeast supports the concept that central metabolism in ethanol-shocked yeast is struggling, and therefore so are the energetics of the cell, due to an imbalance in the redox state of the cell. This suggests that the ability of the cell to cope with ethanol stress might be improved if metabolic flux could be redirected toward alternative, NAD^+-generating pathways *i.e.* strains that are more adaptable in maintaining redox balance could have substantial advantage under conditions of high ethanol concentrations.

1.4.3 Mitochondrial-based NADH oxidation

The mitochondrion can have a significant role in NADH oxidation in aerobically-grown *S. cerevisiae*. 'Internal' NADH dehydrogenase, encoded by *NDI1*, is an enzyme that specifically reacts with NADH and is highly glucose repressible (de Vries and Grivell 1988). Also, highly increased transcription of this gene was observed when cells entered diauxic shift (De Risi *et al.*, 1997). Mitochondria isolated from *nad1Δ* mutants failed to oxidize substrates, such as ethanol, that generate intra-mitochondrial NADH. The growth of *nad1Δ* mutants on ethanol was however unaffected, which was surprising because dissimilation of ethanol would require reoxidation of intramitochondrial NADH (Marres *et al.*, 1991). This observation suggests that other mechanisms are involved in intramitochondrial NADH oxidation. Glucose repression of this gene suggests it has a minor contribution to cytosolic redox balance during growth at high glucose concentrations. Growth of a *nad1Δ* yeast strain in shake-flasks cultures on glucose as a sole carbon source did not appear to be compromised compared to the wild-type strain, suggesting a minor or no contribution of Ndi1 to the overall redox equilibrium under excess glucose conditions (Marres *et al.*, 1991).

Two isoforms of external NADH dehydrogenase, *NDE1* and *NDE2*, have been identified in yeast (Luttik *et al.*, 1998). Similarly to *NDI1*, transcription levels of *NDE1* and *NDE2* increased after diauxic shift, when cells start to utilize ethanol (Risi *et al.*, 1997), suggesting that these genes are also glucose repressed and do not have a significant role in yeast exposed to high-glucose growth conditions. Both *nde1Δ* and

nde2Δ strains grown in shake-flasks with glucose as a sole carbon source grew at a maximum growth rate that was very similar to the wild-type strain (Luttik *et al.*, 1998). This suggested that during growth on glucose, when glucose dissimilation occurs predominantly via its fermentative oxidation, external NADH dehydrogenases do not play a significant role in NAD^+ regeneration. Luttik *et al.*, (1998) also showed that mitochondria isolated from *nde1Δ* and *nde2Δ* strains failed to oxidize external NADH; the growth of such mutants was entirely respirative in aerobic glucose-limited chemostats. These observations suggest that cellular systems other than NADH dehydrogenases participate in the oxidation of cytosolic NADH (Bakker *et al.*, 2001), although their role in ethanol-stressed cultures, which may be in a pseudo-starvation state and therefore possibly not subjected to glucose repression, has not been investigated.

Under aerobic conditions, NADH can also be re-oxidised by the mitochondrial matrix using an ethanol-acetaldehyde shuttle (Bakker *et al.*, 2000; von Jagow and Klingenberg 1970). It has been suggested that acetaldehyde diffuses from the cytosol into the mitochondrion where it is reduced to ethanol by an NADH-dependant mitochondrial alcohol dehydrogenase (Adh), which is encoded by *ADH3*. This contributes to the mitochondrial redox balance as 1 mol of NADH is reduced to NAD^+ inside the mitochondrion. The produced ethanol then diffuses back into the cytosol removing redox equivalents. Valadi *et al.*, (2004) investigated the intracellular localisation of Gpd1 and Gpd2 and found that Gpd2, a cytosolic protein, also has partial mitochondrial localisation. The same authors suggested that Gpd2 is targeted to the intermitochondrial membrane space, where it can drive glycerol phosphate production in the intermembrane space, generating a NAD^+/NADH gradient that provides a driving force for the ethanol-acetaldehyde shuttle (Fig. 1.4).

Excess cytoplasmic NADH can also be oxidized under aerobic conditions by transferring electrons from cytosolic NADH to the mitochondrial electron transport chain. This can be achieved via shuttle systems such as the glycerol 3 phosphate (G3P) shuttle (Fig. 1.5). The G3P shuttle also supports ATP production from cytoplasmic NADH through oxidative phosphorylation. Dihydroxyacetone phosphate (DHAP) in the cytosol is reduced to G3P followed by NADH oxidation to NAD^+. G3P then crosses

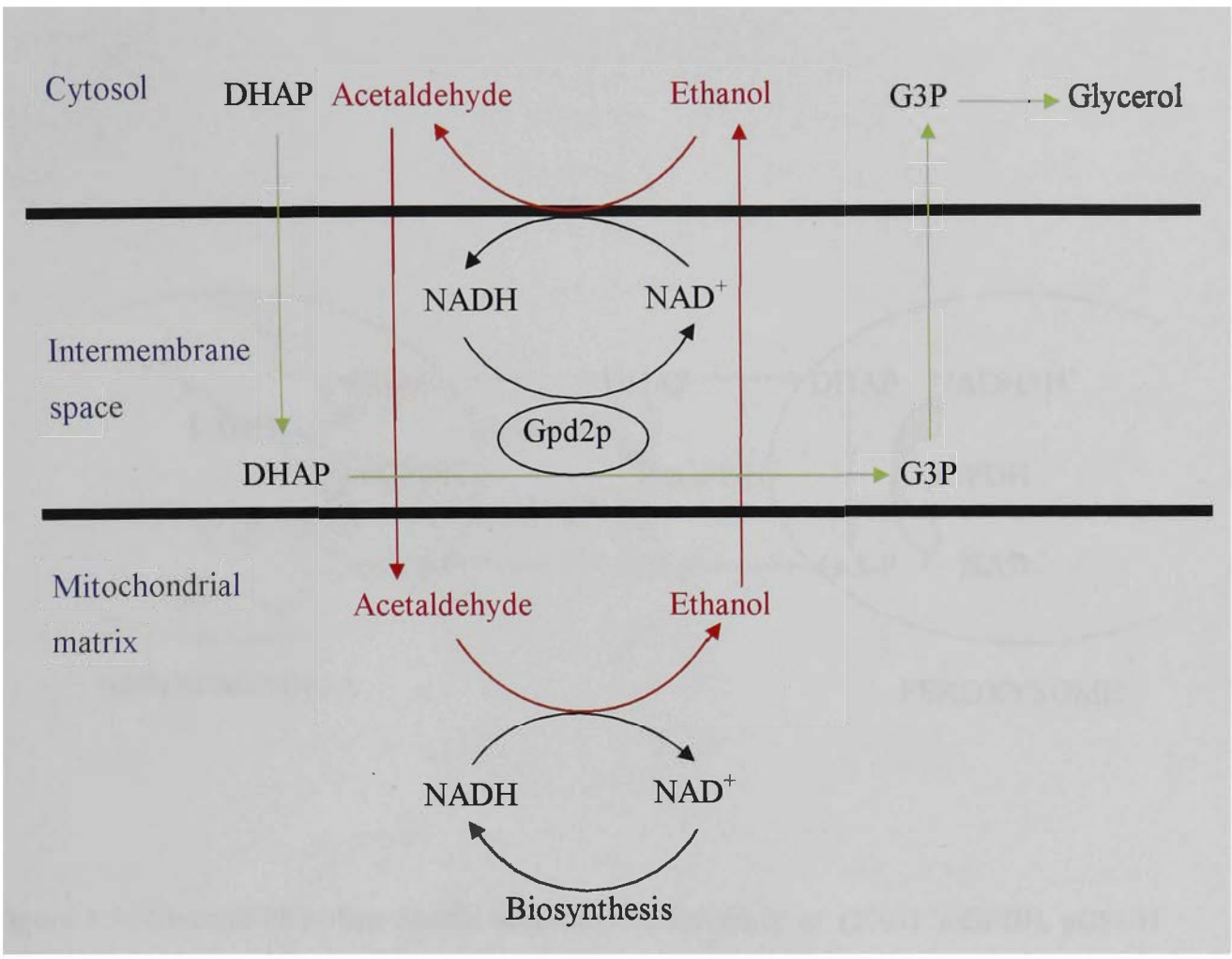

Figure 1.4: The driving force for the Ethanol-Acetaldehyde Shuttle is provided by Glycerol phosphate (G3P) production in the mitochondrial intermembrane space through Gpd2p (mitochondrial glycerol 3-phosphate dehydrogenase) (Valadi *et al.*, 2004).

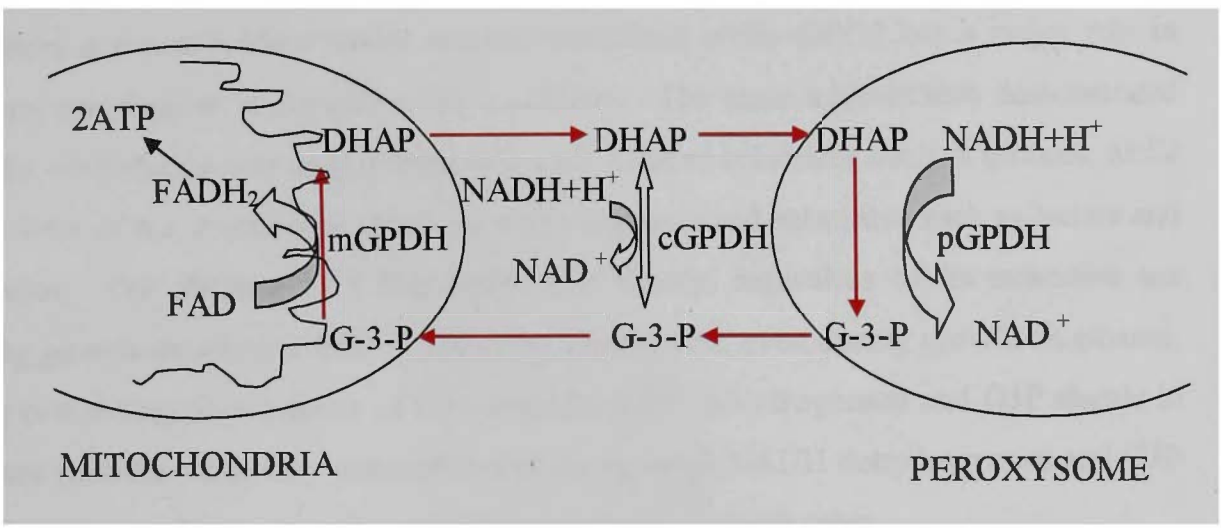

Figure 1.5: Glycerol Phosphate Shuttle adapted from Brisson *et al.* (2001): mGPDH, pGPDH and cGPDH are mitochondrial, peroxisomal and cytoplasmic GPDH.

the mitochondrial membrane where it is oxidised to DHAP via mitochondrial FAD-dependant Gut2 dehydrogenase and returned to cytosol where it is reduced again using NADH as a cofactor, generating more NAD^+ (Larsson *et al.,* 1998; Overkamp *et al.,* 2002). Larsson *et al.,* (1998) demonstrated that the dehydrogenase that catalyses the cytosolic reaction of the G3P shuttle is encoded by the *GPD1* gene. It was found that *GPD2* had no significant role in the shuttle. This is contrary to previous results observed under anaerobic conditions where the deletion of *GPD1* did not affect anaerobic growth while deletion of *GPD2* resulted in a considerable reduction in growth rate (Ansell *et al.,* 1997). The *GPD1*-encoded enzyme is therefore important for maintaining redox balance under aerobic conditions while *GPD2* has a major role in glycerol metabolism under anaerobic conditions. The same authors also demonstrated that the G3P shuttle was used extensively with reduced substrates such as ethanol, while no activity of the shuttle was observed with more oxidised substrates such as lactate and pyruvate. The absence of a functional G3P shuttle, regardless of its extensive use during growth on ethanol, did not affect the growth rate, even during growth on ethanol. After comparing performance of the external NADH dehydrogenase and G3P shuttle in isolated mitochondria they concluded that the external NADH dehydrogenase and G3P shuttle operate additively with capacity to substitute for each other.

Pahlman *et al.,* (2001) measured enzyme and respiratory activities in isolated mitochondria and found that both external NADH dehydrogenase and the G3P shuttle activity diminished at elevated dilution rates, and therefore glucose concentrations, in chemostat-grown cultures. They suggested that adjustment of the activities of these two systems is achieved by activation or inhibition of existing enzymes rather than changing the amount of enzyme present. Both the external NADH dehydrogenase and the G3P shuttle were sensitive to inhibition by ATP, with G3P activity being more affected than the former. The authors suggested that external NADH dehydrogenase is the main system employed for oxidation of cytosolic NADH.

1.4.4 Glycerol production and redox balance

Glycerol, a small and simple polyalcohol, can have a significant role in yeast sugar metabolism. It can cross the yeast plasma membrane although this can only occur via active transport through a Stl1 glycerol/H^+ symporter (Ferreira *et al.*, 2005) or by the constitutively expressed Fps1 channel (Oliveira *et al.*, 2003). It is the main compatible solute in *S. cerevisiae*. The key enzyme in the glycerol pathway is encoded by *GPD2* with its isoform, *GPD1*, being associated primarily with the osmotic stress response (Ansell *et al.*, 1997). Its production is increased with heat shock treatment (Kajiwara, Y. *et al.*, 2000), salt stress (Carvalheiro *et al.*, 1999) and during osmotic stress (Krantz *et al.*, 2004; Mager and Siderius 2002; Mager and Varela 1993; Modig *et al.*, 2007; Wojda *et al.*, 2003). Glycerol is also known to maintain redox balance during fermentation at the expense of ethanol production, moreover it is considered to have a major role in intracellular redox balance (van Dijken *et al.*, 1986). Glycerol is involved in NADH oxidation and the restoration of cytoplasmic redox balance during both aerobic and anaerobic cultivation of yeast (van Dijken *et al.*, 1986). This is supported by the observation that mutants having a blocked glycerol pathway are unable to grow anaerobically (Ansell *et al.*, 1997; Bjorkqvist *et al.*, 1997; Pahlman *et al.*, 2001).

The dynamics of redox-linked interplay between ethanol and glycerol production has been the basis of the design of *S. cerevisiae* strains for either increased or decreased ethanol production. Reducing ethanol production by increasing glycerol production in wine yeast strains is highly desirable in winemaking for the production of low-alcohol wines (Michnick *et al.*, 1997). The concept being that higher glycerol production will increase intracellular NAD^+ supply, which in turn reduces ethanol production either by NAD^+ inhibition of alcohol dehydrogenase activity, or due to limited NADH availability (Fig. 1.3). These investigations mostly study the effect of over-expression or deletion of *GPD1* on yeast metabolism and in most cases it was found that overexpression of *GPD1* caused an increase in glycerol production, a decrease in ethanol yield, increases in the production of acetaldehyde, pyruvate, acetate and lower biomass yields (De Barros Lopes *et al.*, 2000; Michnick *et al.*, 1997; Nevoigt and Stahl 1996; Remize *et al.*, 1999). Remize *et al.*, (1999) suggested that the increase in acetate production in strains overproducing glycerol was due to redox maintenance by providing additional NAD(P)H, since 1 mol of acetate from glucose generates 2 mol of

NAD(P)H. It was also suggested that the decrease in biomass could be due to an increase in ATP consumption resulting from the diversion of carbon towards glycerol.

A number of studies have been conducted using the reverse approach to that described above *i.e.* engineering strains with decreased glycerol metabolism to increase ethanol production. Ethanol production was found to increase in *gpd2Δ* mutants (Valadi *et al.*, 1998), in strains with overexpressed *GLT1* in a *gpd1Δ* background (Kong *et al.*, 2007) and in strains with deleted *FPS1* and overexpressed *GLT1* (Kong *et al.*, 2006). Valadi *et al.* (1998) compared the growth and metabolic profiles of *gpd1Δ* and *gpd2Δ* mutants under anaerobic conditions. The *gpd1Δ* mutant had a slight reduction in glycerol formation, while the *gpd2Δ* mutant showed a 40% reduction in glycerol production. As a consequence of the reduction in glycerol formation, the *gpd2Δ* strain produced 13% more ethanol than the wild-type strain. Although the growth profiles of the wild-type and *gpd1Δ* mutant were indistinguishable, the *gpd2Δ* mutant appeared stressed with an extended lag phase and reduced specific growth rate. The authors also noted that *GPD1* expression increased in the *gpd2* deletion mutant, suggesting that GPD1 is partly compensating for the loss of *GPD2* even under anaerobic conditions. This was consistent with a previously reported decrease in glycerol production by a *gpd1Δ* mutant of *S. diastaticus,* with the *gpd1* deletion strain producing 50% less glycerol and slightly higher ethanol production (Wang *et al.*, 1994).

Kong *et al.*, (2006) created two mutants of *S. cerevisiae* KAM-2, with one of the mutants, KAM-3, containing a *FPS1* gene deletion, which encodes a channel responsible for glycerol export. This was expected to cause accumulation of glycerol in the cell, potentially causing feedback inhibition of glycerol biosynthesis. The second mutant, KAM-11, also had an *FPS1* gene deletion but, this time, combined with over-expression of *GLT1*, whose product is the enzyme glutamate synthase. For KAM-11, it was speculated that decreased glycerol production could create an excess of NADH, which may be decreased by the formation of glutamate; *GLT1* controls glutamate expression therefore over-expression of *GLT1* should reduce intracellular NADH levels. The authors found no change in glucose consumption, biomass or protein content between the 3 strains. Both mutants showed similar higher intracellular glycerol levels compared to the parent strain, consistent with inability to export glycerol. The KAM-3

and KAM-11 mutants produced 10% and 14% more ethanol respectively than the parent KAM-2 strain and both mutants produced substantially less acetate and pyruvic acid.

In subsequent work, Kong *et al.*, (2007) created more *S. cerevisiae* KAM-2 mutants; KAM-4 contained a *GPD1* gene deletion to reduce glycerol synthesis, and KSM-12 contained a *GPD1* deletion as well as *GLT1* overexpression, again to increase NADH oxidation in a glycerol-compromised cell. The growth rate of KAM-4 was slightly lower than the parent and KAM-12. Glucose consumption, biomass and protein content were unchanged in the mutants compared to the wild-type; glycerol production by KAM-4 and KAM-12 was reduced by 21% and 25% and ethanol production increased by 4.8% and 10.8% respectively; a substantial reduction in the formation of acetate and pyruvate was also measured. The improved growth and ethanol production by KAM-12 compared to KAM-4 suggests that NADH can be inhibitory in cells without a functioning glycerol metabolism.

It is clear that the NADH/NAD^+ redox balance in *S. cerevisiae* has significant influence on the functioning of cell metabolism. Central metabolism in particular is tightly regulated by this redox balance, with low NAD^+ levels inhibiting glycolytic flux, as appears to be the case during ethanol stress although the latter requires experimental confirmation. It may be that the ability of a particular strain to oxidise NADH, by mitochondrial activity and/or glycerol and ethanol production, during times of ethanol stress could considerably influence its ability to respond to the stress.

1.5 GENERATING ETHANOL-TOLERANT *S. cerevisiae* MUTANTS

The use of site-directed mutagenesis to improve the ethanol-tolerance of *S. cerevisiae* is largely not effective due to our limited knowledge, and the complexity, of the stressor-related mechanisms that inhibit cell performance, and the intricate nature of the stress response. The creation of strains that are more stress tolerant is however achievable using random mutagenesis approaches such as chemical mutagenesis or evolutionary engineering, and an appropriate selection pressure to isolate stress-tolerant variants. Evolutionary engineering works on the principle that cells will adapt to their environment by the process of natural selection. Most evolutionary engineering experiments in recent times have used continuous culture to bring about evolution-based

change in the organism. Chemostat-based experiments have a number of advantages, such as automated control and monitoring of variables, the ability to apply selective pressure over thousands of generations and improved stability of the isolated mutants.

A number of studies have used evolutionary engineering to create yeast mutants that are tolerant to various stressors, such as freeze-thaw (Takagi *et al.,* 1997), temperature (Wati *et al.,* 1996) salt concentration (Matsutani *et al.,* 1992), acetic acid concentration (Aarnio *et al.,* 1991) and multiple stressors (Cakar *et al.,* 2005). Given the potential of evolutionary engineering to create mutants with improved stress-tolerance, it is surprising that there is only one reported study that uses this approach to create more ethanol tolerant yeast strains (Brown and Oliver 1982). These authors created ethanol-tolerant mutants of *Saccharomyces uvarum* using a continuous culture with frequent, semi-continuous ethanol addition. By measuring CO_2 released from the culture, the system determined the 'fitness' of the culture and this information was used to modulate the selection pressure by increasing the ethanol concentration as the culture evolved increased levels of stress-tolerance. After 344 generations had been exposed to the ethanol stress, five mutants were isolated on plates containing 12% (v/v) ethanol. These mutants showed higher CO_2 production rates compared to the parent strain in the presence and absence of ethanol, suggesting that the mutants had evolved by increasing their fermentation rate. Growth and metabolic phenotypes of the variants were not determined since the purpose of the study was to demonstrate the technique of evolutionary engineering and the use of ethanol as a selection pressure for creating ethanol-tolerant mutants; it was followed by a subsequent paper that presented a mathematical analysis of the technique (Lane *et al.,* 1999).

Chemical mutagenesis is effective at generating genetic diversity, however, the subsequent isolation of stable mutants with desired characteristics can be time consuming and, in some cases, unproductive. This process can be made considerably more effective by using a adaptive evolution and an appropriate selection pressure to enrich ratio of chemically-mutated variants with desired characteristics. The benefits of using adaptive evolution enrichment include the removal of uncompetitive mutants from the variant mix and, having endured the selection pressure over a number of generations, the subsequent isolates are generally more stable. Jimenez and Benitez (1988) used a chemostat to enrich ethanol-tolerant yeast hybrid strains that were created

by crossing ethanol-tolerant wine yeast strains with laboratory yeast strains having low ethanol-tolerance. The authors created 25 yeast hybrids and subjected the strains to competitive growth under semi-continuous culture conditions in which the ethanol concentration in the feed was slowly increased. A hybrid strain was eventually isolated that was more ethanol tolerant than the parental wine strains and also showed a slight increase in ethanol production rate and ethanol yield compared to the parental strains. Although chemostat-based selection of stress-resistant mutants offers many advantages over traditional selection methods, it is not always productive. Cakar *et al.*, (2005) tested a number of different selection procedures on an EMS-treated *S. cerevisiae* strain to isolate mutants that had acquired improved tolerance to multiple stressors, in particular ethanol, heat, oxidative and freeze-thaw stress. Following EMS-mutagenesis, they applied various stress conditions at periodic intervals in a continuous culture inoculated with the variants but they were unable to successfully isolate mutants with improved stress tolerance. After a second round of EMS treatment, they used batch cultures and stress conditions to enrich stress-tolerant mutants and observed some improvement in the freeze-thaw stress resistance of some isolates. The authors found that the best selection strategy for obtaining mutants with improved multiple stress resistance was to incubate the EMS-treated cells batchwise and subject the strains to a freeze-thaw stress. The isolates obtained from this procedure were not only improved in their freeze-thaw stress resistance, but also in their resistance to other stressors such as temperature, ethanol and oxidative stress. It was suggested that a freeze-thaw approach could be a useful strategy for industrial strain improvement if strains with multiple stress resistance were sought. Although only limited characterization of their stress-resistant strains was performed, the authors indicated that more detailed strain analysis, especially transcriptomic, proteomic and phenomic, was being performed to determine how the strains had improved their stress tolerance; a subsequent study has not yet been reported by the authors.

1.6 AIMS AND OBJECTIVES

Ethanol stress can inhibit the performance of yeast cultures resulting in low fermentation productivities and ethanol yields. Although limited, the yeast stress response can provide some protection from the inhibitory effects of ethanol stress, however, the cellular mechanisms involved in this protection are not well understood. The objective of this project was to create ethanol-tolerant mutants of *S. cerevisiae* and to determine the molecular and metabolic factors that conferred stress tolerance. Such information could inform strategies for the construction of yeast strains with improved fermentation rates, a longer productive life and higher ethanol yields, which would be of considerable importance to fermentation industries.

The specific aims were to:

i) Create ethanol-tolerant *S. cerevisiae* mutants using adaptive evolution strategies.

ii) Identify metabolic changes in the mutants that may be responsible for adaptively evolved ethanol-tolerance.

iii) Identify areas of cell function that appear to have a significant role in ethanol-tolerance by comparing gene expression profiles of *S. cerevisiae* parent and mutant strains in the absence and presence of ethanol stress.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Yeast strain

The strain used in this study was *Saccharomyces cerevisiae* W303-1A, (*MAT***a** *leu2-3, 122 trp1-1 can1-100 ura 3-1 ade2-1 his3-11, 15*) a haploid laboratory strain. The strain was developed from parent *S. cerevisiae* W303 (see Replogle *et al.*, 1999; Thomas and Rothstein 1989). W303-1A possesses a ybp1-1 mutation (17L, F328V, K343E, N571D) which abolishes Ybp1p function, therefore increasing sensitivity to oxidative stress (Veal *et al.*, 2003). W303-1A was chosen due to its good track record for work in yeast genetics and molecular biology research; it has a stable genotype and a range of good auxotrophic markers making both easy to work with and reliable. The impact of the ybp1-1 mutation on ethanol tolerance was not determined for my thesis, but it is known that W303 (the parent of W303-1A, which lacks the ybp1-1 mutation) has the same level of ethanol tolerance phenotype as W303-1A (Paul Chambers, Personal Communication), and therefore it is unlikely that the ybp1-1 genotype impacted on the project results. The spontaneous mutant (SM1) was created using adaptive evolution by subjecting wild-type W303-1A strain to ethanol selection pressure in a chemostat for approximately 500 generations. The Chemical Mutant (CM1) was created by initially employing chemical mutagenesis of wild-type W303-1A using Ethyl Methane Sulphonate (EMS) and then enriching the variant population using adaptive evolution with ethanol as the selective pressure. Mutagenesis is described to more detail in Section 2.3.

2.1.2 Buffers and solutions

All buffers and solutions were made using Analytical grade chemicals unless otherwise stated. Distilled and de-ionised Milli-Q water (Milli-Q Plus Ultra Pure Water System, Millipore, Billerica, MA, USA) was used for making up all solutions. The formulae for all buffers and solutions are provided in Appendix 1.1. Buffers and solutions were

sterilized by autoclaving (121°C/15 min) or, where indicated, filter sterilizing using a 0.22 μm or 0.45 μm Millipore membrane filter. Buffers and solutions for RNA work were prepared in RNase-free glassware using diethyl pyrocarbonate-treated water (DEPC 0.1%). All of the glassware and metal components for preparing solutions for RNA work were baked overnight at 200°C. Plasticware was soaked according to safety regulations in a fumehood overnight in 0.1% DEPC and autoclaved the next day. All of the solutions used in the microarray experiments were prepared as for RNA work, including 0.22 μm filtration. All pH adjustments were made using NaOH (1M) or HCl (1M) unless stated otherwise.

2.1.3 Equipment

The incubation of yeast cultures was performed using Erlenmeyer flasks or sidearm flasks in an orbital-shaker incubator (Innova 4231 refrigerated incubator, New Brunswick Scientific, Edison, New Jersey). Chemostats were also used for adaptive evolution experiments. The chemostats comprised 1.2 litre fermentor bowls operated with a 1 litre working volume. A dilution rate of 0.073 h^{-1} was maintained by a Masterflex C/L Pump System (Extech Equipment Pty. Ltd.). Culture broth was removed from bioreactor via small exit port (sited at a level to maintain a 1 litre working volume) which was under continuous vacuum by Masterflex Console Drive peristaltic pump (Extech Equipment Pty. Ltd.).

The spectrophotometer used for all experimental analysis was the LKB Ultraspec Plus, 4054 UV/Visible spectrophotometer (Pharmacia). Centrifugation was conducted using either a Beckman CS-15R swinging rotor centrifuge, an Eppendorf 5415C bench top microfuge (Eppendorf, GmbH, Englesdorf, Germany) or a vacuum centrifuge Savant Speedvac SC116 (Selby Scientific and Medical). PCR was performed in a PTC-100 programmable thermocycler with a heated lid (MJ Research Inc., Waltham, MA, USA). Gels were photographed with a UVP Laboratory Products gel documentation system (Upland, CA, USA) or with UV/visible Darkroom (Pathtech Pty Ltd) connected to Labwork™ analysis software and Digital Graphic printer Up-D890 and Intelligent Dark box II. Microarray slides were hybridised in an Extron HI 2001 incubator (Bartelt Instruments Pty Ltd) and scanned using a GenePix-Pro 4000 scanner (Axon).

2.2 PHYSIOLOGY EXPERIMENTS

2.2.1 Medium

Cultures of *S. cerevisiae* were grown in a defined or nutrient-rich YEPD medium. Medium and culture vessels were autoclaved at 121°C for 20 minutes. The glucose component of the medium was autoclaved separately. In defined medium preparations, all components were filter sterilized using a 0.22 μm Millipore membrane filter for liquid medium or, in the case of solid medium, glucose and agar components were autoclaved separately and mixed with remaining filter-sterilised components prior to pouring plates. All water used for the growth medium was distilled and de-ionised Milli-Q water.

Defined medium (liquid) contained per litre: 20 g D-glucose, 5 g ammonium sulphate and 1.7 g yeast nitrogen base, without amino acids and ammonium sulphate (Difco). The yeast nitrogen base was prepared according to the manufacturer's instructions as a 10× solution (1.7 g nitrogen base in 100 ml sterile water). This solution was filter sterilized using a 0.22 μm filter prior to adding 900 ml of autoclaved glucose and ammonium sulphate. Amino acids and uracil were prepared as stock solutions (Table 2.1), filter sterilised and stored at 4°C; the exceptions were uracil and adenine which were stored at room temperature to prevent precipitation.

Defined medium (solid) was prepared as for liquid medium but with the addition of bacto-agar (15 g). Control plates were made comprising the addition of all 5 supplements listed in Table 2.1. Drop-out plates were made by omitting one of the supplements.

YEPD medium comprised per litre: 10 g yeast extract, 20 g bacto-peptone, 20 g D-glucose and, in case of solid medium only, 15 g bacto-agar. The components were dissolved in distilled de-ionised water and autoclaved at 121°C for 20 minutes.

Table 2.1: Defined medium components.

Defined medium component	Final concentration (mg l^{-1})	Stock solution: (per 100 ml ddH_2O)	Stock solution added to 1 litre of defined medium (ml)
Adenine sulphate	20	200 mg	10
Uracil	20	200 mg	10
L-tryptophan	20	1 g	2
L-histidine-HCL	20	1 g	2
L-leucine	60	1 g	6

Glycerol storage medium comprised 2 × YEPD per litre: 40 g bacto-peptone, 20 g yeast extract and 40 g glucose, with the addition of 15% (v/v) glycerol. All of the components were dissolved in distilled and de-ionised water and autoclaved at 121°C for 20 minutes. This medium was used for long-term storage of all yeast strains at -20°C or -80°C.

2.2.2 Yeast cultivation

2.2.2.1 Standard culture conditions

Yeast cultures were grown under aerobic conditions in YEPD medium at 30°C and shaken at 110 rpm in an orbital-shaker incubator, unless otherwise stated. The culture vessels were Erlenmeyer or sidearm flasks (500 ml) with cotton wool plugs and with working volumes of 200 ml. To harvest cells for RNA extractions, cultures were grown in sidearm flasks (2 L), which had working volumes of one litre.

2.2.2.2 Yeast strain preservation

Long-term storage of yeast cultures was done in 2 ml vials containing 1 ml of sterile glycerol storage medium at -20°C or -80°C. Strains were initially grown on YEPD

plates, yeast colonies were then collected with sterile applicator sticks, avoiding petite mutants, and suspended in the glycerol storage medium. For short-term storage, YEPD agar plates were streaked using -80°C glycerol stock, incubated at 30°C for 2-3 days and then stored at 4°C for up to 2 weeks, at which time they were replaced.

2.2.2.3 Inoculum preparation

To prepare inocula for growth curve experiments, a loopful of cells was taken from YEPD culture plates and aseptically transferred into 200 ml of YEPD medium in a 500 ml sterile Erlenmeyer flask. The yeast cells were incubated overnight at 30°C in an orbital shaker at 110 rpm. On the following day, three parent cultures, each containing 200 ml of fresh YEPD medium, were inoculated to an initial OD_{620} of 0.03, 0.05 and 0.1 to ensure that, at the time of inoculation of experimental cultures, at least one of the parental cultures was in late exponential phase (OD_{620} of 1) at 30°C/110 rpm. The parent culture closest to an OD_{620} of 1 was used as an inoculum whereas the other two cultures were discarded. Selected parent culture cells were collected by centrifugation at 4,000 rpm (3,313×g) in a swinging rotor centrifuge (Sorvall® RT 7) for 5 minutes. The supernatant was discarded and the cells washed in pre-warmed (30°C) fresh medium. The temperature in the centrifuge was maintained at 30°C during the washing procedure. Following washing of the cells, the OD_{620} of the parent culture was used to determine the inoculum size required to achieve an initial OD_{620} reading of 0.1 in the experimental cultures.

2.2.2.4 Ethanol stress experiments (batch growth and survival experiments in the presence of ethanol)

Before batchwise ethanol stress experiments commenced, fresh YEPD medium and all glassware was pre-warmed to 30°C to minimise the effect of temperature shock and to ensure that ethanol was the principal stressor of the cells. For each ethanol stress experiment (batch incubation), a control culture was prepared using the same medium and conditions but without added ethanol. A calculated volume of exponential phase parent culture was inoculated to achieve an initial OD_{620} of 0.1 (approximately 2×10^6 cell ml^{-1}) into the control and experimental flasks containing pre-warmed YEPD

medium. The cultures were immediately transferred to the shaker incubator and grown under aerobic conditions at 30°C/110 rpm. Samples for optical density and viable plate counts were taken at regular intervals during incubation, serially diluted and the appropriate dilutions were plated on duplicate YEPD plates.

2.2.2.5 Sampling and harvesting cells in ethanol stress experiments

The sidearm of an Erlenmeyer flask was wiped with ethanol (70% v/v) and the initial 5-10 ml of culture removed via a sterile syringe was discarded before the sample volume was collected. Sampling was performed initially at time 0 (time of inoculation) and then at regular intervals during the course of the incubation. Samples (around 3-5 ml) were taken for optical density measurements and viable plate counts.

For extracting RNA and for metabolite analysis, culture samples were harvested with special care. Sample volumes were increased to 100 ml, separated into two 50 ml Falcon™ tubes, and the cells pelleted by centrifugation for 10 minutes at 30°C in a swing rotor centrifuge (Sorvall® RT 7 Centrifuge) at 4,000 rpm (3,313 g). This was done in order to ensure quantitative cell recovery, since equal cell numbers were used for RNA extraction as explained in Section 2.5.1. The supernatant and pellet were separated, snap-frozen in liquid nitrogen and stored at -80°C. The supernatant was used for metabolite analysis and the pelleted cells for RNA extractions.

2.2.2.6 Competition experiments

Competition experiments were performed in 500 ml Erlenmeyer flasks containing 200 ml of YEPD medium with or without added ethanol. All of the cultures were grown under aerobic conditions at 30°C with continuous agitation at 110 rpm. To create a mixed population, equal cell numbers of the two competing strains, from late exponential phase parent cultures (OD_{620} of approximately 1), were inoculated into YEPD medium in the presence or absence of ethanol. Samples were taken on a regular basis and plated, as described for batch experiments, on duplicate YEPD plates and the viable populations determined. The selection criteria (used to distinguish between the

strains) were based on the unique culture physiology of each strain when grown on YEPD plates, as described in Section 3.6.4.

2.3. MUTAGENESIS AND SELECTION OF ETHANOL TOLERANT MUTANTS

2.3.1 Chemical mutagenesis

Chemical mutagenesis was performed using the mutagen ethyl methane sulphonate (EMS). The toxicity and mutagenicity of EMS required all of the EMS experiments to be performed in the fume hood. All glassware, plasticware and solutions that came in contact with EMS were rinsed in 5% sodium thiosulphate to inactivate the EMS and disposed of in the mutagenic waste container according to safety regulations. Prior to performing mutagenesis experiments, the optimisation of mutagenesis conditions was performed using EMS kill curves.

2.3.1.1 EMS kill curves

EMS kill curves were performed using overnight cultures grown under standard conditions (30°C/110 rpm). Equal volumes of culture, containing around 1×10^8 cells, were transferred in 5 ml aliquots into centrifuge tubes, centrifuged and the culture broth replaced with 0.01M phosphate buffer. One culture was grown for sacrifice at each time-point to avoid opening EMS-containing cultures as required for classical sampling. The tubes were loosely screwed and wrapped in parafilm. The cultures were incubated horizontally in a shaking incubator. Shaking was adjusted so that slight rolling of tubes occurred. One culture was removed from incubator for each time-point. The culture was centrifuged and the EMS supernatant discarded into the liquid mutagen waste disposal unit. This was followed by a thorough washing of the mutagenised yeast pellet, firstly with 5% sodium thiosulphate, then with autoclaved distilled water and finally with 10% ascorbic acid. The remaining pellet was resuspended in YEPD to the original volume and each suspension diluted and plated onto YEPD plates in duplicate. Plate incubation and viable population determination was performed as previously described.

2.3.1.2. Mutagenesis

EMS-based mutagenesis was found to have optimum results using 1% EMS and an incubation time of 1 hour. The EMS mutagenesis experiments were performed as follows. *S. cerevisiae* W303-1A was grown overnight to a population of approximately 1-1.2 × 10^8 cells ml^{-1}. A portion of the culture (50 ml) was transferred into Falcon™ tubes, the cells were centrifuged and culture broth was replaced with 0.01M phosphate buffer. EMS was added to a concentration of 1% (v/v) and the tubes wrapped in parafilm and placed in small plastic bags sprayed with 5% sodium thiosulphate. The samples were incubated for 1 hour, centrifuged and subsequently washed with 5% sodium thiosulphate, then with autoclaved distilled water and finally with 10% ascorbic acid. The pellet was resuspended in YEPD and inoculated into the 1 L Chemostat fermentors (containing YEPD and 7.5% (v/v) ethanol) and grown batchwise. If the batch culture recovered and reached stationary phase, adaptive evolution experiments were started by turning on the feed pump containing 7.5% (v/v) ethanol in the medium at a dilution rate of 0.076 h^{-1}. Ethanol selection pressure was maintained as described in Chapter 3, Section 3.4. Samples were taken daily and measured for ethanol tolerance as described below.

2.3.2. Adaptive evolution

Adaptive evolution experiments were performed by exposing a chemostat-grown yeast culture to constant ethanol selection pressure (as described in Chapter 3, Section 3.4). The chemostat bioreactor was inoculated to an optical density (620 nm) of 0.1 and initially cultured batch-wise without added ethanol. Upon reaching the late exponential phase, the feed pump was turned on and fresh medium containing ethanol (initially at 7% v/v) was fed into the bioreactor at a dilution rate of 0.073 h^{-1}. After 14 days the biomass level in the culture had substantially decreased (to an OD_{620} of 0.005) and the cell population was close to being totally washed out. To revive the culture, the selection pressure was reduced by decreasing the ethanol concentration to 4% (v/v); when the cell population had recovered, the ethanol concentration was increased to 5% (v/v). This was the operational procedure for the adaptive evolution experiment over a continuous 6-month period. The maximum ethanol concentration that could be endured

by the culture before washout gradually increased during this time. After 6 months (192 days) of continuous operation at a dilution rate of 0.073 h^{-1} (*i.e.* doubling time of 9.49 hrs), the culture had experienced 486 generations and was able to grow at a relatively high optical density with 8.5% (v/v) ethanol in the feed, indicating that the population had evolved over this time.

During each chemostat experiment, the culture was sampled daily for optical density and viable cell population. In the later stages of the experiment, colonies from chemostat samples grown on solid medium were subjected to phenotype analysis (see below). After 6 months of chemostat operation, a significant increase in the ethanol tolerance of yeast isolates from the culture was observed. The chemostat experiment was stopped when ethanol-tolerant isolates were found to be stable, retaining their ethanol-tolerant phenotype after storage at -20 and -80°C and growth in the absence of an ethanol selective pressure for five overnight serial cultures. Selected isolates (see Section 3.5) were cultivated to generate a healthy parent culture, which was then used in growth and survival experiments for comparison with the wild-type phenotype under the same conditions.

2.3.3. Selection and isolation of ethanol tolerant mutants

Samples (10 ml) were taken from chemostat cultures on a daily basis, and plated on YEPD plates. These samples were inoculated directly into fresh YEPD medium (200 ml) and grown for a minimum of five successive overnight cultures without added ethanol. The last (fifth) overnight culture was subjected to an ethanol stress experiment using 18% and 20% (v/v) ethanol as described in Section 2.2.2. The samples were taken hourly and a minimum of 8 plates were plated for each time-point. Colonies from the plates that survived for the longest period of time were selected, grown for three successive overnight cultures without added ethanol and subjected to an ethanol stress experiment using a range of ethanol concentrations (0 - 20% v/v). Specific growth rates and lag period duration were measured and compared to that of the wild-type, the best performing cultures were selected and stored at -80°C and -20°C.

2.4 METABOLITE ANALYSIS

Analysis of glucose, glycerol, acetic acid and ethanol concentrations was performed on a Varian Star Chromatography Workstation, using a BIORAD organic acid column HPX-87H and RI detector ERC-7515A by ERMACR.INC. Culture supernatants were thawed from -80°C, filtered (0.22 μm) and diluted in sterile miliQ water to an appropriate dilution. Isovaleric acid (the internal standard) was added to each sample vial to achieve a final concentration of 0.25% (v/v) in both sample and standard solutions. Standards were prepared by diluting stock solutions of acetic acid, glycerol, glucose and ethanol to concentrations that covered the full range of detection sensitivity as specified for the column. All standards were prepared using the highest purity (HPLC grade) chemicals. The final volume in all HPLC vials was 1ml.

The mobile phase comprised filtered (0.45 μm) 5 mM sulphuric acid pumped at a pressure of 50 atm. Helium was used for continuous degassing of the mobile phase. Each sample was injected (20 μl) into the column operating at 60°C and with a mobile phase flow rate of 0.6 ml min^{-1}; each run lasted 30 minutes. All of the peaks in both standards and samples were well resolved as shown in Figure 3.17. The results were viewed and analysed using Star 6.41 Chromatography Workstation software.

2.5. MOLECULAR METHODS

2.5.1. RNA extraction

All procedures for extraction and handling of RNA were carried out under RNase-free conditions. All glassware was baked overnight at 200°C. All solutions were prepared using 0.1% DEPC treated ddH_2O and filter-sterilised (0.22 μm). Benches and other work areas including fumehoods were sprayed with RNase ERASE (ICN) or treated with RNase ERASE wet wipes (ICN). All Plastic ware was purchased RNase-free or soaked overnight in DEPC (0.05%) followed by autoclaving. Gel tanks were sprayed with RNase ERASE and rinsed with DEPC-treated water. Certified RNase-free barrier pipette tips were used for all procedures. Glass beads (0.4 μm, B. Braun Biotech International) were acid washed and baked overnight at 200°C. RNA samples were kept on ice at all times and centrifugation was performed at 4°C.

RNA for microarray analysis was extracted from an equal number of cells representing each experimental condition. Frozen cell pellets (-80°C) from 50 ml of culture were quickly resuspended in pre-calculated volume of cold RNA buffer to give final concentration of $2x10^7$ yeast cells per 400 µl. RNA extraction was carried out using the glass bead extraction method, essentially as described by Ausubel *et al.*, (1997).

Resuspended cells (400 µl) were distributed in 2 ml boil-proof eppendorf tubes containing 400 µl Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1, pH 8. 0), 20µl Tris (0.5M, pH 8.0) and 300 µl glass beads (0.4 µm). The contents of the tube were vortexed for 1 minute and then placed on ice for 1 minute. Vortexing procedure (1minute vortex, 1 minute on ice) was repeated a further three times followed by centrifugation at 12,000×g for 1 minute to separate the cell debris and the glass beads from the aqueous phase. The aqueous phase was transferred into a clean eppendorf tube containing a further 400µl Tris-saturated phenol:chloroform:isoamyl alcohol pH 8.0. The mixture was vortexed for 20 seconds, centrifuged at 12,000 g and aqueous phase collected. The phenol extraction procedure was repeated 3 times in total and the aqueous phase transferred into clean eppendorf tube containing 25 µl 3M sodium acetate pH 5.2 for the ethanol precipitation at -80°C overnight. Next day the RNA was recovered by centrifugation (14,000 g at 4°C) for 15 minutes and the pellet washed with 1 ml of 75% chilled ethanol. After centrifugation, the ethanol was carefully removed and the pellet air dried before resuspending in 25 µl of DEPC treated water. All RNA samples were stored at -80°C.

To determine quality and quantity of RNA, 2 µl of RNA were diluted with 598 µl of DEPC treated water and absorbance was determined at 260 and 280 nm. Quality and integrity of extracted samples was immediately visualised by gel electrophoresis in 1% non-denaturing agarose gel using 1 × TAE as the electrophoresis buffer. The gels were prepared by microwaving 50 ml of water and 0.5 g of DNA-grade agarose (Progen) for 1 minute on high. After cooling, 2 µl of ethidium bromide (1g ml^{-1}) was added and the gel poured. RNA samples were mixed with RNase-free loading buffer. Electrophoresis was conducted at 60V for 60 minutes and gels viewed on a UV transilluminator (UVP Laboratory Products gel documentation system; Upland, CA, USA).

2.5.2 DNase treatment

RNA extracted as described above is contaminated with genomic DNA. Therefore for microarray analysis, all RNA samples were DNase treated using the Ambion DNA-Free™ kit. Briefly, 20 µl of each RNA sample was mixed with 2 µl of DNase buffer and 1.5 µl DNase-I enzyme (both provided in the kit). The reactions were incubated at 30°C for 45 minutes. Inactivation of the DNase enzyme was achieved by adding 3 µl of the DNase-inactivating suspension, also provided with the kit, and mixing the sample by occasional flicking for 3 minutes at room temperature. Samples were then centrifuged at 12,000 g for 2 minutes and supernatant was carefully recovered. The efficiency of the DNA removal, and the quality and integrity of the remaining RNA was inspected spectrophotometrically and by agarose gel electrophoresis, as described previously. The DNase treated RNA samples were used immediately; however, this RNA can be safely stored at -80° if required.

2.5.3. Gene array

The microarrays were obtained from The Clive and Vera Ramaciotti Centre for Gene Function Analysis (Ramaciotti Centre). The Glass slides (SchottNexterion Slide A+ amino link) were spotted with 13824 elements across 6 vertical and 4 horizontal arrays and comprised duplicates of the 6,528 *S. cerevisiae* PCR-amplified open reading frames (ORF). These unmodified 50-mer oligonucleotides were spotted using aminosilane attachment protocol. However, a change in the printing protocol at the Ramaciotti Centre during the course of this study required a change of slide blocking protocol. Initially slides required baking prior to use. Only three slides of this type were used and these and were hybridised to cDNAs labelled with cyanine dyes. The remaining 18 slides were all from the same printing batch and did not require baking prior to use. These slides were used with cDNAs labelled with AlexaFluor dyes.

2.5.3.1 cDNA synthesis and labelling using cyanine dyes (Cy3 and Cy5)

DNase treated RNA (18.7 µl) was added into RNA free PCR tube followed by 13.5 µl of microarray mix 1 (Table 2.2). The tube was placed into PCR cycler and the mix

heated to 65°C for 5 min. After 5 min incubation at 45°C, 5.95 µl of microarray mix2 (Table 2.2) and 2 µl of Superscript II enzyme were added and cDNA synthesis was allowed to proceed for 2 h and 15 min at 42°C. After this time, 4 µl of EDTA (50 mM pH 8.0) and 2 µl of NaOH (10 M) were added and the mixture incubated for 20 min at 65°C. Finally, 4 µl of acetic acid (5 M) was added to neutralise the reaction mix prior to purification of the cDNA.

Table 2.2: Mastermix recipes used in microarray experiment

Microarray MIX1			
Ingredient	**Concentration**	**Supplier**	**Volume (µl) for one slide (2 reactions)**
First strand buffer	5×	Invitrogen superscript kit (#18064-014)	19.2
Oligo dT	0.5 µg μl^{-1}	Invitrogen (#18418-012)	3.6
DTT	0.1 M	Invitrogen superscript kit	9.6
Microarray MIX2			
dNTPs (dGTP; dCTP and dATP) **each**	10 mM	Invitrogen (#10297-108)	4.8 (each)
dTTP	2.5 mM	Invitrogen (#10297-108)	6.24
aa-dUTP	10 mM	Sigma (#A0410)	3.24
Hybridisation MIX			
DigEasy HYB		Roche (#11603558001)	100
Yeast tRNA	10 mg ml^{-1}	Ambion (#7119)	5
Samon sperm DNA	10 mg ml^{-1}	Invitrogen (#15632011)	2.75

2.5.3.2 cDNA synthesis for labelling with AlexaFluor dyes

For cDNA to be labelled with AlexaFluor dyes, the Invitrogen SuperScript ™ Plus Indirect cDNA Labeling Module was used. The AlexaFluor dyes were supplied by Invitrogen as part of this kit (#L1014-04). Detailed protocols, followed without modification can be found on Invitrogen website on following link: http://www.invitrogen.com/content/sfs/manuals/superscript_plus_indirectcdnalabeling_man.pdf Briefly, for cDNA synthesis 16 µl of DNase treated RNA and 2 µl of Anchored Oligo $(dT)_{20}$ primer were mixed and the tube placed into pre-programmed thermal PCR cycler. The tube was incubated at 70°C for 5 minutes and then following reagents were added on ice:

5 × First-Strand buffer	6 µl
0.1 M DTT	1.5 µl
dNTP mix (including amino-modified nucleotides)	1.5 µl
RNaseOUT™ (40 U μl^{-1})	1 µl
SuperScript™ III RT (400 U μl^{-1})	2 µl

The contents were gently mixed and returned to the thermocycler for 3 hours at 46°C. After incubation the samples were hydrolysed to degrade the original RNA by addition of 15 µl of 1 N NaOH. The contents were mixed and incubated at 70°C for 10 minutes. The reaction mix was neutralised by addition of 15 µl of 1 N HCl and the cDNA prepared for purification by addition of phosphate buffer from Qiagen purification kit as described below (Section 2.5.3.5)

2.5.3.3 Slide processing (blocking protocol) for slides labelled with cyanine dyes

Slides were baked for 30 min at 120°C. After cooling to room temperature, slides were placed in a slide jar and washed with gentle agitation, in a series of solutions according to following protocol:

- 0.1% Triton X-100 for 5 min
- 4.38 mM HCl for 2 min

- Repeat 4.38 mM HCl for 2 min
- 100 mM KCl for 10 min
- DEPC treated water for 1 min

After washing, blocking was performed in blocking buffer (25% Ethylene glycol and 0.01% HCl) for 30 min at 50°C, and finally rinsed in DEPC treated water for 1 min. Slides were carefully placed into 50 ml Falcon™ tube with cotton wool on the bottom, and dried by centrifugation in a Beckman centrifuge (CS-15R swinging rotor centrifuge) at 1000 rpm for 5 min. These slides were used immediately for hybridisation.

2.5.3.4. Slide processing (blocking protocol) for slides labelled with AlexaFluor dyes

The microarray slides used for cDNAs labelled with AlexaFluor dyes were prepared by the Ramaciotti institute using a different procedure to crosslink the oligonucleotides to the glass slide. These microarray slides were baked by the supplier before distribution and therefore only required washing for 20 seconds in 0.1% SDS and 20 seconds in DEPC treated water prior to blocking.

For blocking, 200 µl of blocking buffer was placed over the array and a large microarray lifterslip (Grale Scientific) was gently lowered into position. The slide was placed in a Corning microarray chamber (Edward Keller Australia #2551) and incubated for 45 minutes at 45°C in the Extron HI 2001 incubator with gentle rocking of the platform. The blocking buffer was prepared as follows:

- 178 µl of 5 × SSC and 0.1% SDS
- 20 µl of 45 to 55 mg ml^{-1} BSA (Invitrogen)
- 2 µl of salmon sperm DNA 10 mg ml^{-1} (Invitrogen,)

Following the incubation, the slides were rinsed with DEPC treated water and dried briefly by centrifugation at 2000 rpm in 50 ml Falcon™ tube. Slides were used immediately.

2.5.3.5. PCR clean up, labelling and hybridisation

Purification of cDNAs (irrespective of cDNA synthesis protocol), was performed using the QiaQuick purification kit supplied by Qiagen (#28104). Firstly, 150 µl of phosphate buffer (Qiagen) was added to the cDNA. The mixture was then transferred onto the QiaQuick purification column and centrifuged in desktop centrifuge for 1 min at 13,000 g. All further centrifugation steps were carried out under the same conditions (13,000 g, 1 min). The columns were washed with 700 µl of 70% ethanol and the flowthrough discarded. The ethanol wash was repeated and centrifugation used to remove all traces of ethanol prior to cDNA elution. Finally the cDNA was eluted in 25 µl of DEPC treated water following 5 min incubation at room temperature. To ensure optimal cDNA recovery the elution step was repeated with a further 10 µl of DEPC treated water. Finally the volume of each eluted cDNA was reduced from 35 µl to approximately 2 µl using a Savant Speedvac SC116 vacuum centrifuge.

2.5.3.6 Labelling with AlexaFluor or Cyanine dyes

AlexaFluor dyes (AF555 as green and AF647 as red) from Invitrogen or Cyanine dyes (Cy3 or Cy5) from Amersham were dissolved in 2 µl of DMSO and used immediately. Firstly 5 µl of coupling buffer (Invitrogen kit#L1014-04) was added to each cDNA sample (approximate volume 2 µl). This was followed by the 2 µl of the appropriate AlexaFluor or Cyanine dye. Each cDNA labelling reaction was then incubated in Extron HI 2001 incubator 2 hours at 30°C with gentle rocking.

Labelled cDNAs were purified in darkness using the QuiaQuick purification kit, the cDNA being applied to the QuiaQuick column after addition of 65 µl of phosphate buffer (Qiagen) The purification procedure was performed as described above and each labelled cDNA was finally eluted in a volume of 35 µl which was then reduced to 10 µl in Savant Speedvac SC116. The two labelled cDNA samples representing the control and test samples were then combined and the volume further reduced to approximately 5 µl in Savant Speedvac vacuum centrifuge.

2.5.3.7 Hybridisation

To prepare the cDNA for hybridisation to the microarray, 85 µl of freshly prepared hybridisation mix (Table 2.2) was added to the labelled cDNA mix and incubated at 65°C for 5 min. These samples were allowed to cool to the room temperature. The cDNA mix was then carefully applied to the blocked slides, covered with microarray lifterslip with elevated sides (Grale Scientific) and placed into coning microarray chamber (Edward Keller Australia #2551). These steps were performed with minimal illumination to protect the fluorescent dyes. The chambers had two wells which were filled with 25 µl of DEPC treated water to prevent the slides from drying. The chambers were then incubated at 37°C overnight (minimum 16h) in the Extron HI 2001 incubator with gentle rocking of the platform.

2.5.3.8. Washing

The washing steps were also performed in the dark or with minimal exposure to light to ensure safe slide handling. The slides were washed sequentially with pre-warmed solutions in a temperature controlled shaking incubator also at 42°C. The coverslips were first removed by placing the slide in 2 × SSC/0.1% SDS solution. The slides were then washed as follows:

1. 2 × SSC/0.1% SDS	10 min
2. 1 × SSC/0.1% SDS	2 × 5 min
3. 1 × SSC	5 min
4. 0.2 × SSC	2 × 2 min
5. 0.2 × SSC/0.05% TritonX-100	final rinse

The final rinse step with 0.2 × SSC with Triton X-100 needed to be performed rapidly as the solution tends to evaporate leaving streaks if not removed immediately by centrifugation. This step was performed by centrifugation for 5 min at 2000 ×g in a 50 ml Falcon™ tube with compressed Kim-wipes at the base to collect the excess liquid. Immediately after spinning the slide was removed allowing rapid evaporation of any

remaining liquid on the slide surface and placed in an amber light protected 50 ml Falcon™ tube for immediate scanning.

2.5.3.9 Analysis of microarray data

Following hybridization, the glass slides were scanned using a GenePix-Pro 4000 scanner and analysed using Genowiz™ 4.0.2.1 software (Ocimum Biosolutions) as described in Chapter 4. Microarray data was normalised before statistical analysis using Lowess (Yang *et al.*, 2001). Other software packages used during the analysis of the microarray data were freely available online. These included: FUNSPEC (Robinson *et al.*, 2002) which was used to investigate functional significance by gene ontology (GO) analysis; variations of BLAST as described in Balachrishnan *et al.*, (2005) and GenMAPP and MAPPFinder (Doniger *et al.*, 2003) for pathway analysis.

CHAPTER 3

GENERATION AND CHARACTERISATION OF ETHANOL-TOLERANT MUTANTS OF *Saccharomyces cerevisiae*

3.1 INTRODUCTION

The purpose of this project was to create ethanol-tolerant mutants of *Saccharomyces cerevisiae* and to then study the molecular changes responsible for their acquired higher ethanol tolerance. Before doing this, it was important that the ethanol tolerance of the wild-type strain *S. cerevisiae* W303-1A was physiologically characterised so that it could be used to benchmark the ethanol tolerance of W303-1A variants isolated from the mutation experiments. This chapter describes the profiling of *S. cerevisiae* W303-1A ethanol tolerance by determining the change in its viable population over time after inoculation into fresh medium containing various ethanol concentrations. The results from such experiments provided insight on the ability of W303-1A to acclimatise to non-lethal ethanol concentrations and its ability to remain viable in lethal ethanol concentrations.

With the ethanol tolerance of the wild-type characterised, the next step was to perform mutagenesis of W303-1A and then select ethanol-tolerant mutants. Adaptive evolution on mutagenised and non-mutagenised cultures of W303-1A was used; both approaches included extensive use of chemostats for the purpose of providing a selection pressure in a growth competitive environment. The subsequently isolated mutants were then physiologically profiled to confirm and characterise their improved tolerance to ethanol.

The aims of this chapter were to:

1. characterise the ethanol-tolerance of wild-type *S. cerevisiae* W303-1A.
2. determine the conditions to be used in both mutagenesis and molecular work.
3. create and isolate ethanol-tolerant mutants using adaptive evolution of chemically mutagenised and non-mutagenised populations of W303-1A.
4. characterise the phenotypes of ethanol-tolerant variants.

3.2 GROWTH PROFILE OF *S. cerevisiae* W303-1A IN THE PRESENCE OF ETHANOL

All growth experiments were initiated by inoculating yeast into fresh medium in the absence or presence of ethanol. A number of conditions were met in the experimental design to ensure that results were reproducible and accurate, and that the only significant stress encountered by the inoculum was due to sudden exposure to ethanol. It was important to ensure that each inoculum was prepared from pre-stationary phase parent culture to avoid the subsequent lag period being influenced by inoculation of stationary phase cells *ie.* cells that have already been stress challenged. Also, all inocula were washed with pre-warmed fresh medium to avoid carryover of lag-influencing by-products present in the parent culture. To reduce the influence of inoculum size on the lag period, all experimental cultures were inoculated to approximately the same initial cell population. Finally, all experimental cultures within each experiment were inoculated from the same parent culture, and at the same time, to ensure that the cells were in the same physiological state at the time of inoculation.

Reproducibility of culture profiles under the various environmental conditions used in this chapter was determined by repeating each experiment a minimum of 4 times. Figure 3.1 shows a representative growth profile of the wild-type W303-1A where mean viable counts from an average of 8 replicates for each ethanol concentration were plotted against time. Statistics for these growth curves are presented in Appendix 1.2, where the high reproducibility of results is demonstrated. Statistical software KY-Plot 2 beta 15 was used to calculate the statistical data presented in this thesis, specific growth rates were calculated using the Unconstrained Linear Least Squares Method and Modified Gram-Schmidt Method. Accurately determining the specific growth rates was especially important since they were used to determine critical dilution rate and the optimum dilution rate used in the chemostat-based experimental work described later in this chapter.

W303-1A was inoculated into medium containing ethanol concentrations ranging from 5-20% (v/v) ethanol (Figure 3.1). All cultures were sampled regularly for viable plate counts and optical density. The cultures without added ethanol had no detectable lag

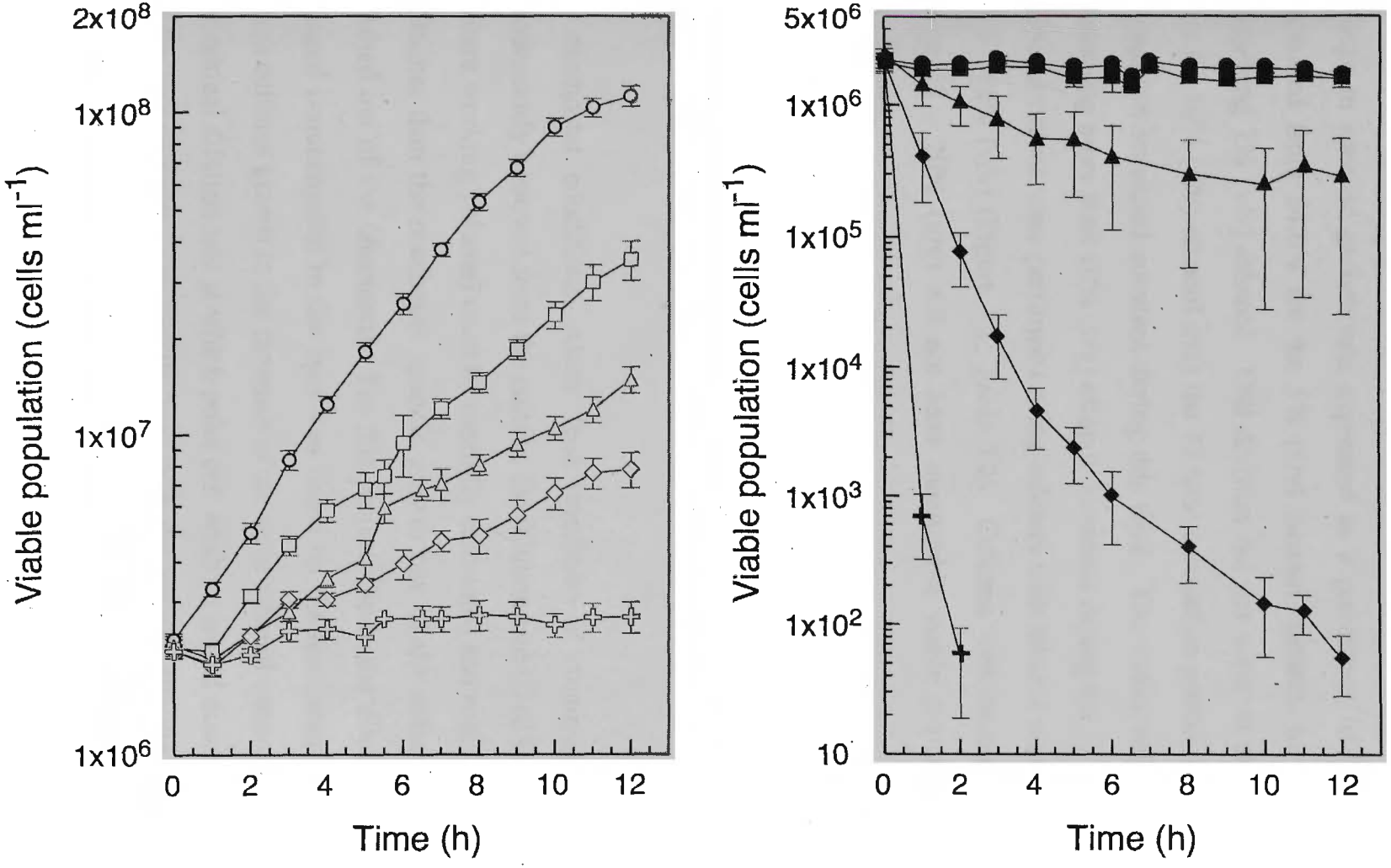

Figure 3.1: The effect of ethanol concentration on the viable cell population of *S. cerevisiae* W303-1A. Late exponential phase cells were inoculated in the absence (O) or in presence of added ethanol at (v/v) 5% (□), 6% (△), 7% (◇), 8% (✞), 9% (●), 10% (■), 12% (▲), 14% (♦) or 16% (✚) ethanol (v/v). The cultures were incubated at 30°C/110 rpm. The error bars represent the standard error from the mean of 8 replicate experiments for each condition. When not visible, error bars are smaller than the symbol.

period and a mean specific growth rate (SGR) of 0.388 h^{-1} (Table 3.1). Cultures with ethanol concentrations in the range of 5-7% (v/v) had increasing lag periods and decreasing SGR as the added ethanol concentration increased. The lag period increased from around 1 hour at 5% (v/v) ethanol to around 4 hours at 7% (v/v) ethanol; the change in specific growth rate expressed as a percentage of the non-stressed culture decreased from 54.6% for the 5% (v/v) ethanol cultures, to 23.3% for the cultures containing 7% (v/v) ethanol. Cell division did not occur in cultures containing more than 8 - 10% (v/v) ethanol over the 12 hour incubation period, however, the viable cell population remained constant during this time. The viable cell population of cultures containing more than 10% (v/v) ethanol decreased during the 12 hour incubation period. Survival curves were performed using cultures with ethanol concentrations in the range of 9 - 16% (v/v) (Figure 3.1; Table 3.2). Cultures with ethanol concentrations in the range 17 - 20% (v/v) did not have measurable viable populations after 1 hour of incubation.

3.3 CHEMOSTAT ASSEMBLY AND OPERATION

3.3.1 Chemostat set-up

In chemostat conditions, where feed (medium) is continuously added and waste continuously removed from the culture, the dilution rate (feed flow rate compared to the culture working volume) must be carefully calculated and managed. If the dilution rate is higher than the maximum specific growth rate of the culture, then all cells will be washed out of the chemostat. For this project, the initial dilution rate for a particular ethanol concentration in the feed was based on the maximum specific growth rate of batch cultures grown in the presence of the same ethanol concentration; this represented the critical dilution rate at which point cell washout would occur. The dilution rate used in the experiments was slightly less than the critical dilution rate to avoid washout. Chemostats used in this project had a working volume of 1 litre, which means that the feed flow rate (in l h^{-1}) was equal to the dilution rate *ie.* if the maximum specific growth rate of a culture was 0.5 h^{-1} (and therefore the critical dilution rate was 0.5 h^{-1}), then the feed flow rate needed to be less than 0.5 l h^{-1} to avoid washout.

Table 3.1: Growth parameters for *S. cerevisiae* W303-1A in the presence of non-lethal ethanol concentrations.

Ethanol Concentration (% v/v)	Mean SGR[1] (h^{-1})	Standard Error (for SGR)	Variance (for SGR)	Mean Doubling Time[2] (h)	Lag Period[3] (h)	Growth rate relative to growth rate in 0% ethanol culture
0	0.388	0.0023	$8.3x10^{-05}$	1.8	0	100%
5	0.212	0.0146	0.00107	3.3	1	55%
6	0.178	0.0089	0.00048	3.9	2	46%
6.5	0.134	0.0126	0.00096	5.2	3	34%
7	0.093	0.0038	$5.7x10^{-05}$	7.5	3.5	23%

[1]The Specific Growth Rate (SGR) was calculated using statistical software KY-Plot 2 beta 15, and the Unconstrained Linear Least Square Method and Modified Gram-Schmidt Method from an average of 6 replicate experiments for each ethanol concentration. Time-points within the lag and stationary phase were not taken into consideration in SGR calculations.

[2]The doubling time was calculated from the Specific Growth Rate.

[3]The lag period was calculated from the mean growth curves as an intercept point of the SGR line and lag line (Stanley *et al.*, 1997).

Table 3.2: Survival parameters for *S. cerevisiae* W303-1A in the presence of lethal ethanol concentrations.

Ethanol Concentration (% v/v)	Mean Specific Survival Rate[1] (h^{-1})	Standard Error (for SSR)	Variance (for SSR)	Mean Halving Time[2] (h)
8	-0.005	0.0104	0.0011	145.7
9	-0.020	0.0139	0.0025	34.5
10	-0.027	0.0174	0.0024	25.4
11	-0.315	0.1305	0.0681	2.2
12	-0.555	0.1258	0.0791	1.25
13	-0.886	0.0427	0.0055	0.78
14	-0.915	0.0434	0.0075	0.76
15	-2.395	0.1236	0.0611	0.29
16	-6.500	0.1655	0.0821	0.11

[1]Mean Specific Survival Rate was calculated in the same way as SGR in Table 3.1. Negative values indicate reducing viable cell population.

[2]Mean Halving Time represents the time taken for the viable counts to reduce by half and is calculated in the same way as Doubling Time

Pump calibrations were initially required to determine the actual flow rate for each setting on the pump dial. Two pumps (Masterflex C/L) were used to feed YEPD medium into the two chemostats. The flow rate also depended on the diameter of the silicon tubing therefore calibration was performed using different tube sizes. The tube size that provided the appropriate flow rates was 2 mm in diameter, which enabled flow rates in the range, 0.073 - 0.384 l h^{-1} and, 0.076 - 0.405 l h^{-1} for the two pumps used. Smaller diameter tubing was found to cause clogging and connection problems and larger diameter tubing was unable to support the low dilution rates required for cultures with high ethanol concentrations.

All growth experiments used for determining specific growth rates (Table 3.1) were performed in sidearm Erlenmeyer flasks, recognising that these conditions slightly differ from the environment in the fermentor bowl used for the chemostat cultures. In practice it was found that the limiting ethanol concentration in the feed for W303-1A, in a chemostat operating at a dilution rate of 0.073 h^{-1}, was 6.8% (v/v) instead of the estimated (from Figure 3.2) 7.3% (v/v). This was considered acceptable given the differences in experimental set-up. From a practical perspective, the dilution rates in both chemostats were regularly monitored during operation by measuring the change in medium volume in the calibrated feed tank over time. A photograph of the chemostat arrangement used for adaptive evolution experiments is shown in Figure 3.3.

3.3.2 Chemostat operation

Operation of the chemostat over extended periods was conducted as follows. Autoclaved fresh medium (YEPD) containing the desired ethanol concentration was pumped (using pump B) from Schott bottle A through a sterile filter C (0.22 μm filter) into feed tank D (Figure 3.3). Medium from feed tank D was pumped (using pump E) at an appropriate constant dilution rate into the 1.2 litre chemostat bowl F. The culture in the chemostat was maintained at 30°C and 110 rpm. Waste was pumped out of chemostat bowl F (leaving via an exit port that maintained a height of culture broth in the chemostat bowl corresponding to a working volume of 1 litre) using waste pump G that was maintained at a higher flow rate than feed pump E. Waste was collected in waste tank H from which it was aseptically removed into another vessel containing

Figure 3.2: Specific growth rate of *S. cerevisiae* W303-1A relative to ethanol concentration in YEPD medium (from Figure 3.1 and Tables 3.1 and 3.2). Panel B represents an enlargement of panel A to illustrate critical ethanol concentrations under non-lethal conditions. Minimum and maximum dilution rates covered by the two feed pumps used in the chemostat experiments are shown by arrows on the panel B ordinate for a tube diameter of 2 mm. Panel B demonstrates that the critical ethanol concentration for a dilution rate of 0.073 h^{-1} (pump 1) is 7.3% (v/v). This suggests that if pump 1 is operating with a flow rate of 0.073 l h^{-1} or higher (and a 1 litre working volume is used), wash-out will occur at a feed ethanol concentration of 7.3 % (v/v) or higher.

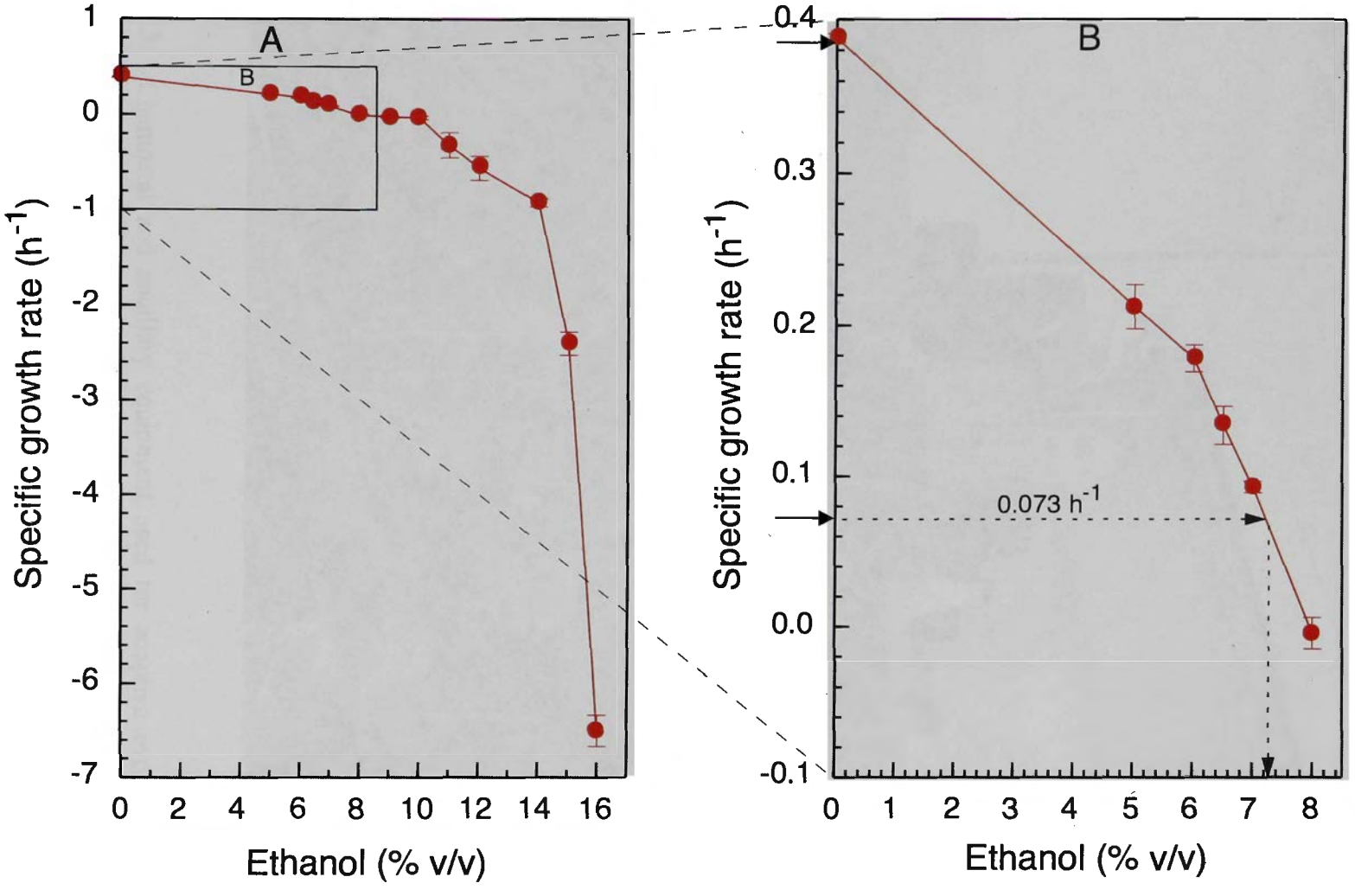
A
B
Specific growth rate (h-1)
Ethanol (% v/v)
1
0
-1
-2
-3
-4
-5
-6
-7
0
2
4
6
8
10
12
14
16
B
0.4
0.3
0.2
0.1
0.0
-0.1
0.073 h-1
0
1
2
3
4
5
6
7
8

Figure 3.3: Chemostat and ancillary equipment used for adaptive evolution experiments. Labels show: Schott bottle with autoclaved medium (A), pump for sterile filtration (B), sterile filter (C), feed tank (D), feed pump (E), fermentor (F), waste pump (G) and waste tank (H).

chemical disinfectant (additional pump and container not shown in Figure 3.3). Waste was removed in this manner from the 20 litre waste container weekly and the waste removal tubing was kept in 70% (v/v) ethanol during out-of-use periods. Sampling port tubing on chemostat bowl F was also kept in 70% (v/v) ethanol during out-of-use periods. All other ports and connectors on the chemostat bowl were checked for leakage (using compressed air) prior to chemostat operation. Lids on the feed tank could not be kept airtight due to pressure changes caused by the pumping of medium in and out of the vessel so, to maintain sterility, sterile cotton wool was wrapped around the lids. This method of chemostat operation proved to be effective in maintaining culture sterility as evidenced by its continuous sterile operation for over 6 months in the adaptive evolution experiments.

3.4 MUTAGENESIS

3.4.1 Generation and enrichment of spontaneous mutants

An Evolutionary Engineering approach was used to create spontaneous mutants by subjecting *S. cerevisiae* W303-1A (wild-type) to ethanol stress over an extended time period; this was performed in a chemostat where the only selection pressure was a gradually increasing ethanol concentration in the feed. The ethanol concentration was manually adjusted on a regular basis to ensure an optimum selection pressure was maintained. The procedure for generating spontaneous mutants is described below.

The Chemostat bioreactor was inoculated to an OD620 of 0.1 and initially cultured batchwise without added ethanol (Figure 3.4). After reaching the stationary phase, the feed pump was turned on and fresh medium containing ethanol (initially at 7% v/v) was fed into the bioreactor at a dilution rate of 0.073 h^{-1}. After 14 days the biomass level in the culture had substantially decreased (OD_{620} of 0.005) and the cell population was close to total wash out. To revive the culture, the selection pressure was reduced by decreasing the ethanol concentration to 4% (v/v); when the cell population had recovered, the ethanol concentration was increased to 5% (v/v). This was the operational procedure for the chemostat over a continuous 6-month period. The maximum ethanol concentration that could be endured by the culture before washout

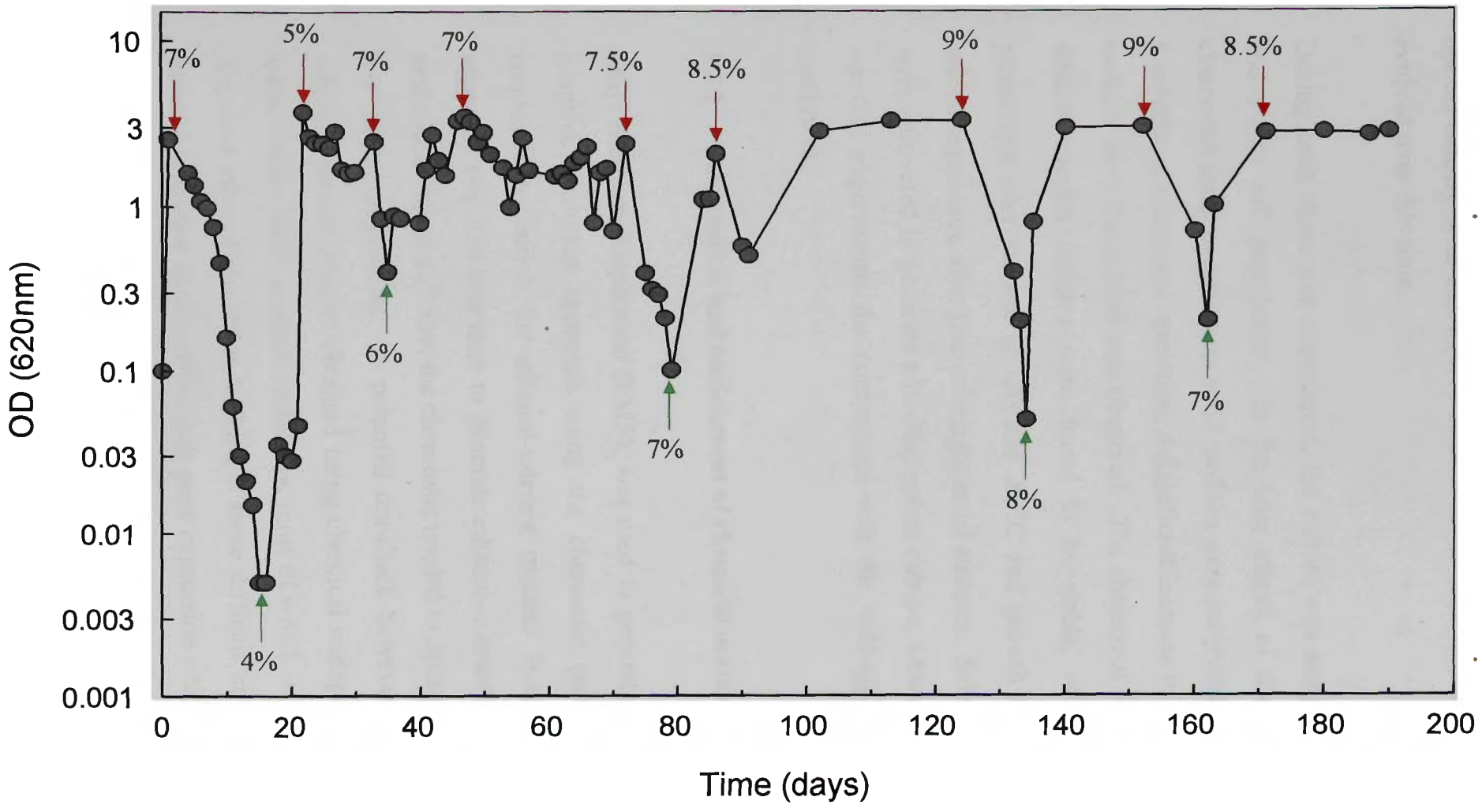

Figure 3.4: Biomass profile from the adaptive evolution experiment used to create ethanol-tolerant mutants of *S. cerevisiae* W303-1A. Ethanol provided the selection pressure in the chemostat and changes in ethanol concentration of the YEPD feed are represented by arrows. If the selection pressure was too high, risking wash out, the ethanol concentration in the feed was reduced to allow the culture to recover after which the ethanol selection pressure was increased again. This approach resulted in the isolation of ethanol-tolerant mutant SM1 after 192 days of continuous cultivation. The chemostat was operated at 30°C/110 rpm with a constant dilution rate of 0.073 h^{-1}.

gradually increased during this time. After 6 months (192 days or 4,608 hrs) of continuous operation at a dilution rate of 0.073 h^{-1} (*i.e.* doubling time of 9.5 hrs), the culture had gone through 486 generations and was able to grow at a relatively high optical density with 8.5% (v/v) ethanol in the feed, indicating that the population had evolved over this time.

During each chemostat experiment, the culture was sampled daily for optical density and viable cell population. In the later stages of the experiment, colonies from chemostat samples grown on solid medium were subjected to phenotype analysis. After 6 months of chemostat operation, a significant increase in the ethanol tolerance of yeast isolates from the culture was observed. The chemostat experiment was stopped when ethanol-tolerant isolates were found to be stable, retaining their ethanol-tolerant phenotype after storage at -20 and -80°C and growth in the absence of an ethanol selective pressure after five overnight serial cultures. Selected isolates (see Section 3.6) were cultivated to generate a healthy parent culture, which was then used in growth and survival experiments for comparison with the wild-type phenotype under the same conditions.

3.4.2 Generation and enrichment of chemical mutants

Ethyl Methane Sulphonate (EMS), was used to generate an array of mutants, then an adaptive evolution approach using the chemostat (with ethanol in the feed) was employed to enrich for ethanol-tolerant mutants from the variant mixture. This approach required less time to generate ethanol-tolerant mutants than was required for non-mutagenised cultures; the chemostat needed to operate for only 2-4 weeks to enrich the variant population. A potential drawback however with this approach was that ethanol-tolerant isolates obtained using chemical mutagenesis would be likely to have substantially more genome mutations, most of which would not be associated with the acquired ethanol tolerance, making it more difficult to identify genes associated with ethanol tolerance in the subsequent gene expression work described in Chapter 4. This is one of the reasons that both methods of creating ethanol-tolerant mutants were used in this project. The conditions used for EMS mutagenesis were designed such that around 30 - 50% of the starting cell population was still viable at the end of the

exposure period; typically the most effective mutagenesis strategies assume a survival range of 10 - 50% (Barbour *et al.*, 2006).

An experiment was performed using different EMS concentrations in a series of flasks, each containing a suspension of W303-1A; the inocula comprised overnight-grown stationary phase cells. Eight cell suspensions (*i.e.* one suspension for each time-point), inoculated with cells from the same parent culture, were used for each EMS concentration (Figure 3.5) to avoid the health risks associated with a classic sampling approach for EMS-containing cell suspensions. At each time-point, one of these cell suspensions for each EMS concentration was removed from the incubator, washed and a sample plated on duplicate YEPD plates for viable counts (see Section 2.3.1). To limit exposure to the mutagen, EMS concentrations of 1, 2 and 3% (v/v) are the result of a single experiment (Figure 3.5). Exposure of the cells to EMS concentrations of 2% and 3% (v/v) resulted in a relatively rapid decline in viable counts after 1 h of exposure suggesting that the viability of these cells was severely compromised by such EMS concentrations (Figure 3.5). The cell death rate was slower using an EMS concentration of 1% (v/v), with a reduction in viable counts of 37% over the first 60 minutes. This was considered to be a more appropriate EMS concentration for this project considering that the purpose was to create new strains that, although having significant changes in DNA profile, were nonetheless vital and stable. An EMS concentration of 1% (v/v) and an exposure period of 1 h were chosen for all subsequent chemical mutagenesis work.

Overnight *S. cerevisiae* cultures were exposed to EMS (1% v/v) for 1h after which the cell suspension was washed several times with 5% sodium thiosulphate to remove all traces of EMS. This suspension of chemical variants was then inoculated into the chemostat bioreactor containing YEPD and ethanol (7.5% v/v). This ethanol concentration was selected since in batch cultures it was the highest ethanol concentration that wild-type W303-1A could acclimatise to and then commence growth within a 12 hour period. This immediate exposure of the variants to ethanol was a form of pre-selection for screening out variants with compromised ethanol tolerance and also variants with low stability and viability.

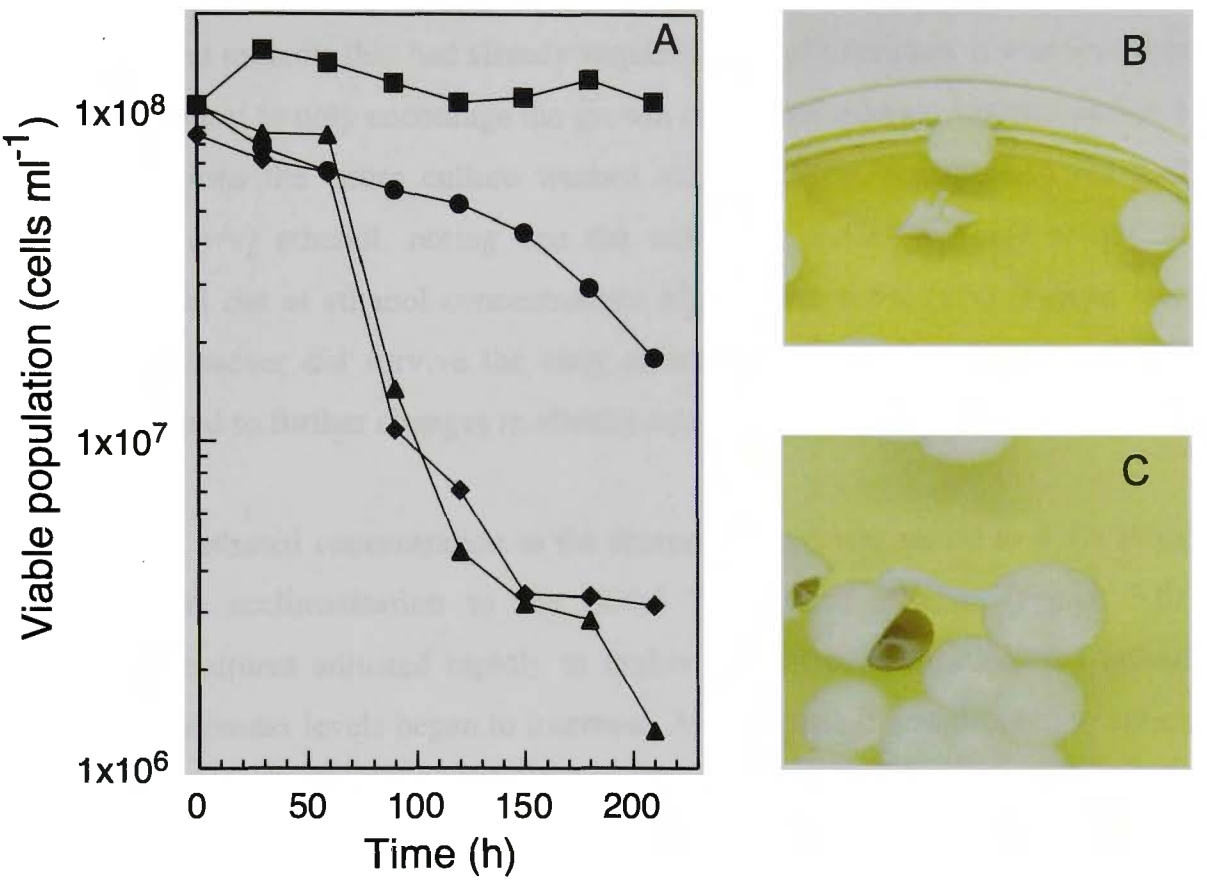

Figure 3.5: Viable cell population of *S. cerevisiae* W303-1A suspensions exposed to various EMS concentrations (Panel A). Cells from stationary phase, overnight grown cultures were exposed to the following EMS concentrations (v/v): no added EMS (control) (■), 1% (●), 2% (▲) and 3% (◆). The suspensions were incubated in Falcon™ tubes at 30°C/50 rpm. Panels B and C show some of the effects of 1% EMS exposure for 1 hour on colony morphology.

The inoculated variant population was grown batchwise in 7.5% (v/v) ethanol and when the culture reached stationary phase, the feed and waste pumps were turned on to commence chemostat operation; the initial ethanol concentration in the feed was 7.5% (v/v) and the dilution rate was 0.076 h^{-1}. The purpose of the chemostat in these experiments was not only to provide an environment for generating ethanol-tolerant strains (as was the case for the adaptive evolution approach), it was also to enrich chemically-derived mutants that had already acquired ethanol tolerance. It was therefore important at the onset to only encourage the growth of ethanol-tolerant variants and on a number of occasions the entire culture washed out of the chemostat when the feed contained 7.5% (v/v) ethanol, noting that the wild-type under the same chemostat conditions washed out at ethanol concentrations higher than 6.8% (v/v) (Figure 3.4). Some cultures however did survive the early chemostat treatment and these cultures were then subjected to further changes in ethanol concentration in the feed[1].

After 6 days, the ethanol concentration in the chemostat feed was raised to 8.5% (v/v), following culture acclimatization to the initial 7.5% (v/v) ethanol (Figure 3.6). Typically these cultures adjusted rapidly to higher ethanol concentration and indeed after 2 days the biomass levels began to increase. At that stage it was decided to enrich for variants with the 'highest' ethanol tolerance by introducing a large step change in the feed ethanol concentration so that the 'least' ethanol-tolerant variants would wash out of the chemostat. The ethanol concentration used for "washing out" was 12% (v/v); the dilution rate was kept constant at 0.076 h^{-1}. After introducing the washout ethanol concentration, only 2 chemostat cultures out of a total of 8 recovered and these continued to grow, maintaining a high viable population of around 8×10^7 cells ml^{-1} with 12% (v/v) ethanol in the feed (Figure 3.6). The ethanol concentration in the feed of cultures that failed to recover after 2-3 days was reduced to 10% (v/v); 3 of these chemostats recovered at 10% (v/v) ethanol. After 8 separate mutagenesis and subsequent chemostat-based selection experiments, 3 chemostat cultures could tolerate 10% (v/v) and 2 chemostat cultures could tolerate 12% (v/v) ethanol in the feed at a

[1]It should be noted that wild-type W303-1A initially failed to survive in a chemostat with the same dilution rate (0.076 h^{-1}) and an ethanol concentration of 7% (v/v) in the feed. The variant cultures that recovered at 7.5% (v/v) were therefore already outperforming the wild-type in their ability to adapt to ethanol.

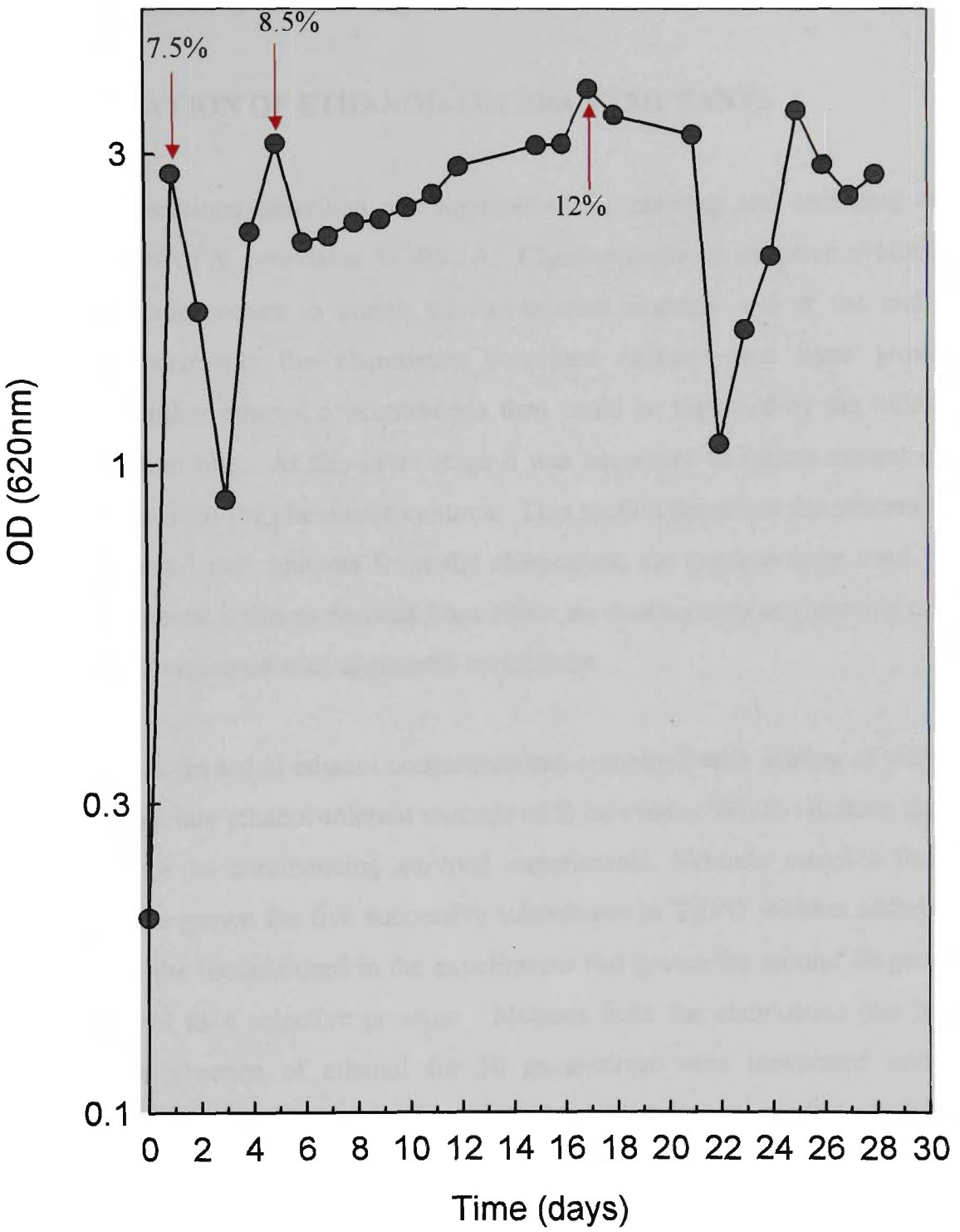

Figure 3.6: An example of a chemostat culture that was used to enrich ethanol-tolerant variants of EMS-treated *S. cerevisiae* W303-1A. A suspension of EMS-treated cells was inoculated into the chemostat bioreactor and initially grown batchwise in the presence of 7.5% (v/v) ethanol; chemostat operation commenced once the batch culture had reached stationary phase. The pump was turned with 7.5% (v/v) ethanol in the YEPD feed as indicated by the arrow; further changes to the feed ethanol concentration during the course of the incubation are shown with arrows. The chemostat was operated at a dilution rate of 0.076 h^{-1} and 30°C/110 rpm.

dilution rate of 0.076 h^{-1}. Ethanol-tolerant isolates were then obtained from these five cultures as described in the following section.

3.5 ISOLATION OF ETHANOL-TOLERANT MUTANTS

The previous sections described two approaches for creating and enriching ethanol-tolerant mutants of *S. cerevisiae* W303-1A. Chemostat-driven adaptive evolution was used in both approaches to enrich ethanol-tolerant mutants and at the end of the respective experiments the chemostats contained cultures that were growing in substantially higher ethanol concentrations than could be tolerated by the wild-type at the same dilution rate. At this latter stage it was necessary to isolate mutant ethanol-tolerant strains from the chemostat cultures. This section describes the process used to isolate ethanol-tolerant mutants from the chemostats; the methodology used was the same for chemostat cultures derived from either an evolutionary engineering approach or chemical mutagenesis with chemostat enrichment.

Survival curves (in lethal ethanol concentrations) combined with plating of viable cells were used to isolate ethanol-tolerant mutants of *S. cerevisiae* W303-1A from chemostat cultures. Prior to commencing survival experiments, biomass samples from each chemostat were grown for five successive subcultures in YEPD without added ethanol to ensure that the inocula used in the experiments had grown for around 30 generations without ethanol as a selective pressure. Mutants from the chemostats that had been grown in the absence of ethanol for 30 generations were inoculated into YEPD containing 18% (v/v) ethanol[2]. Biomass samples were taken regularly over 8-10 hours for viable plating (on YEPD medium without ethanol) with a minimum of 8 time-points being sampled to isolate the longest surviving cells as colonies.

A loopful of cells from a single colony (originating from a sample of cells that survived for the longest time period in the presence of 18% (v/v) ethanol) was grown in YEPD (without ethanol) in several successive subcultures and samples were stored at -80°C and -20°C (see Section 2.3.3). These mutant strains were labelled as either SM1 or

[2]Under these conditions, cultures of the wild-type W303-1A had no viable population after 60 minutes at 18% (v/v) ethanol.

CMX, where S refers to a spontaneous mutant (only one mutant, SM1, was isolated from evolutionary engineering experiments), C refers to mutants isolated from chemical mutagenesis and X refers to the colony number on the plate from which the mutant was isolated.

The five isolates (CM1, CM2, CM3, CM4 and SM1) were then subjected to survival experiments at 20% (v/v) ethanol to test their ethanol-tolerant phenotype (Figure 3.7). SM1 and CM1 cultures both had an initial rapid decrease in viability in the first hour, followed by a lower death rate such that a measurable, viable cell population was present in the cultivations after exposure for 7 hours to 20% (v/v) ethanol; this level of ethanol tolerance was substantially higher than that of the wild-type. CM2, CM3 and CM4 cultivations were similar in their viable population profiles, being represented by an initial rapid decline in cell population to around 1×10^3 cells ml^{-1} during the first 2 hours of incubation and then maintaining a relatively constant viable population for up to 24 hours (only the first 8 hours of incubation are shown in Fig. 3.7). Despite their high ethanol-tolerance, these isolates were found in subsequent experiments to be unstable and temperature sensitive, not being able to survive freezing temperatures. Consequently, it was decided to continue working with confirmed stable and freeze-thaw tolerant strains, SM1 and CM1.

3.6 CHARACTERISATION OF ETHANOL-TOLERANT MUTANTS SM1 AND CM1.

3.6.1 Presence of genetic markers

SM1 and CM1 were tested for the presence of the genetic markers present in their wild-type strain W303-1A. The presence of these auxotrophic markers in the ethanol-tolerant mutant strains would provide some measure of confidence that they are genetic variants of W303-1A, rather than wild yeast contaminants. *S. cerevisiae* W303-1A is only able to grow on minimal medium supplemented with the amino acids leucine, tryptophan, uracil, adenine and histidine. Plates containing minimal medium were prepared lacking one of either leucine, tryptophan, uracil, adenine or histidine. Minimal

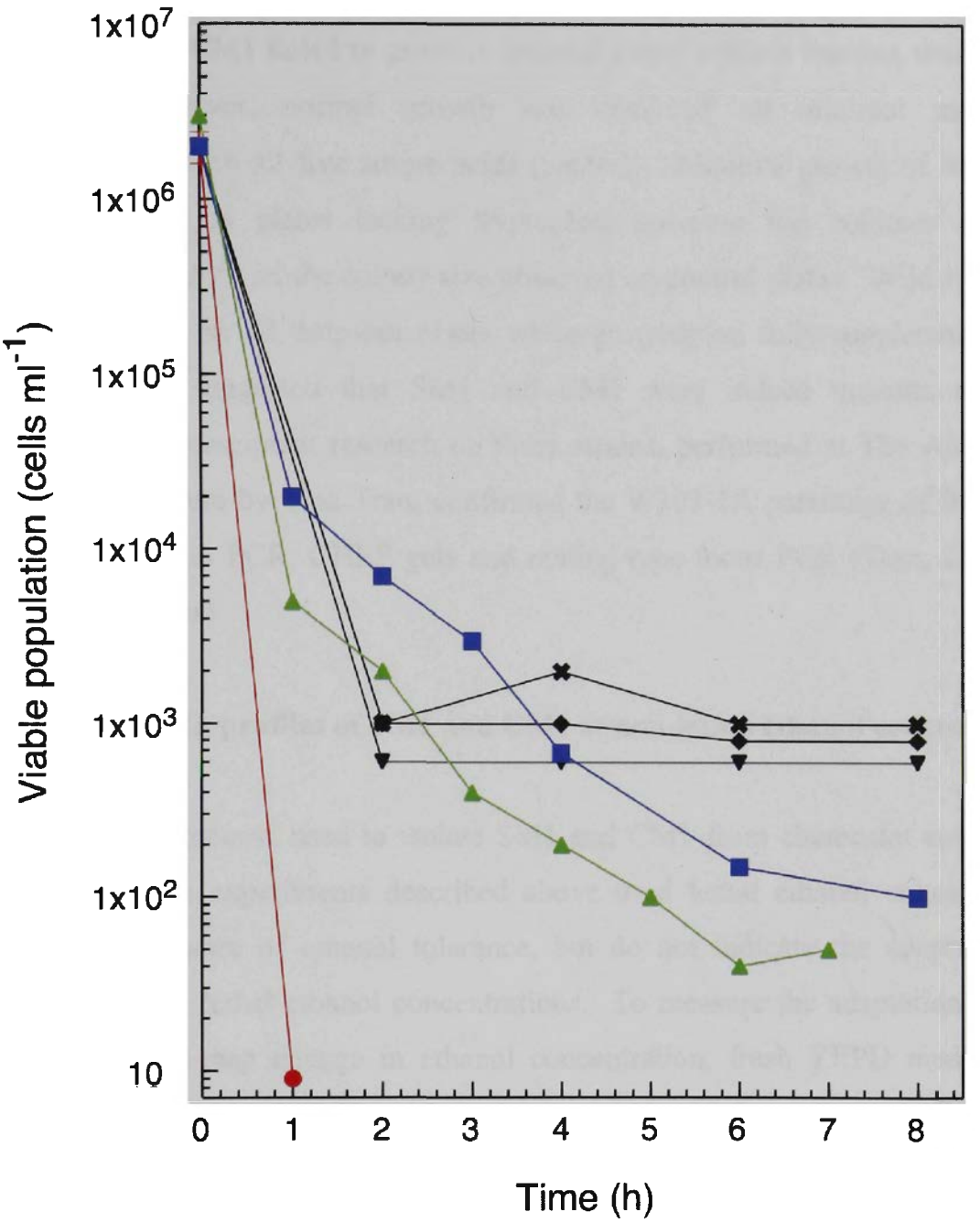

Figure 3.7: Viable population profiles of ethanol-tolerant isolates in YEPD containing 20% (v/v) ethanol. CM1 (▲), CM2 (✖), CM3 (◆), CM4 (▼) and SM1 (■). The viability of wild-type W303-1A (●) is shown at 18% (v/v) ethanol, since at 20% (v/v) ethanol there were no viable cells 30 minutes after inoculation. Incubations were conducted at 30°C/110 rpm.

medium plates containing all five amino acids were also prepared as a control (see Chapter 2, Section 2.2.1).

Both SM1 and CM1 failed to grow on dropout plates without leucine, uracil, adenine or histidine, however, normal growth was observed on minimal medium plates supplemented with all five amino acids (control). Minimal growth of SM1 and CM1 was observed on plates lacking tryptophan however the colonies only reached approximately 10% of the colony size observed on control plates. Wild-type W303-1A failed to grow on all drop-out plates while growing on fully-supplemented medium. These results suggested that SM1 and CM1 were indeed mutants of W303-1A. Furthermore, subsequent research on these strains, performed at The Australian Wine Research Institute by Tina Tran, confirmed the W303-1A parentage of SM1 and CM1 using transposon PCR, CHEF gels and mating-type locus PCR (Tran, 2006, personal communications).

3.6.2 Growth profiles of SM1 and CM1 at non-lethal ethanol concentrations.

The selection process used to isolate SM1 and CM1 from chemostat cultures and the characterisation experiments described above used lethal ethanol concentrations that provide a measure of ethanol tolerance, but do not indicate the adaptability of the mutants to non-lethal ethanol concentrations. To measure the adaptation rate of SM1 and CM1 to a step change in ethanol concentration, fresh YEPD medium, with or without added ethanol (6.5% v/v), was inoculated with late exponential phase cells of SM1 or CM1 and their growth profiles were determined; the control culture comprised wild-type strain W303-1A.

SM1 and CM1 had similar growth profiles to wild-type W303-1A in the absence of ethanol, however, they were able to adapt more quickly to, and grow more rapidly in, 6.5% (v/v) ethanol compared to W303-1A (Fig. 3.8). When exposed to 6.5% (v/v) ethanol, SM1 and CM1 had lag periods of less than 1 hour compared to W303-1A which had a lag period of around 3.5 hours, representing an increase of at least 75% in the adaptation rate by the mutants compared to that of the wild-type. Once adapted, SM1 and CM1 also had higher specific growth rates (0.240 h^{-1} and 0.182 h^{-1}

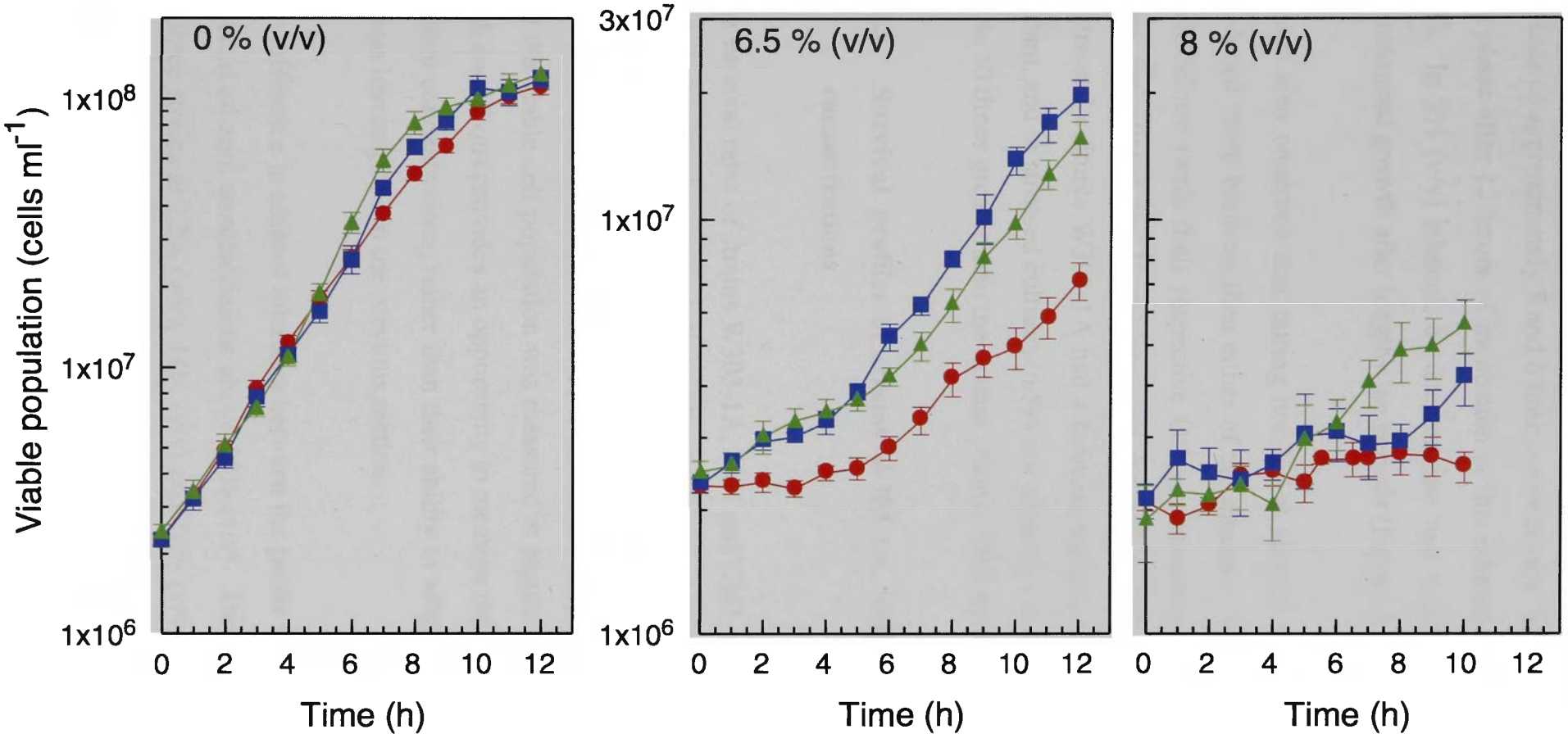

Figure 3.8: Viable cell population profiles of the wild-type W303-1A (●) and mutant strains SM1 (■) and CM1 (▲) under various ethanol stress conditions. The cultures were incubated in YEPD at 30°C/110 rpm. The error bars represent the standard error for the mean of 10 replicate experiments. When not visible, error bars are smaller than the symbol.

respectively) than W303-1A (0.134 h^{-1}) in the presence of 6.5% (v/v) ethanol (Table 3.3; Figure 3.9). SM1 and CM1 commenced growth in 8% (v/v) ethanol after lag periods of approximately 8 and 6 hours respectively, however, W303-1A was still in the lag phase after 12 hours of incubation at this ethanol concentration (Table 3.3; Figure 3.8). In 9% (v/v) ethanol, a concentration that is lethal for W303-1A, both mutants commenced growth after lengthy lag periods (Figure 3.10).

It was also observed that during non-lethal ethanol stress, W303-1A (the wild-type) produced more biomass than either of the mutants. Falcon tubes containing 50 ml of each culture (with their respective OD_{620} measurements adjusted to the same value) were centrifuged for 10 minutes and the volume of their cell pellets compared. In unstressed cultures W303-1A had a biomass volume up to 10 times higher than either mutant, and in stressed cultures (6.5% v/v ethanol) the wild-type had cell pellet volumes up to 50 times greater than the mutant strains. This trend was highly reproducible.

3.6.3 Survival profiles of strains W303-1A, SM1 and CM1 at lethal ethanol concentrations

The survival rates of strains W303-1A, SM1 and CM1 were investigated in the presence of lethal ethanol concentrations. Late exponential phase cells were inoculated into YEPD medium containing ethanol concentrations that are known to be lethal *i.e.* identified as lethal for the wild-type, W303-1A. The cultures were incubated at 30°C and the viable cell population was measured at regular intervals. Incubating cells under such conditions provides an opportunity to measure the tolerance of these strains to high ethanol concentrations, rather than their ability to adapt and grow during ethanol stress as was investigated in the previous section.

The difference in ethanol tolerance between the parent and mutant strains is also evident at lethal ethanol concentrations above 10% (v/v). Survival curves were performed for all three strains at 12% (v/v), 14% (v/v) and 16% (v/v) ethanol (Figure 3.11 and Table 3.3). The mutant strains were also incubated in ethanol concentrations of 20% (v/v), conditions under which the wild-type could not survive for longer than 30 minutes of incubation (Table 3.3). The viable population of the mutant strain cultures was always higher than W303-1A cultures at all lethal ethanol concentrations used. For example,

Table 3.3: Comparison of growth/survival rates in ethanol for *S. cerevisiae* strains W303-1A, SM1 and CM1.

Ethanol Concentration % (v/v)	W303-1A			SM1			CM1		
	SGR[1] (SSR[2]) (h^{-1})	Standard Error	Doubling (Halving) Time (h)	SGR (SSR) (h^{-1})	Standard Error	Doubling (Halving) Time (h)	SGR (SSR) (h^{-1})	Standard Error	Doubling (Halving) Time (h)
0	0.388	0.0029	1.79	0.431	0.0067	1.61	0.473	0.0202	1.46
6	0.178	0.0089	3.90	0.247	0.0364	2.80	0.268	0.0359	2.58
6.5	0.134	0.0126	5.19	0.240	0.0171	2.88	0.182	0.0190	3.80
8	(-0.005)	0.0104	(145.72)	0.113	0.0827	6.15	0.142	0.1298	4.86
10	(-0.027)	0.0174	(25.42)	(-0.022)	0.0214	(31.38)	(-0.030)	0.0209	(22.99)
12	(-0.555)	0.1258	(1.25)	(-0.023)	0.0058	(30.08)	(-0.067)	0.0201	(10.26)
14	(-0.915)	0.0434	(0.76)	(-0.162)	0.0919	(4.28)	(-0.434)	0.0734	(1.60)
16	(-6.499)	0.1655	(0.11)	(-0.435)	0.1329	(1.59)	(-0.735)	0.0708	(0.94)
20				(-1.876)	0.2602	(0.37)	(-1.664)	0.0645	(0.42)

[1]Specific Growth Rate

[2]Specific Survival Rate

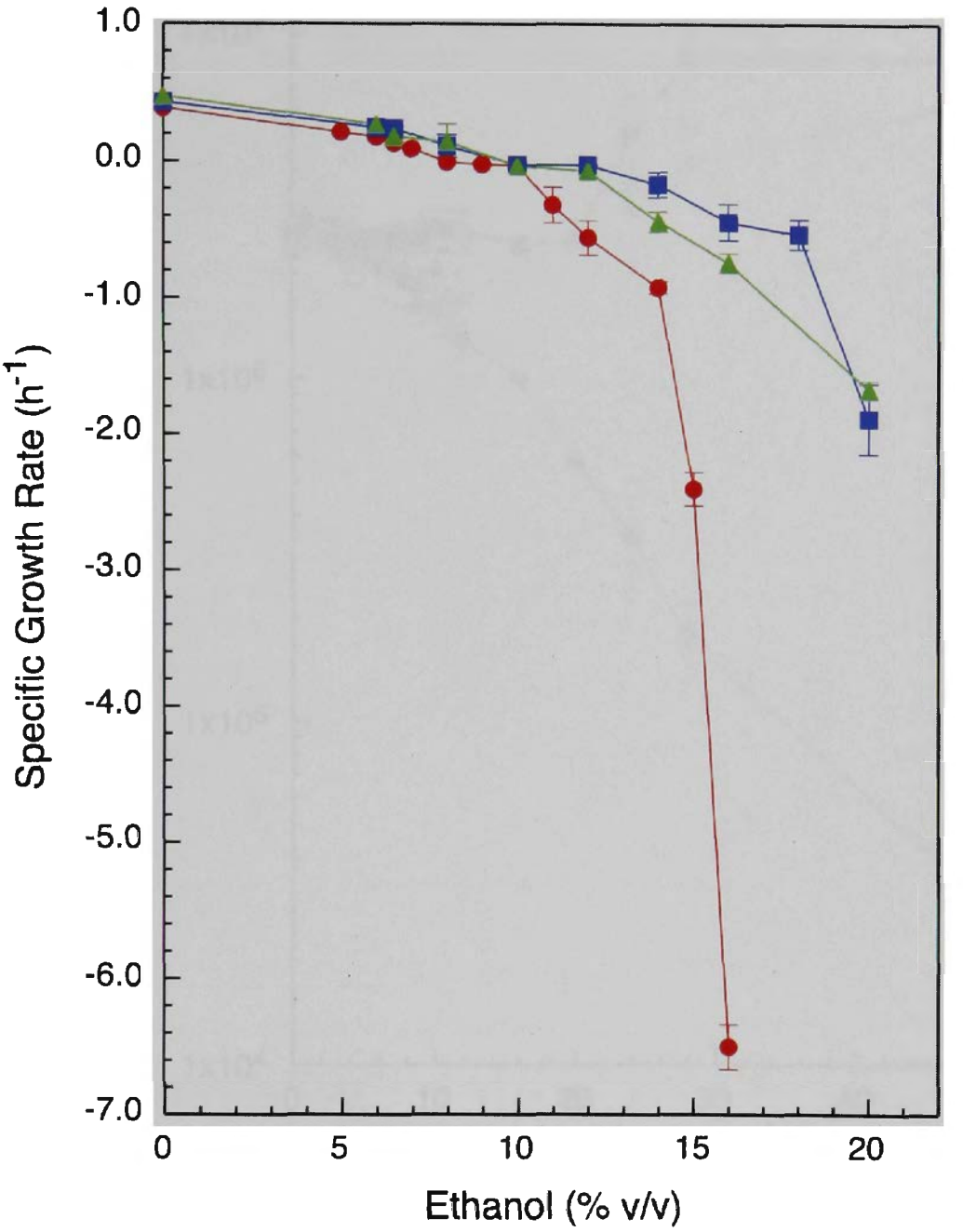

Figure 3.9: Comparison of Specific Growth Rates (SGR) for strains W303-1A (●), SM1 (■) and CM1 (▲) over a range of ethanol concentrations in YEPD at 30°C/110 rpm. Error bars represent standard errors and if not visible on the graph they are smaller than the symbol. Data associated with the graph is presented in Tables 3.1, 3.2 and 3.3.

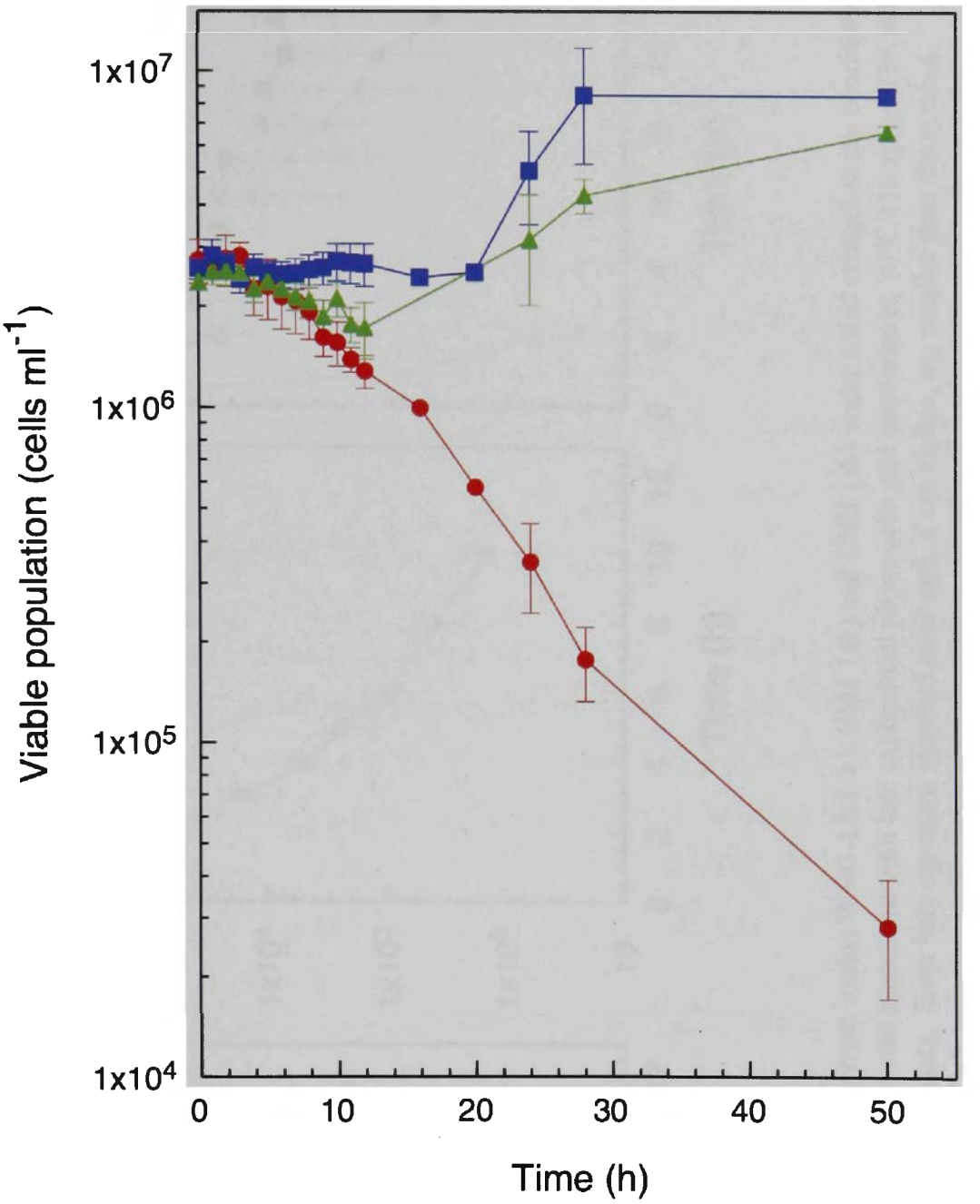

Figure 3.10: Viable cell population profiles of strains W303-1A (●), SM1 (■) and CM1 (▲) in YEPD containing 9% (v/v) ethanol; cultures were incubated at 30°C/110 rpm. Mean values from a minimum of 3 experiments are shown. Error bars represent standard error and are smaller than the symbol if not visible.

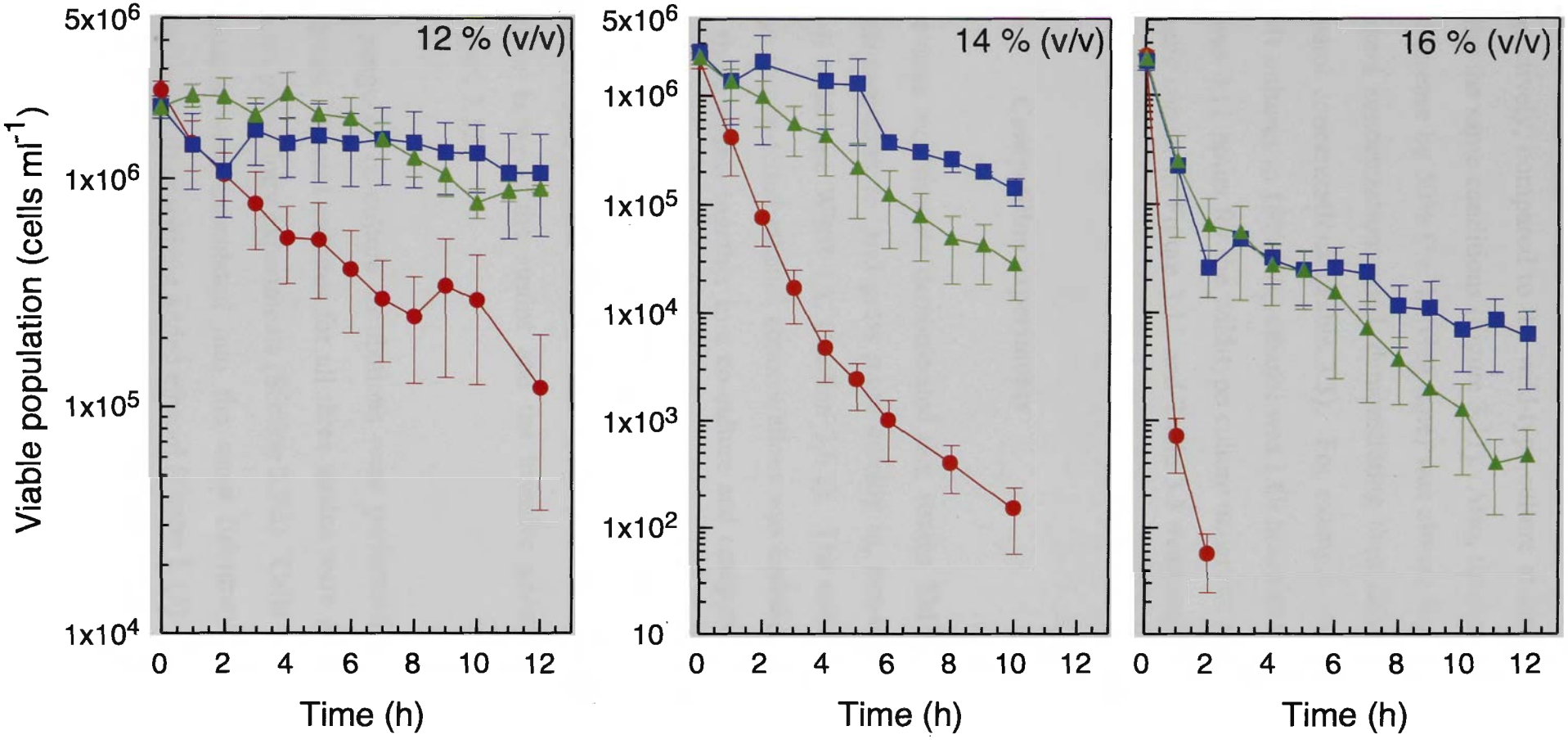

Figure 3.11: Viable cell population profiles of *S. cerevisiae* strains W303-1A (●), SM1 (■) and CM1 (▲) under lethal ethanol stress conditions. Ethanol concentration is indicated on the graph. YEPD medium was inoculated with late exponential phase cells and incubated at 30°C/110 rpm. The values shown represent the mean of a minimum of 3 replicate experiments. Error bars represent standard error and, if not visible, are smaller than the symbol.

the viable population (expressed as a percentage of the initial population) of the SM1 and CM1 cultures after 12 hours incubation in 12% (v/v) ethanol was 52% and 44.4% respectively, compared to the wild-type culture at around 5% of the initial population under the same conditions (Figure 3.11). Also, the time taken for the viable population to decrease by 50% (*i.e.* halving time) was always higher for the mutant cultures at all ethanol concentrations used, demonstrating their enhanced ability to cope with lethal ethanol concentrations (Table 3.3). For example, the halving time for the SM1 and CM1 cultures in 16% (v/v) ethanol was 1.60 hours and 0.94 hours respectively, whereas it was 0.11 hours for the wild-type culture under the same conditions (Table 3.3). The results shown in Figure 3.11 and Table 3.3 demonstrate the superior ability of strains SM1 and CM1 to tolerate lethal ethanol concentrations compared to the wild-type.

3.6.4 Competition experiments

Previous experiments demonstrated that strains SM1 and CM1 have higher rates of acclimatisation to, and grow more quickly in, non-lethal ethanol concentrations than their wild-type W303-1A, (Section 3.6.2). The competitiveness of mutant and wild-type in non-lethal ethanol concentrations was investigated further by growing any two of these strains together in a co-culture and comparing the cell population profile for each strain. This was possible because the colonies of each strain had a unique morphology on plates. Demarcation of the colonies for each strain on solid medium containing a mixture of two strains from a co-culture was based on the difference in colour between the strains and the invasive growth characteristics of the mutants (Figure 3.12).

A range of co-culture incubations were performed in the presence and absence of ethanol. Parent cultures for all three strains were grown as described previously for batch physiology experiments (Section 2.2.2). Cells from late exponential phase parent cultures were inoculated into the same Erlenmeyer flask containing fresh YEPD medium with or without added ethanol (Figure 3.13). Each co-culture was inoculated to an initial cell population of around 2×10^6 cells ml^{-1} for each strain *ie.* a total cell population of 4×10^6 cells ml^{-1}. Samples for biomass analysis were taken regularly over the course of the incubation and prepared in duplicate on YEPD plates. Triplicate

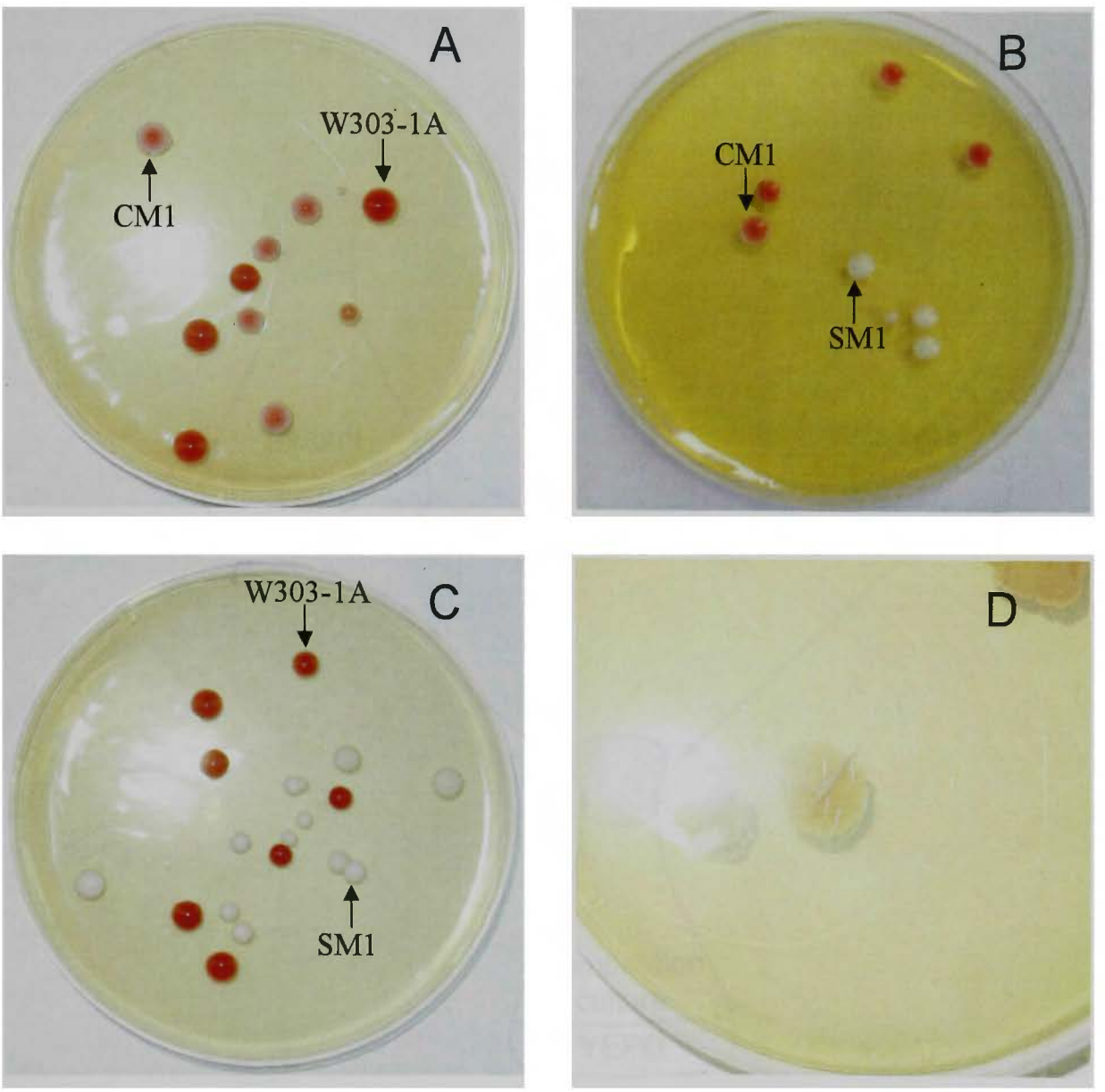

Figure 3.12: Colony morphology of *S. cerevisiae* strains W303-1A, SM1 and CM1. Colonies of the 3 strains displayed differences in colour; during plate incubation W303-1A changed from pink on day 2 to burgundy on day 5 while, over the same period, CM1 changed from cream to pink and SM1 changed from white to off-white. Pink CM1 colonies had a cream-coloured ring on the colony outskirts. Both mutants also showed invasive growth which could be observed by inverting the plate. CM1 displayed star-shaped invasive growth (Panel **D**) into the depth of agar, whereas SM1 grew into the agar causing needle-thin prick-like scars under the colony; the wild-type did not display any forms of invasive growth. Colony morphologies are shown for YEPD plates after incubation for 5 days at room temperature.

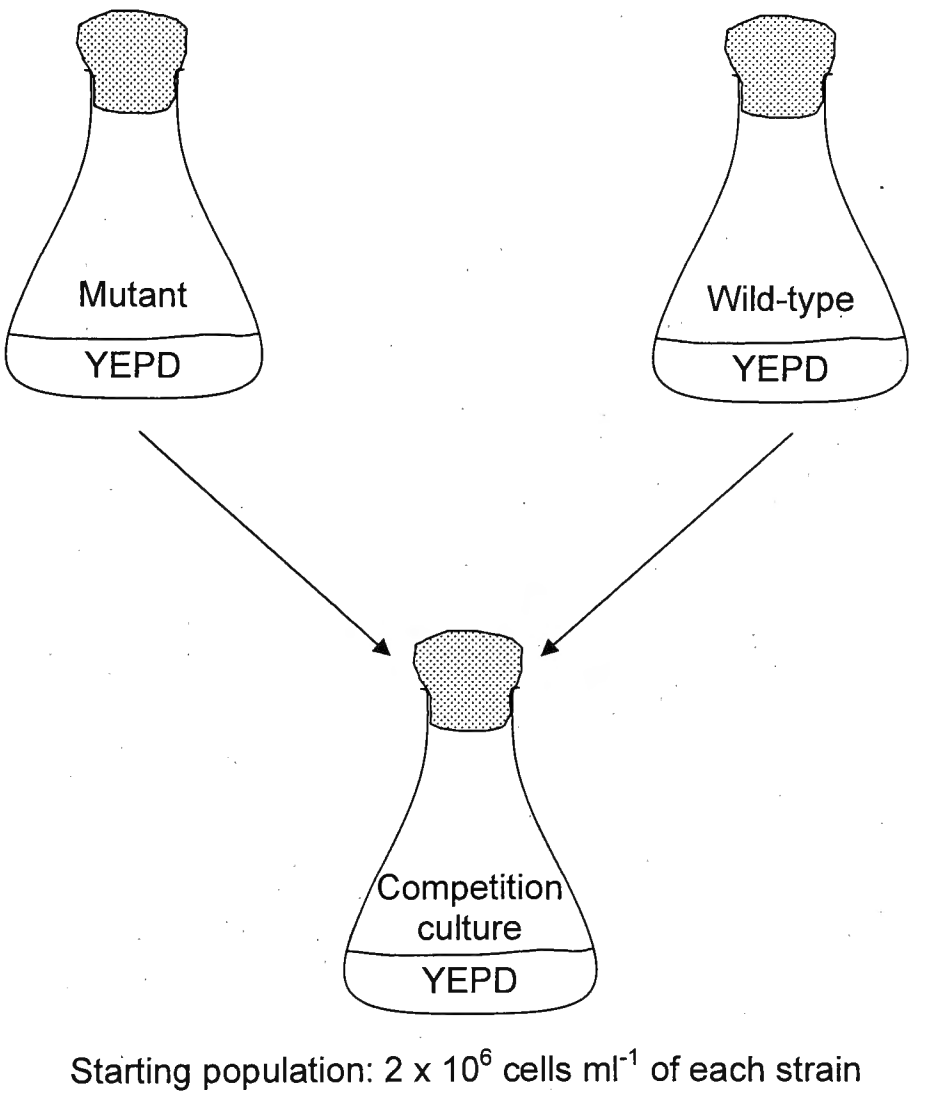

Figure 3.13: Schematic diagram representing the inoculation procedure for competitive growth experiments.

experiments were performed for each set of conditions.

3.6.4.1 W303-1A and SM1 co-cultures

There was no significant difference in the growth profiles of strains W303-1A and SM1 when co-incubated in the absence of ethanol (Figure 3.14). The specific growth rate and final cell population were comparable over a 55 hour incubation. In the presence of ethanol, however, SM1 was considerably more competitive than W303-1A with the former strain dominating the total cell population in the co-cultures after 55 hours of incubation. When the co-cultures were incubated in 6% (v/v) ethanol, SM1 reached a final cell population that was approximately 4-fold greater than the final cell population achieved by W303-1A. A significant difference was observed in the viable cell population profiles of these two strains when incubated in 9% (v/v) ethanol, with SM1 growing to a final cell population of around 3×10^7 cells ml^{-1} after 55 hours incubation while the viable cell population of W303-1A decreased during this time *i.e.* this strain was slowly dying with no remaining viable cells 50 hours after inoculation (data not shown).

3.6.4.2 W303-1A and CM1 co-cultures

Strain CM1 appears to have a higher specific growth rate than W303-1A when coincubated in the absence of ethanol, however, after 25 hours of incubation both strains had similar cell populations (Figure 3.15). In the presence of 6% (v/v) ethanol, strain CM1 was substantially more competitive than strain W303-1A with the former strain dominating the total cell population in the co-cultures after 55 hours of incubation. CM1 reached a final cell population that was approximately 25-fold greater than the final cell population achieved by W303-1A. A more unusual feature of this co-culture was the significant decline in viable cell population of W303-1A after 10 hours of co-incubation with CM1, appearing to plateau at around 1.4×10^6 cells ml^{-1} somewhere between 12-28 hours of incubation. As with the SM1 and W303-1A co-culture, a significant difference was observed in the response of CM1 and W303-1A when coincubated in 9% (v/v) ethanol, with CM1 growing to a final cell population of

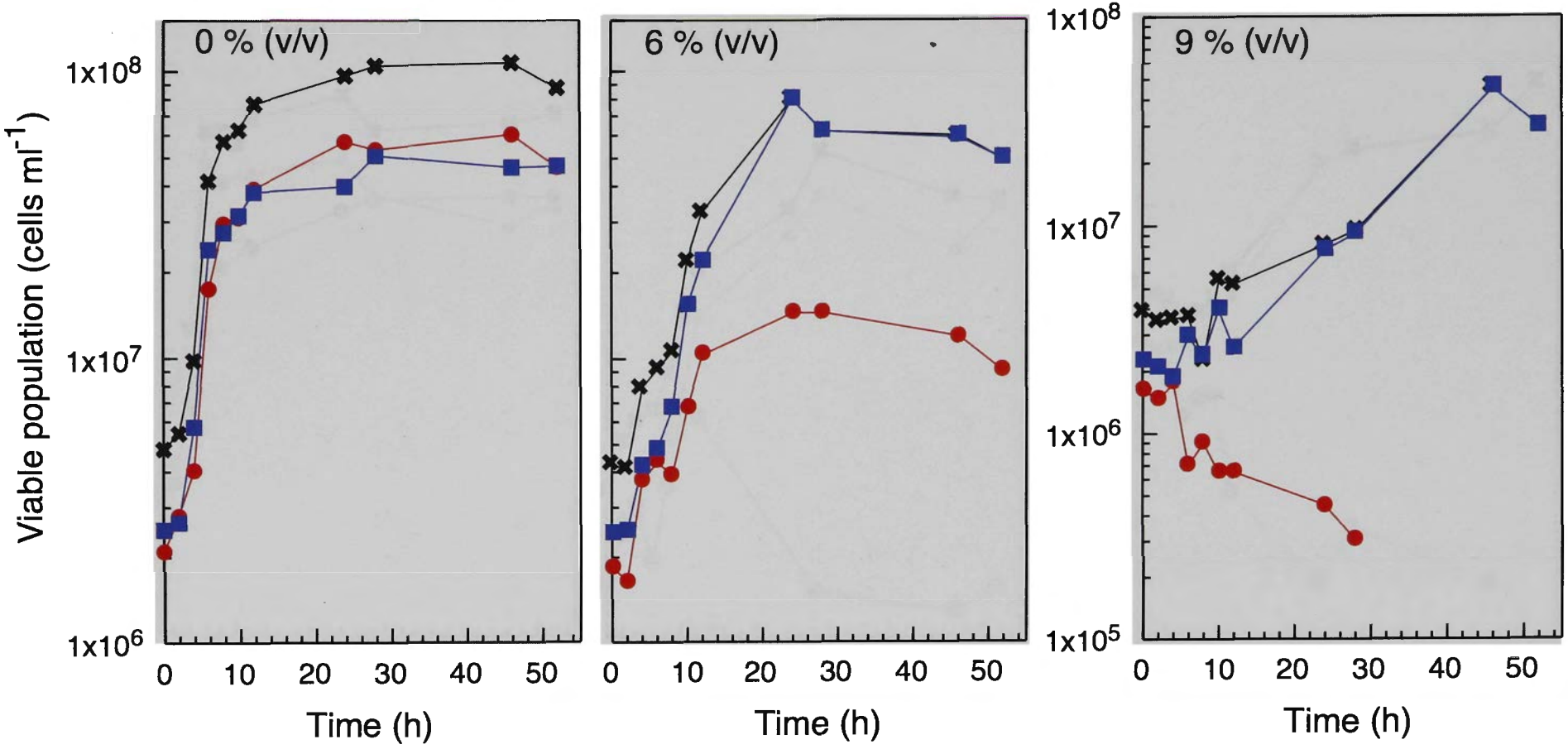

Figure 3.14: Cell population profiles of batch co-cultures containing *S. cerevisiae* strains W303-1A and SM1 in the presence or absence of ethanol. The graphs show total cell population (×), strain W303-1A only (●) and strain SM1 only (■) for each set of co-culture conditions. YEPD medium was inoculated with late exponential-phase cells and the culture incubated at 30°C/110 rpm.

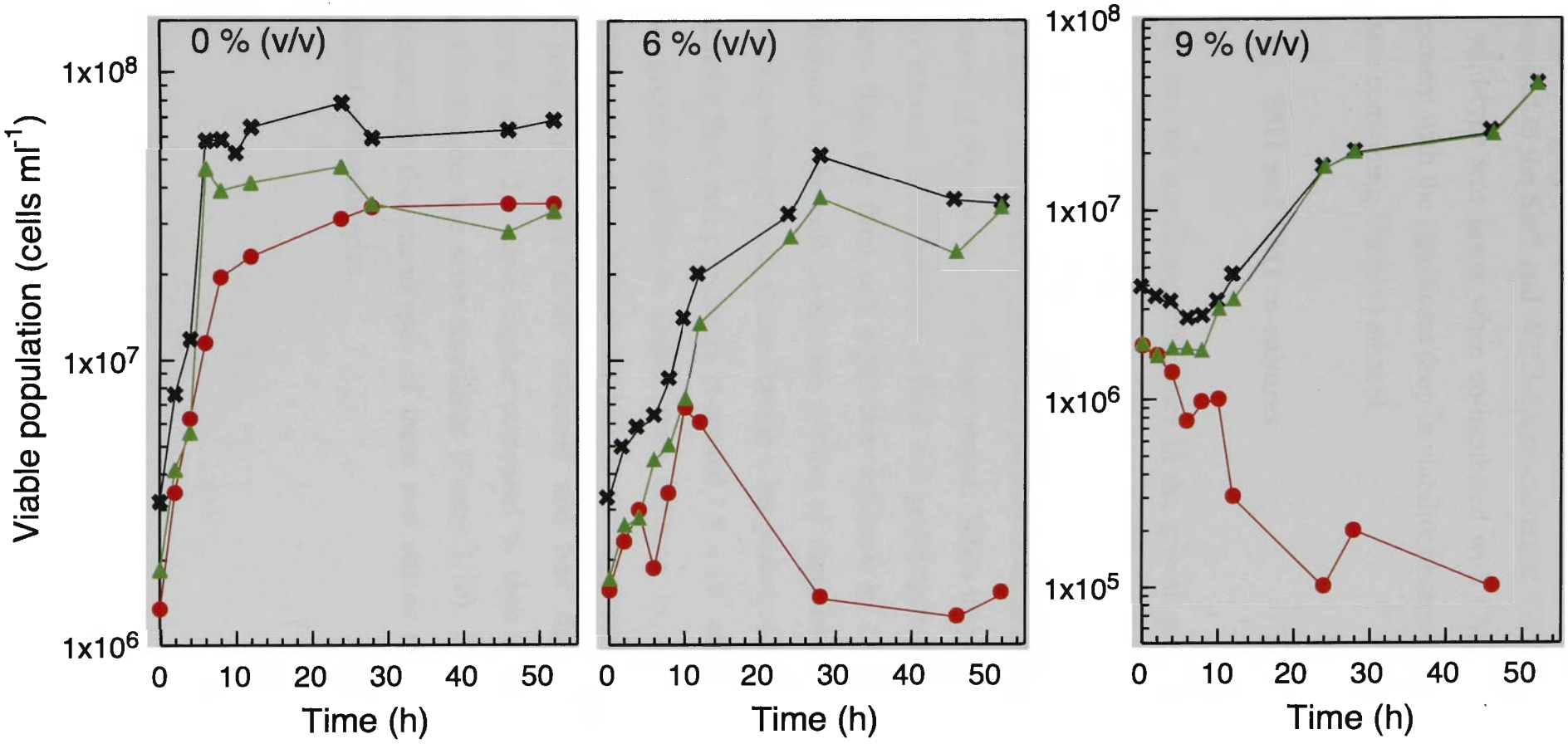

Figure 3.15: Cell population profiles of batch co-cultures containing *S. cerevisiae* strains W303-1A and CM1 in the presence or absence of ethanol. The graphs show total cell population (×), strain W303-1A only (●) and strain CM1 only (▲) for each set of co-culture conditions. YEPD medium was inoculated with late exponential-phase cells and the culture incubated at 30°C/110 rpm.

around 4×10^7 cells ml^{-1} after 55 hours incubation (and it appeared to still be growing at that stage) while the viable cell population of W303-1A decreased during this time. Compared to the SM1 and W303-1A co-culture at 9% (v/v) ethanol, the survival rate of the wild-type was lower when co-incubated with CM1 in 9% (v/v) ethanol, which is consistent with the significant drop in viability observed for the W303-1A and CM1 co-culture containing 6% (v/v) ethanol.

3.6.4.3 SM1 and CM1 co-cultures

There was no significant difference in the growth profiles of strains SM1 and CM1 when co-incubated in the absence of ethanol (Figure 3.16). The specific growth rates and final cell populations were the same over a 55 hour incubation period. There were only small differences in the growth profiles of these two strains when incubated in the presence of ethanol over a 55 hour period. When the co-cultures were incubated in 6% (v/v) ethanol, SM1 reached a final cell population that was approximately 1.8-fold greater than the final cell population achieved by CM1. There was no significant difference in the cell population profiles of these two strains when incubated in 9% (v/v) ethanol with both strains having a lag period of around 12 hours and each strain reached a final cell population of around 1.8×10^7 cells ml^{-1}. However, compared to their growth profiles in single strain cultures, in a co-culture of SM1 and CM1 containing 9% (v/v), both strains had shorter lag periods (12-13 hours compared to 15-20 hours in single strain cultures) and had final cell populations that were approximately 2.5 times higher compared to their respective single strain cultures incubated under the same conditions (Figure 3.10). Overall there appears to be little difference in the phenotypes of these two strains despite being created using two different methodologies.

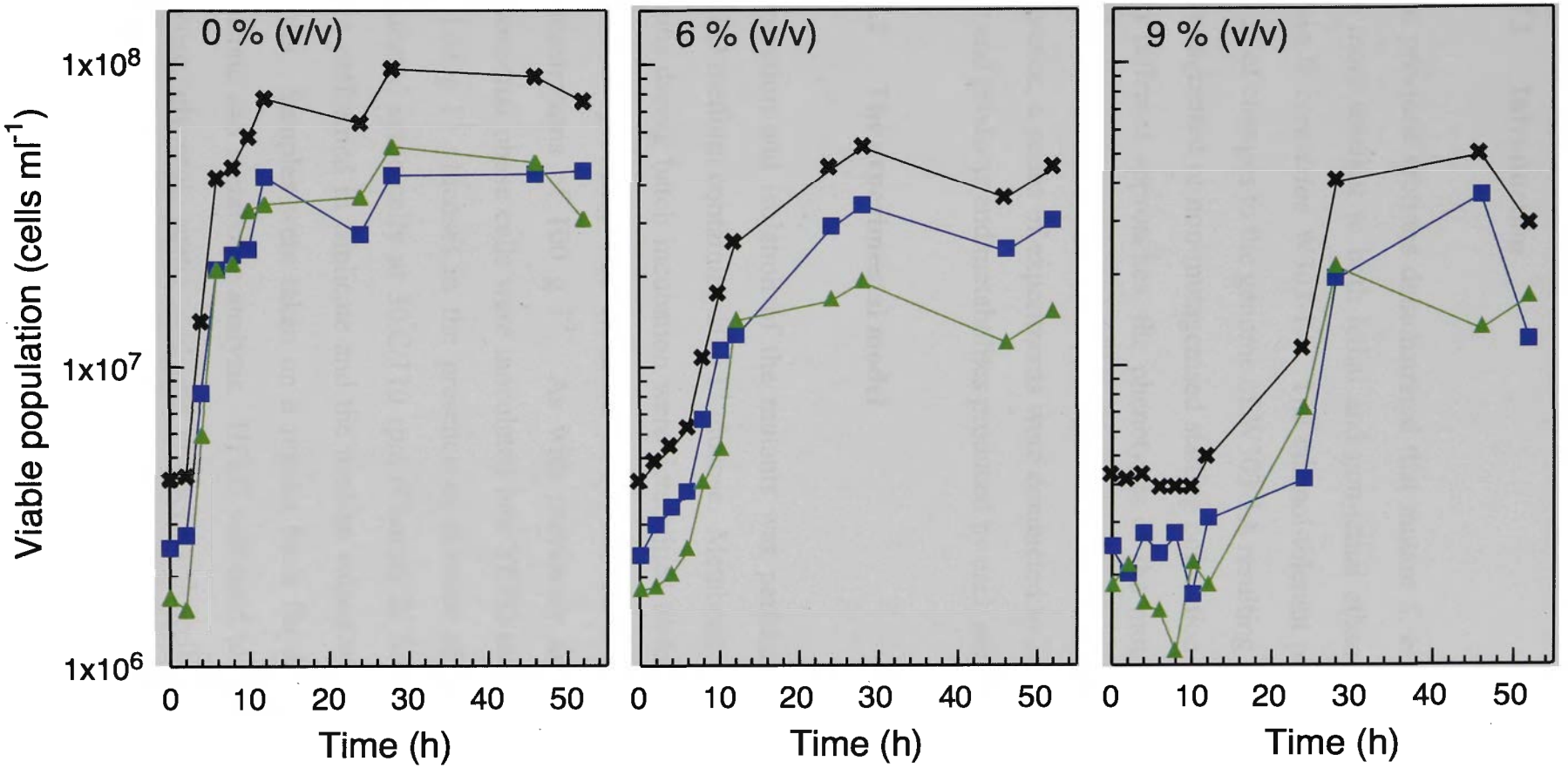

Figure 3.16: Cell population profiles of batch co-cultures containing *S. cerevisiae* mutant strains CM1 and SM1 in the presence or absence of ethanol. The graphs show total cell population (✖), strain SM1 only (■) and strain CM1 only (▲) for each set of co-culture conditions. YEPD medium was inoculated with late exponential-phase cells and the culture incubated at 30°C/110 rpm.

3.7 METABOLIC PROFILES OF ETHANOL-TOLERANT MUTANTS AND WILD-TYPE W303-1A

3.7.1 Introduction

The previous sections demonstrated that mutant *S. cerevisiae* strains, SM1 and CM1, are more tolerant to both lethal and non-lethal ethanol concentrations than wild-type strain *S. cerevisiae* W303-1A. The ethanol-tolerant phenotypes of the mutants are the result of changes to the genome of W303-1A resulting from adaptive evolution of either a mutagenised or non-mutagenised starting population. Despite having been created by two different approaches, the phenotypes of the mutants are very similar. To better understand the changes that occurred in the mutants which lead to improved ethanol tolerance, a series of experiments were conducted to measure changes in the profiles of key end products and metabolites produced by each strain.

3.7.2 The experimental model

Generation and isolation of the mutants was performed under aerobic conditions in YEPD medium containing 20 g l^{-1} glucose. Metabolic profiles of the parent and mutant strains during batch incubation were determined under the same conditions, however, metabolic profiling was extended to include cultures grown under higher glucose concentrations of 100 g l^{-1}. As with previously described batch incubations, late exponential phase cells were inoculated into YEPD medium (containing either 20 g l^{-1} or 100 g l^{-1} glucose) in the presence or absence of added ethanol (6.5% v/v) and incubated aerobically at 30°C/110 rpm (Chapter 2, Section 2.2.2). These incubations were performed in triplicate and the median values were used to generate the growth curves. Samples were taken on a regular basis for determination of cell population, substrate and metabolite analysis. HPLC was used to determine the concentrations of glucose, glycerol, acetic acid and ethanol as described in Chapter 2 (Section 2.4). Under the conditions used for HPLC analysis, all four compounds gave good quality peaks; isovaleric acid (IVA) was used as the internal standard (Figure 3.17).

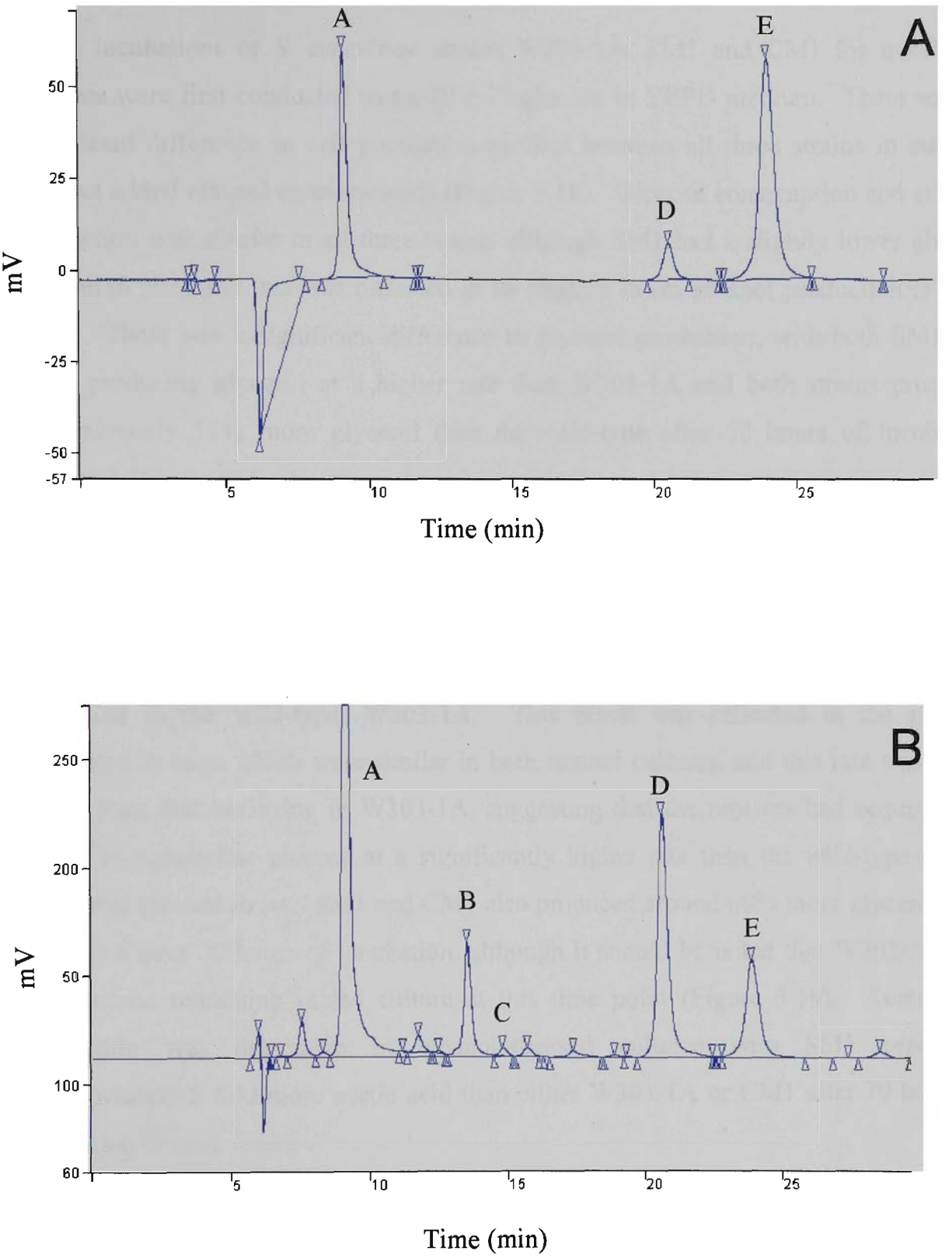

Figure 3.17: Representative HPLC chromatograms using a Biorad HPX-87H HPLC column for the analysis of culture samples. The two chromatograms demonstrate the quality of the peaks obtained from authentic culture samples for glucose (A), glycerol (B), acetic acid (C), ethanol (D) and internal standard IVA (E). Due to their lower concentrations, undiluted or low dilution samples were used to measure glycerol and acetic acid (Panel B).

3.7.3 Cultivations of wild-type, SM1 and CM1 in 20 g l^{-1} glucose

Batch incubations of *S. cerevisiae* strains W303-1A, SM1 and CM1 for metabolite analysis were first conducted using 20 g l^{-1} glucose in YEPD medium. There was no significant difference in cell population profiles between all three strains in cultures without added ethanol (non-stressed) (Figure 3.18). Glucose consumption and ethanol production was similar in all three strains although SM1 had a slightly lower glucose utilization rate, and this was reflected in its slightly lower ethanol production (Figure 3.18). There was a significant difference in glycerol production, with both SM1 and CM1 producing glycerol at a higher rate than W303-1A and both strains produced approximately 55% more glycerol than the wild-type after 50 hours of incubation (Figure 3.18).

Differences in metabolism were more apparent when the strains were incubated in 6.5% (v/v) ethanol (Figure 3.19). As observed previously, SM1 and CM1 cultures had higher specific growth rates, shorter lag periods and reached higher final cell populations compared to the wild-type, W303-1A. This result was reflected in the glucose consumption rates which were similar in both mutant cultures, and this rate was much higher than that occurring in W303-1A, suggesting that the mutants had acquired the ability to metabolise glucose at a significantly higher rate than the wild-type during non-lethal ethanol stress. SM1 and CM1 also produced around 60% more glycerol than W303-1A over 70 hours of incubation, although it should be noted that W303-1A still had glucose remaining in the culture at this time point (Figure 3.19). Acetic acid production was detectable in ethanol-stressed cultures with SM1 producing approximately 5-fold more acetic acid than either W303-1A or CM1 after 70 hours of incubation (Figure 3.19).

3.7.4 Cultivations of wild-type, SM1 and CM1 in 100 g l^{-1} glucose

Batch incubations of *S. cerevisiae* strains W303-1A, SM1 and CM1 for metabolite analysis were also conducted using 100 g l^{-1} glucose in YEPD medium. In the absence of ethanol stress, strains W303-1A and SM1 grew similarly, although SM1 had a higher final cell population; CM1 grew more slowly and had a significantly lower final cell

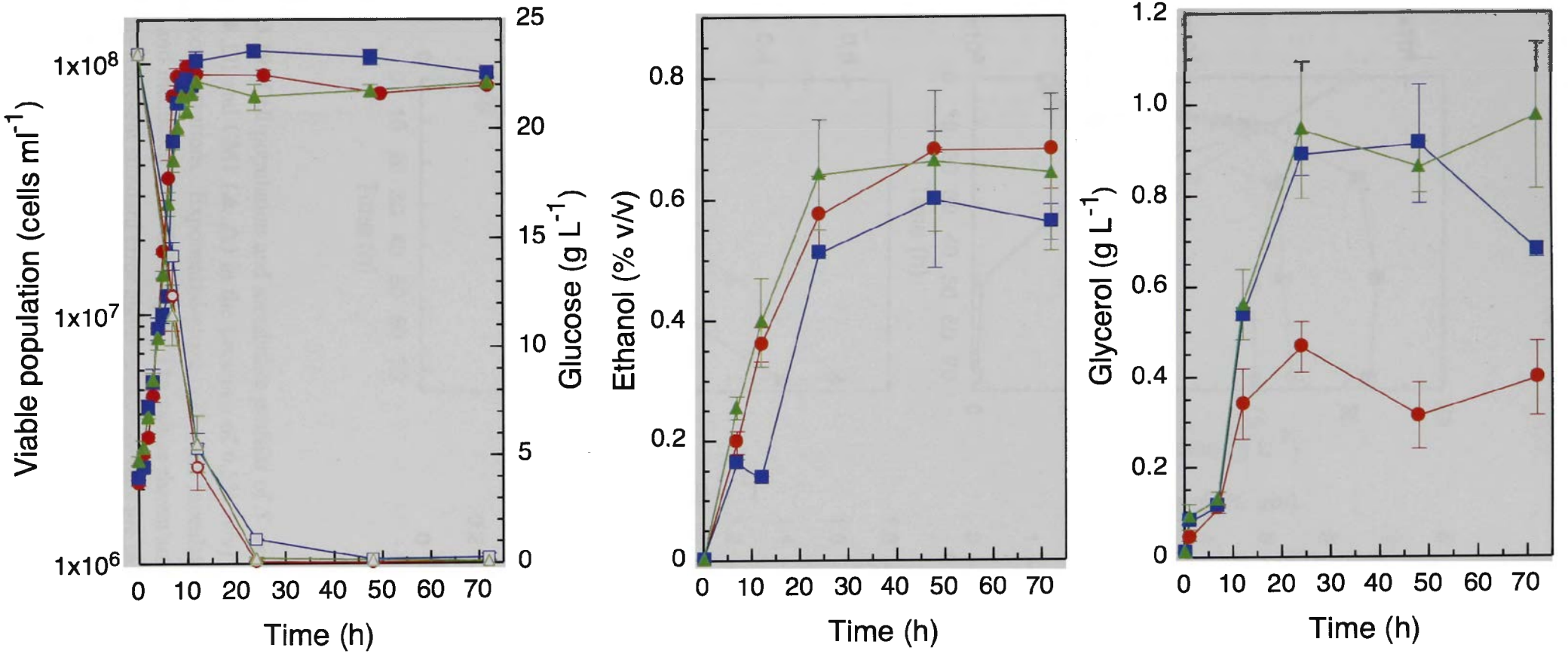

Figure 3.18: Cell population and metabolite profiles of *S. cerevisiae* strains W303-1A (●,○), SM1 (■,□) and CM1 (▲,△) in the absence of added ethanol; open symbols represent glucose concentrations. Exponential-phase cells were inoculated into YEPD containing 20 g l^{-1} glucose and incubated at 30°C/110 rpm. Acetic acid concentrations were not detectable. The values shown are means of triplicate experiments. Error bars represent standard error and if not visible they are smaller than the symbol.

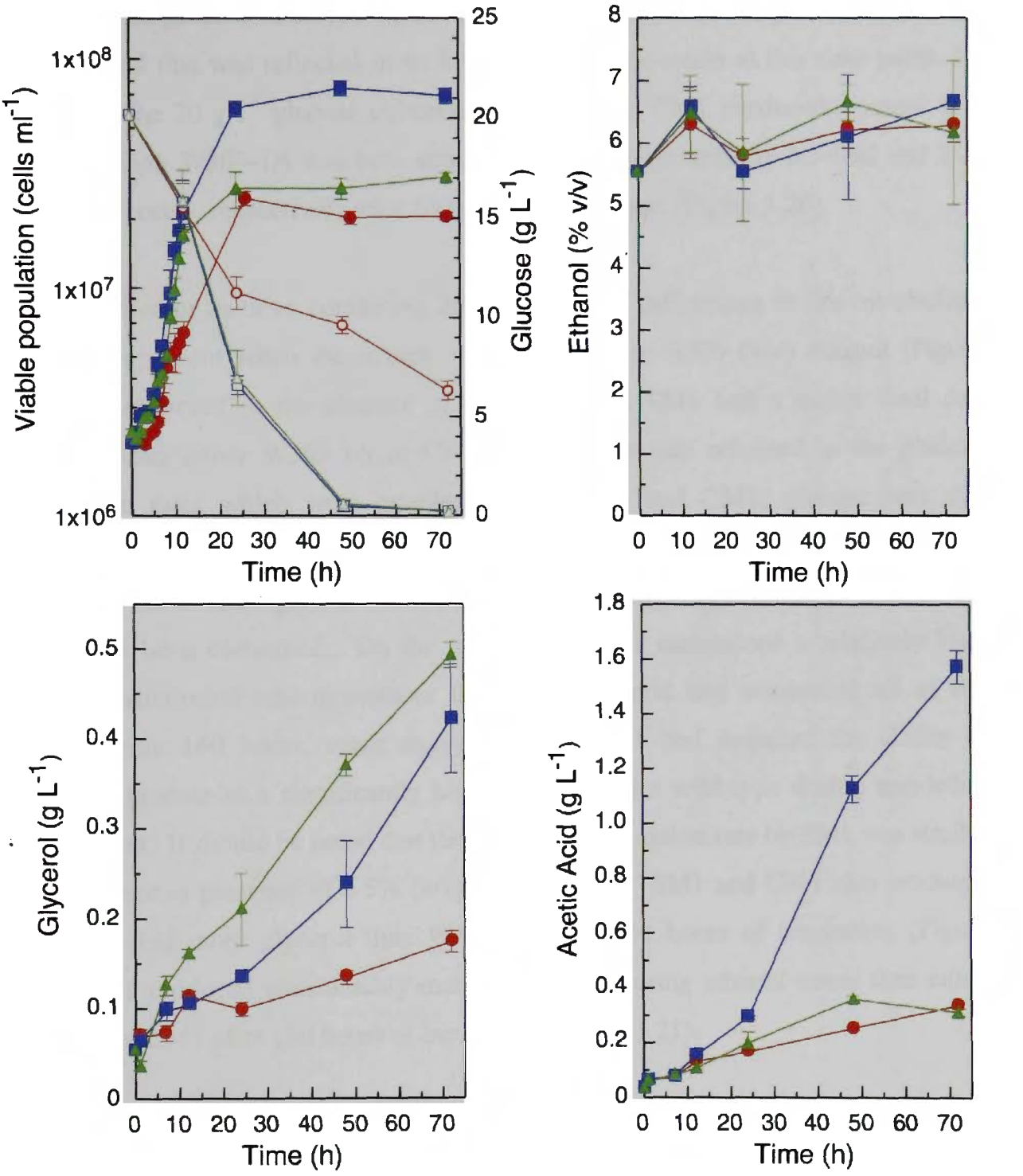

Figure 3.19: Cell population and metabolite profiles of *S. cerevisiae* strains W303-1A (●,○), SM1 (■,□) and CM1 (▲,△) in the presence of 6.5% (v/v) ethanol; open symbols represent glucose concentrations. Exponential-phase cells were inoculated into YEPD containing 20 g l^{-1} glucose and incubated at 30°C/110 rpm. The values shown are means of triplicate experiments. Error bars represent standard error and if not visible they are smaller than the symbol.

population than the wild-type and SM1 (Figure 3.20). W303-1A and SM1 also had similar rates of glucose consumption and ethanol production, however, CM1 had not used all of the glucose after 160 hours of incubation (approximately 25 g l^{-1} glucose remained) and this was reflected in its lower ethanol production at this time point. As observed in the 20 g l^{-1} glucose cultures, both SM1 and CM1 produced glycerol at a higher rate than W303-1A and both strains produced approximately 3.3-fold and 2.2-fold more glycerol, respectively after 50 hours of incubation (Figure 3.20).

As seen earlier in cultures containing 20 g l^{-1} glucose, differences in the metabolism were more apparent when the strains were incubated in 6.5% (v/v) ethanol (Figure 3.21). As observed in the absence of added ethanol, SM1 had a higher final cell population than either W303-1A or CM1. This result was reflected in the glucose consumption rates which were retarded in W303-1A and CM1; glucose was still available after 160 hours of incubation. Furthermore, both strains appeared to significantly slow their glucose utilization rate when only approximately half of the glucose had been consumed. On the other hand, SM1 maintained a relatively high glucose consumption rate throughout the incubation and had consumed all of the glucose within 160 hours, again suggesting that SM1 had acquired the ability to metabolise glucose at a significantly higher rate than the wild-type during non-lethal ethanol stress. It should be noted that the glucose consumption rate by SM1 was similar in the absence or presence of 6.5% (v/v) ethanol stress. SM1 and CM1 also produced around 2.3-fold more glycerol than W303-1A over 160 hours of incubation (Figure 3.21). SM1 produced considerably more acetic acid during ethanol-stress than either W303-1A or CM1 after 160 hours of incubation (Figure 3.21).

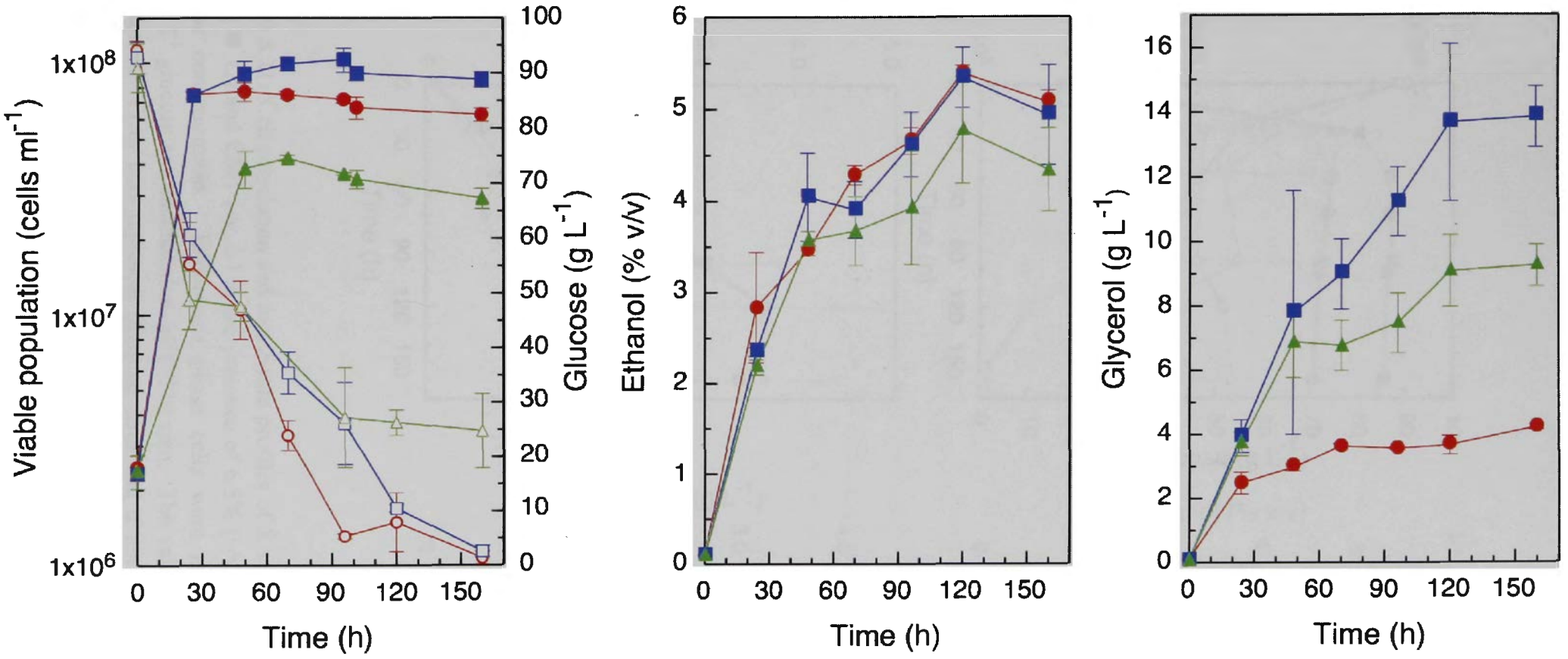

Figure 3.20: Cell population and metabolite profiles of *S. cerevisiae* strains W303-1A (●,○), SM1 (■,□) and CM1 (▲,△) in the absence of added ethanol; open symbols represent glucose concentrations. Exponential-phase cells were inoculated into YEPD containing 100 g l^{-1} glucose and incubated at 30°C/110 rpm. Acetic acid concentrations were not detectable. The values shown are means of triplicate experiments. Error bars represent standard error and if not visible they are smaller than the symbol.

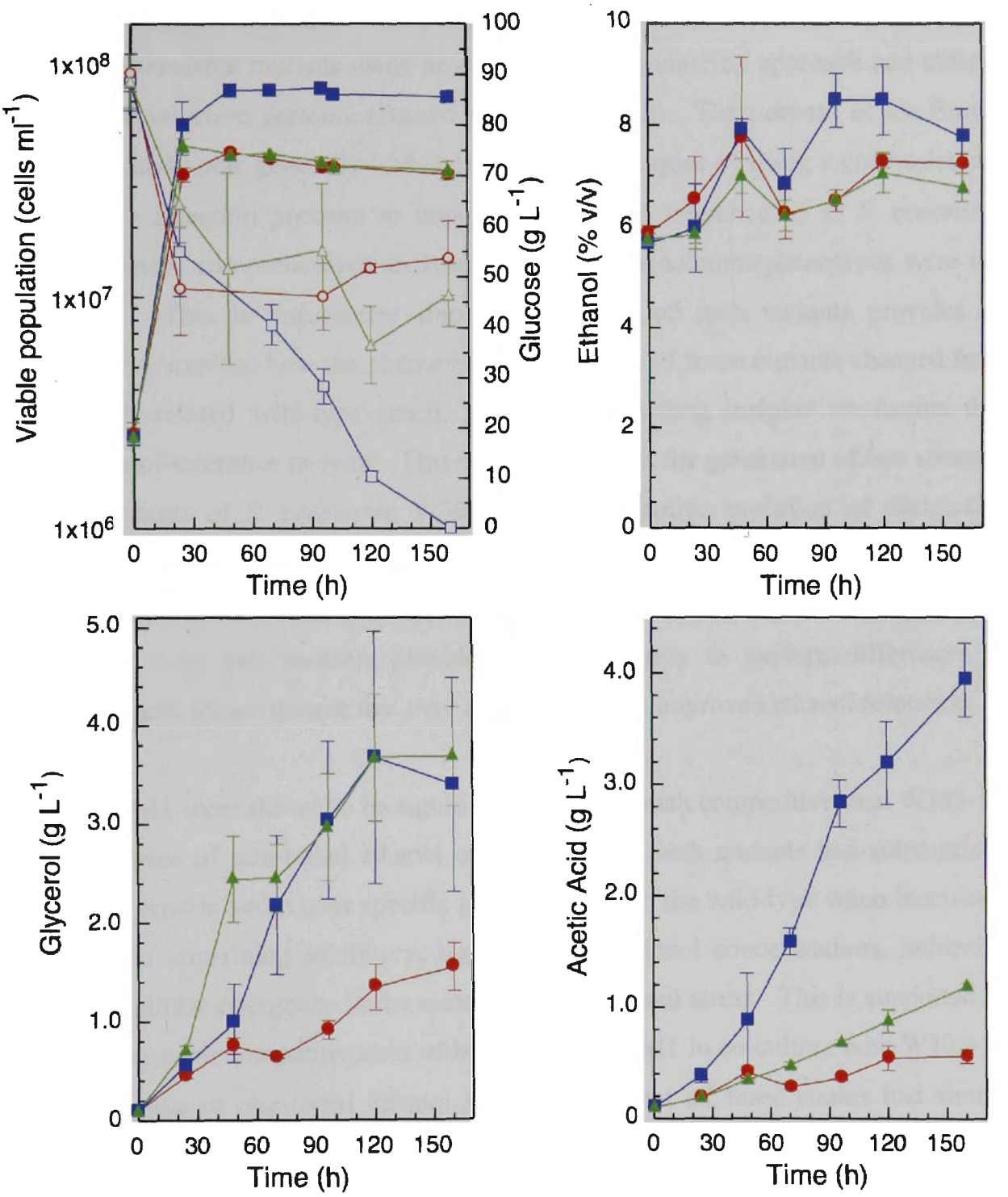

Figure 3.21: Cell population and metabolite profiles of *S. cerevisiae* strains W303-1A (●,○), SM1 (■,□) and CM1 (▲,△) in the presence of 6.5% (v/v) ethanol; open symbols represent glucose concentrations. Exponential-phase cells were inoculated into YEPD containing 100 g l^{-1} glucose and incubated at 30°C/110 rpm. The values shown are means of triplicate experiments. Error bars represent standard error and if not visible they are smaller than the symbol.

3.8 DISCUSSION

There is, to our knowledge, only one study that describes the creation of ethanol-tolerant *S. cerevisiae* mutants using an evolutionary engineering approach and ethanol stress as the selection pressure (Brown and Oliver 1982). The purpose of the Brown and Oliver study was generally to demonstrate the principles of using a chemostat and ethanol as a selection pressure to improve the ethanol tolerance of an *S. cerevisiae* strain, however, comprehensive analyses of the ethanol-tolerant phenotypes were not undertaken. . This is unfortunate since the isolation of such variants provides an opportunity to explore how the phenotype and genotype of these mutants changed from their closely-related wild-type strain, potentially providing insights on factors that confer ethanol-tolerance in yeast. This chapter describes the generation of two ethanol-tolerant mutants of *S. cerevisiae* W303-1A using adaptive evolution of chemically mutagenised (isolate CM1) and non-mutagenised (isolate SM1) populations of W303-1A; both approaches used ethanol in the chemostat feed as selection pressure. The creation of these two mutants provided the opportunity to explore differences in metabolism and transcriptome that may account for their improved ethanol tolerance.

SM1 and CM1 were shown to be significantly more growth competitive than W303-1A in the presence of non-lethal ethanol concentrations. Both mutants had substantially shorter lag periods and higher specific growth rates than the wild-type when inoculated into medium containing inhibitory, but not lethal, ethanol concentrations, indicating improved cellular energetics in the mutants during ethanol stress. This is supported by the greater growth competitiveness of both SM1 and CM1 in co-culture with W303-1A in the presence of non-lethal ethanol stress, noting that all three strains had similar growth phenotypes in the absence of ethanol stress. SM1 and CM1 were also able to survive higher ethanol concentrations than W303-1A, with an ethanol concentration of 9% (v/v) being lethal to the wild-type but not for the two mutant strains. The two mutants also demonstrated considerably higher ethanol-tolerance than W303-1A in lethal ethanol concentrations. Cell growth does not provide a competitive advantage under such conditions, however, SM1 and CM1 were observed to have higher survival rates, suggesting that their ability to resist the damaging and inhibitory effects of lethal ethanol concentrations is markedly improved compared to the wild-type. Overall, despite the differences in approach used to create SM1 and CM1, their ethanol-tolerant

growth phenotypes were remarkably similar across a range of ethanol stress conditions. The ethanol-tolerant phenotypes of the two mutant strains were also stable, being retained after several successive sub-cultures in the absence of stress and after storage in glycerol at -20 and -80°C; this is most likely attributable to using a chemostat-based approach for strain enrichment since unstable mutants do not ultimately survive in the mixed population.

Analysis of the glucose utilization rate by all three strains in the presence and absence of non-lethal ethanol stress provides some insight into how the mutant strains may have improved their ethanol tolerance. Glucose metabolism in the wild-type was noticeably compromised in the presence of 6.5% (v/v) ethanol stress. Glucose utilization during ethanol stress was considerably lower in W303-1A than either SM1 or CM1 in medium containing 20 g l^{-1} glucose. In the presence of 100 g l^{-1} glucose and 6.5% (v/v) ethanol, the SM1 strain showed little change in its glucose utilization rate compared to that in the absence of ethanol stress and the same initial glucose concentration, suggesting that glucose metabolism in SM1 is relatively unaffected by this level of ethanol stress. On the other hand, there was no obvious difference in glucose utilization by either wild-type W303-1A or CM1 in 100 g l^{-1} glucose and 6.5% (v/v) ethanol, with both strains having substantially lower glucose utilization rates than SM1. This is supported by the higher final cell population achieved by SM1 in all ethanol-stressed cultures, suggesting a greater efficiency in glucose metabolism in this strain when exposed to ethanol stress. Although glucose utilization by CM1 is similar to SM1 at low glucose concentrations, its ability to utilize glucose at 100 g l^{-1} is compromised both in the presence and absence of ethanol, which is probably a defect resulting from the use of EMS mutagenesis to create the strain.

An explanation for improved glucose utilization by SM1 and CM1 during ethanol stress may be found in the higher glycerol production rates of both strains. Despite the different techniques used to generate SM1 and CM1, both strains had in common significantly higher glycerol production rates compared to W303-1A. Glycerol metabolism has an important role in the redox state of the cell by oxidising NADH to NAD^+, with the ratio of these two cofactors being influential regulators of central metabolism (Brisson *et al.*, 2001; Nevoigt and Stahl 1997; Vemuri *et al.*, 2007). There is some evidence to suggest that glycolytic flux is inhibited during ethanol stress,

resulting in compromised energetics in the cell (Alexandre *et al.*, 2001; Chandler *et al.*, 2004; Fujita *et al.*, 2004). It has been suggested that one bottleneck in glycolytic metabolism during ethanol stress is glyceraldehyde 3 phosphate dehydrogenase activity, which uses NAD^+ as a cofactor, and for which supply is limited due to the loss of intracellular acetaldehyde across an ethanol-compromised plasma membrane (Barber *et al.*, 2002; Stanley *et al.*, 1997). It has been proposed that a loss of intracellular acetaldehyde during ethanol stress reduces the rate of NADH oxidation by alcohol dehydrogenase activity leading to an imbalance in the $NADH/NAD^+$ ratio (Barber *et al.*, 2002; Stanley *et al.*, 1997); it has been shown that adding acetaldehyde to ethanol-stressed *S. cerevisiae* cultures improves their adaptation to, and growth rate in, non-lethal ethanol concentrations (Barber *et al.*, 2002; Stanley *et al.*, 1993; Vriesekoop *et al.*, 2007; Vriesekoop and Pamment 2005). The higher activity of the glycerol metabolism in SM1 and CM1 could, in part, compensate for the loss of NAD^+ production during ethanol stress by escalating the NADH oxidation rate, subsequently increasing the availability of NAD^+ for glyceraldehyde 3 phosphate dehydrogenase activity, which may lead to improved glycolytic flux and cellular energetics.

Both mutant strains produced more acetate than the wild-type during ethanol stress, with SM1 producing considerably more acetate than either CM1 or W303-1A. This may be a response to the higher glycerol production in the mutants, which increases NAD^+ supply, and/or reduces NADH levels, in the cell, which in turn could stimulate aldehyde dehydrogenase activity. This phenomenon has been observed in glycerol overproduction studies where increased glycerol production coincided with higher acetate production, noting that such studies did not involve investigations using ethanol stress (De Barros Lopes *et al.*, 2000; Michnick *et al.*, 1997; Remize *et al.*, 1999). This speculation is however inconsistent with the observation in the current study that acetate could not be detected in the absence of ethanol stress, despite the two mutant strains producing much higher amounts of glycerol than W303-1A under these conditions.

The above observation suggests that acetate production was a direct response to ethanol stress, in which case several scenarios could potentially account for this effect. One possibility is that increased acetate metabolism may reflect a need by the cell to increase the amount of acetyl-CoA entering the citric acid cycle, at a time when protein turnover is high due to the process of acclimatising to the stress; this would be facilitated by

mitochondrial-based *ALD5* activity (Kurita and Nishida 1999; Saint-Prix *et al.,* 2004). Alternatively, it may be due to increased *ALD6* activity which uses $NADP^+$ as a cofactor, increasing the cytosolic supply of NADP(H). It has been suggested that an increase in acetate production in strains overproducing glycerol could be a way of providing additional NAD(P)H since 1 mol of acetate from glucose leads to the production of 2 mol of NAD(P)H (Remize *et al.,* 1999). This is plausible given that an ethanol-stressed cell adapts to the stress by changing the fatty acid profile of its membrane lipids, and fatty acid metabolism requires NADP(H) as a cofactor. Finally, it could be that high ethanol concentrations stimulate *ALD4* activity, which is required for growth on ethanol as a carbon source. Normally, this would be considered unlikely since gluconeogenic respiration on ethanol by *S.cerevisiae* is glucose repressed (Entian and Barnett 1992; Gancedo 1992; Randez-Gil *et al.,* 1997) and there was excess glucose in the experiments described in this thesis. Recent studies however suggest that *S. cerevisiae* cells enter a pseudo-starvation state when exposed to ethanol stress, even when surplus glucose is present in the medium (Chandler *et al.,* 2004). Under such conditions, the cell may respond to the intracellular 'pseudo-starvation' and high ethanol concentrations by derepression of the ethanol respiration metabolism. Further work on the acetate metabolism would be required to determine which of these mechanisms is responsible for the acetate production observed in the current studies.

This chapter described the creation of two ethanol-tolerant mutants of *S. cerevisiae* W303-1A using adaptive evolution. Compared to the wild-type, it was shown that the mutants had increased acclimatisation and growth rates when cultivated in non-lethal ethanol concentrations, and their ability to survive lethal ethanol concentrations was considerably improved. Metabolite analysis demonstrated that both mutants produce significantly more glycerol than the wild-type, possibly as a means of increasing NAD^+ supply in an ethanol-compromised cell. Acetate was only detectable in ethanol-stressed cultures with both mutants producing more acetate than the wild-type; although a number of possible mechanisms may account for this, further work is required to determine which mechanism(s) is responsible. In this regard, the following chapter describes gene expression analysis of the wild-type and mutant strains under various environmental conditions, the objective being to acquire more information on which functions in the cell are affected by ethanol toxicity and the biochemical mechanisms involved in physiological adaptation.

CHAPTER 4

MICROARRAY PROFILING OF THE ETHANOL STRESS RESPONSE IN WILD-TYPE AND ETHANOL-TOLERANT MUTANTS OF *S. cerevisiae*

4.1 INTRODUCTION

The response of yeast to environmental stress has received much attention over the years. There are a considerable number of publications investigating different stress factors in yeast, including heat and cold shock, ethanol, pH and acid stress, oxidative and reductive stress, hyper-osmotic stress and starvation. However, at the commencement of this project there were only two studies focusing on global gene expression in response to ethanol stress. Microarray analysis by Ogawa *et al.,* (2000) and Alexandre *et al.,* (2001) provided the first insights into the effect of ethanol on gene expression in *S. cerevisiae*. This was later followed by a number of publications directly addressing changes in transcription (Chandler *et al.,* 2004; Fujita *et al.,* 2004; Hirasawa *et al.,* 2007) and others screening deletion libraries for impaired growth under ethanol stress (Aguilera and Benitez 1986; Fujita *et al.,* 2006; Kubota *et al.,* 2004; Takahashi *et al.,* 2001; van Voorst *et al.,* 2006).

This chapter applies microarray technology to identify genes that are differentially expressed in the *S. cerevisiae* W303-1A due to the presence of 6.5% ethanol, together with genes contributing to increased ethanol tolerance in both CM1 and SM1 mutant strains. The aim of this study was to highlight common mechanisms shared by the wild-type and mutant strains for ethanol tolerance and also mechanisms specific for either the CM1 or SM1 mutant. Identifying genes differentially expressed in the ethanol tolerant mutants compared to the wild-type during ethanol stress should provide valuable information on pathways that can be invoked by yeast to ensure survival during ethanol stress. Furthermore, understanding the ways that these mutants have adapted to ethanol stress at a molecular level, combined with detailed metabolic analysis, may help to identify novel approaches for creating ethanol tolerant strains with higher stress tolerance in the future.

The aims of this chapter were to:

- identify genes that are differentially expressed in response to ethanol.
- determine similarities and differences in gene expression profiles between the ethanol tolerant mutants and wild-type in the presence and absence of ethanol stress.
- compare gene expression data with results from metabolic analyses, presented in the previous chapter, to identify specific mechanisms and pathways involved in conferring enhanced ethanol tolerance in the SM1 and/or CM1 mutant strains.

4.2 EXPERIMENTAL CONDITIONS FOR INDUCING ETHANOL STRESS

To identify genes involved in the adaptation of yeast to non-lethal ethanol stress, each strain was grown separately at 0% and 6.5% (v/v) ethanol and their gene expression compared using microarray analysis. Growth curves were analysed prior to RNA extraction as described in Section 3.2. For the inoculum preparation, late exponential cells, grown in rich YEPD medium, were washed to remove any products of metabolism that might interfere with the cells' response to the new conditions. Cultures were then inoculated to an OD_{620} of 0.1 in YEPD medium (1 litre) containing either 0 or 6.5 % (v/v) ethanol. The cultures were incubated at 30°C and 110 rpm, under aerobic conditions. Samples were taken for plate counts on a regular basis.

The growth curves presented in Figures 4.1 and 4.2 show mean viable counts over a period of 12 hours from four replicate experiments used for RNA extraction and subsequent microarray analysis. Three of these experiments were conducted over an extended time course of 72 hours to allow sampling for metabolite analysis, as presented in Section 3.7. Cells for analysis of gene expression were collected after one hour, at which point all three strains were still in the lag phase and were undergoing acclimatisation to their new environment, as previously established in Chapter 3, Section 3.6. The collected cells were immediately frozen in liquid nitrogen.

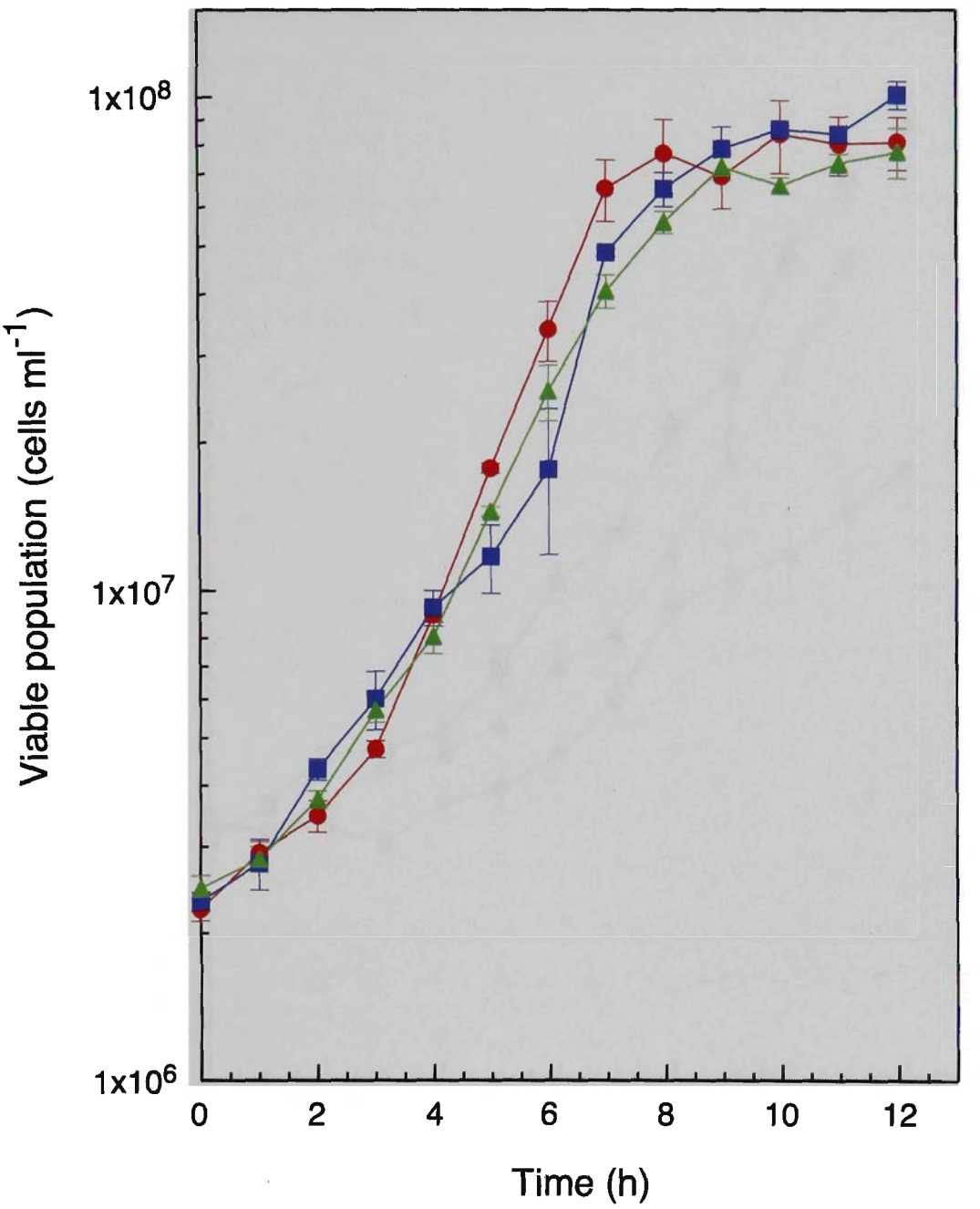

Figure 4.1: Growth profile of *S. cerevisiae* strains W303-1A (●), SM1 (■) and CM1 (▲) in the absence of ethanol. The cultures were incubated aerobically at 30°C/110 rpm. Mean viable populations were determined from four independent experiments for each of the three strains. Error bars represent standard error. When not visible, error bars are smaller than the symbol.

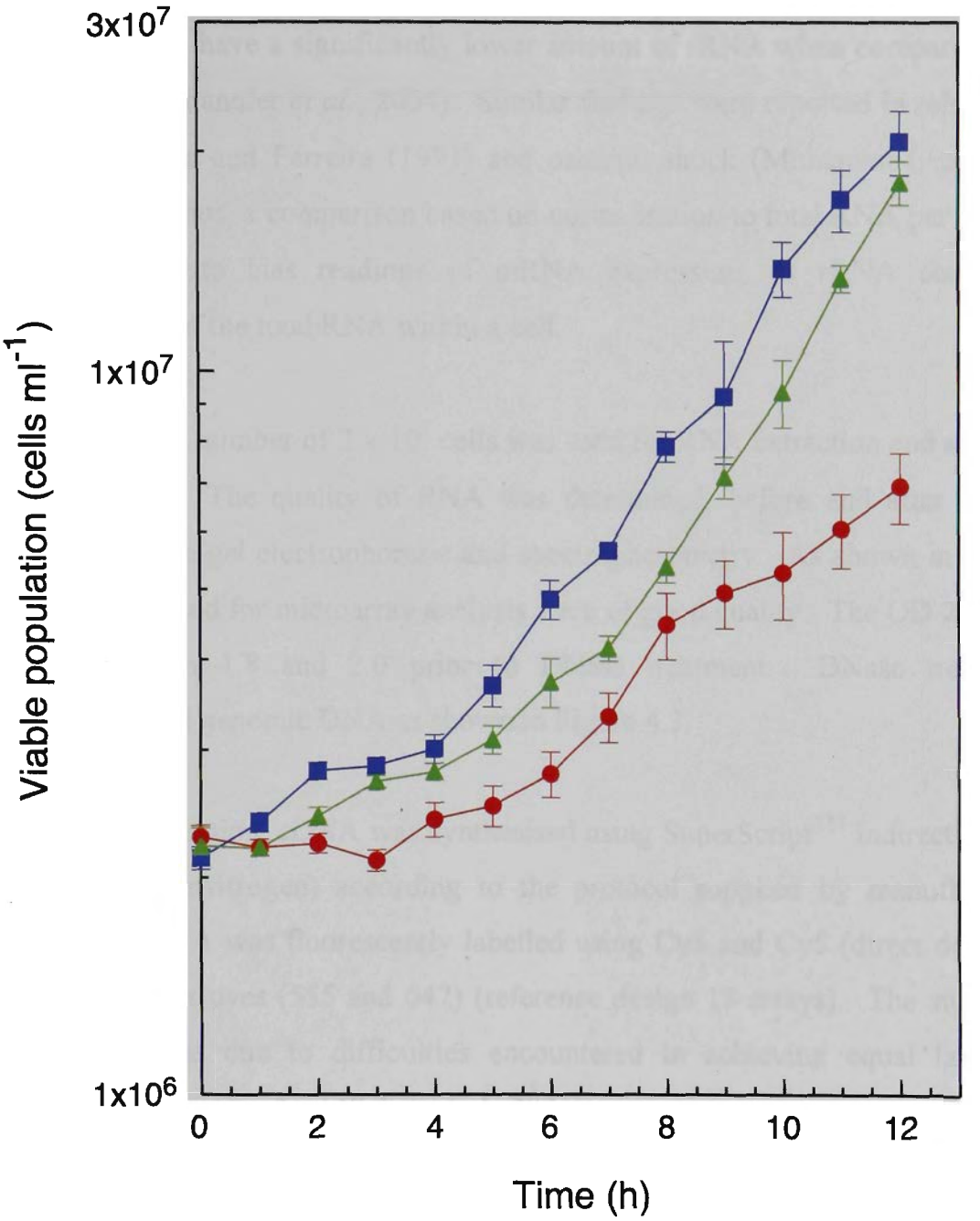

Figure 4.2: Growth profile of *S. cerevisiae* strains W303-1A (●), SM1 (■) and CM1 as (▲) in the presence of 6.5% (v/v) ethanol. The cultures were incubated aerobically at 30°C/110 rpm. Mean viable populations were determined from four independent experiments for each of the three strains. Error bars represent standard error. When not visible error bars are smaller than the symbol.

For comparison of gene expression, RNA was extracted from equal numbers of cells. This approach was adopted as previous research at Victoria University has shown that ethanol stressed cells have a significantly lower amount of rRNA when compared with non-stressed cells (Chandler *et al.*, 2004). Similar findings were reported in relation to heat stress by Mager and Ferreira (1993) and osmotic shock (Mohammad, personal communications). Thus, a comparison based on normalisation to total RNA per sample would be expected to bias readings of mRNA expression, as rRNA comprises approximately 95% of the total RNA within a cell.

In this work an equal number of 2×10^7 cells was used for RNA extraction and analysis of gene expression. The quality of RNA was determined, before and after DNase treatment, by agarose gel electrophoresis and spectrophotometry. As shown in Figure 4.3, RNA samples used for microarray analysis were of good quality. The OD 260/280 ratios were between 1.8 and 2.0 prior to DNase treatment. DNase treatment successfully removed genomic DNA as shown in Figure 4.3.

Following RNA extraction, cDNA was synthesised using SuperScript™ Indirect cDNA Labelling System (Invitrogen) according to the protocol supplied by manufacturer. After synthesis, cDNA was fluorescently labelled using Cy3 and Cy5 (direct design 3 arrays) or AlexaFluor dyes (555 and 647) (reference design 18 arrays). The switch to AlexaFluor dyes was due to difficulties encountered in achieving equal labelling efficiency with the cyanine dyes (Schut *et al.*, 2001).

Microarray slides were obtained from The Clive and Vera Ramaciotti Centre for Gene Function Analysis (Ramaciotti Centre). The glass slides (SchottNexterion Slide A+ amino link) were spotted with 13824 elements across 6 vertical and 4 horizontal arrays, comprising duplicate spots of 6,528 *S. cerevisiae* open reading frames (ORF). The unmodified 50-mer oligonucleotides had been spotted using an aminosilane attachment protocol. Following hybridization, the glass slides were scanned using a GenePix-Pro 4000 scanner and analysed using Genowiz™ 4.0.2.1 software. The quality of the slides is shown in Figure 4.4.

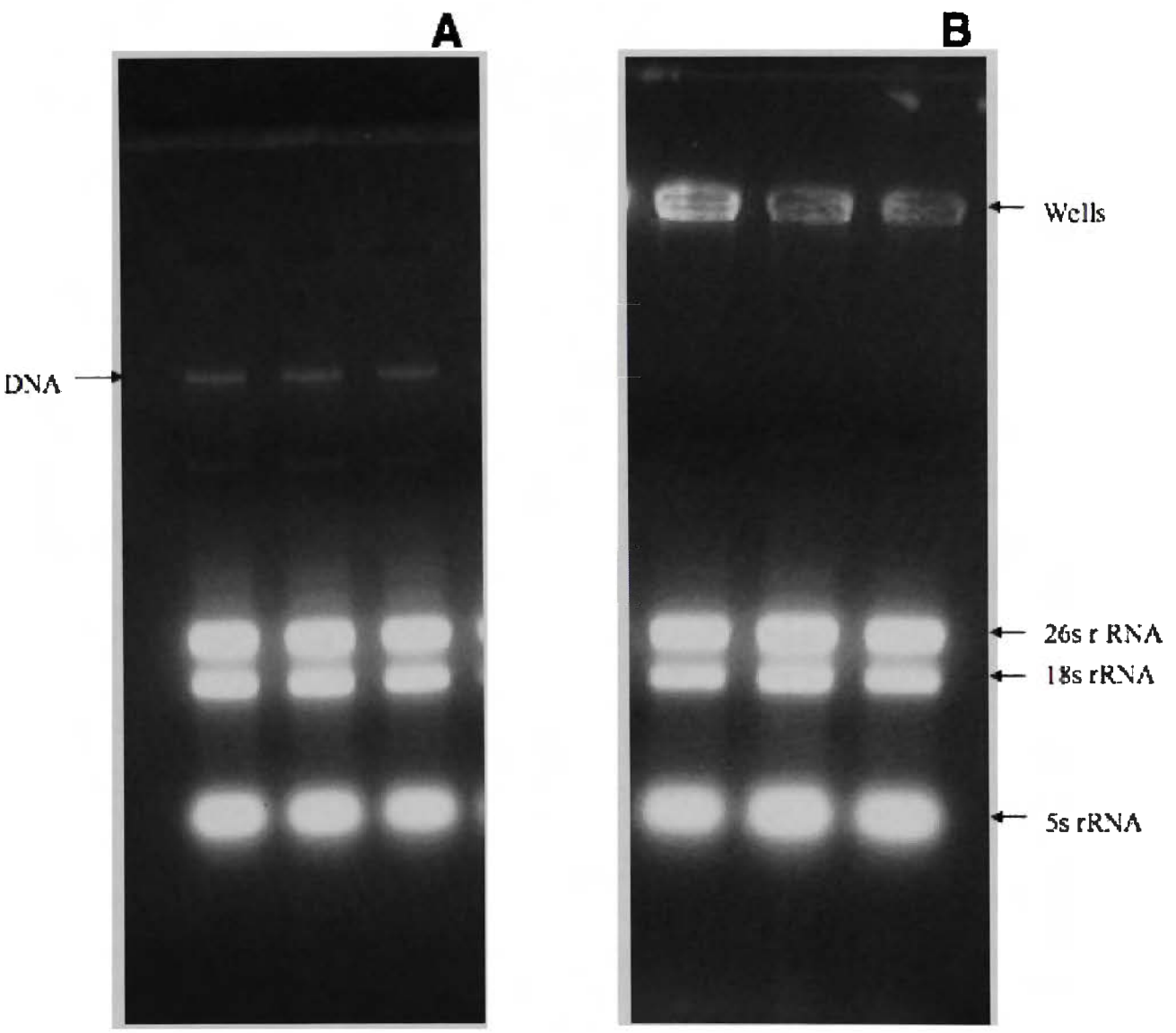

Figure 4.3: Quality of RNA demonstrated by gel electrophoresis: Total RNA was isolated from equal numbers (2×10^7) of cells and visualized on a 1% ethidium bromide-stained agarose gel (A) before DNase treatment and (B) after DNase treatment. The relatively constant RNA yield from the same experimental condition over 3 different growth experiments indicates that the extraction method was consistent. The overall quality of the preparations is apparent from the integrity of the rRNA bands. DNA was removed without compromising RNA quality. It should be noted that gels were overexposed in order to visualise DNA band.

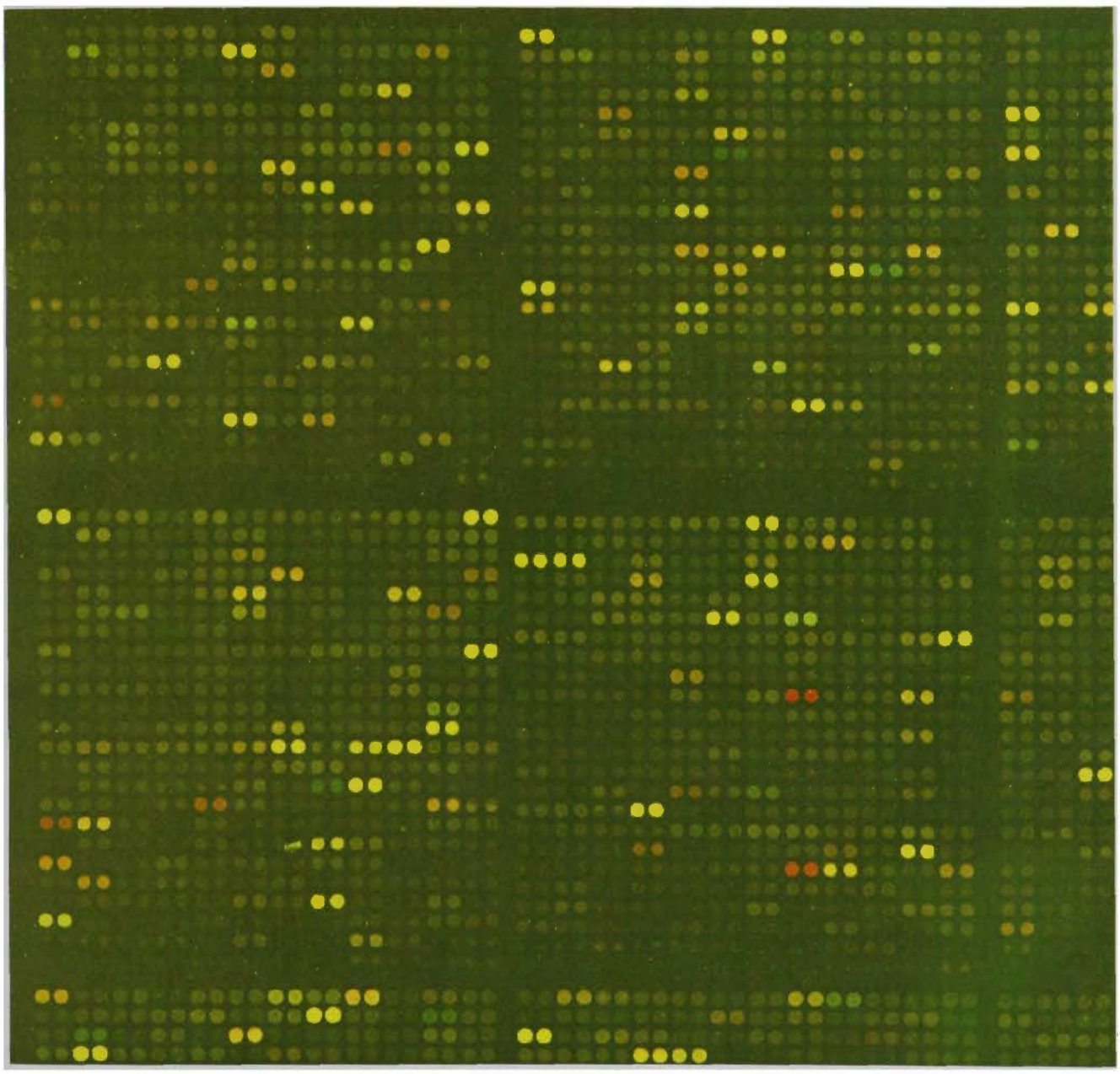

Figure 4.4: A section of a representative microarray demonstrating the efficient labelling of cDNA probes with AlexaFluor dyes, hybridisation and washing efficiency and integrity of the spots.

Due to the complexity of the experimental design and the extent of data produced during this experiment, it was decided to focus on aspects of the analysis considered to be most relevant to this thesis. Detailed results of the analysis, together with original gpr files, are provided on an accompanying Appendix CD.

4.3 EXPERIMENTAL DESIGN

The overall aim of the microarray analysis was to identify genes contributing to ethanol tolerance in *S. cerevisiae.* In contrast to Alexandre *et al.,* (2001) and Chandler *et al.,* (2004) who performed direct comparisons involving one strain under stressed and non-stressed conditions, this study involved 3 strains; the wild-type, the chemical mutant (CM1) and the spontaneous mutant (SM1). The experimental design therefore reflected the need to identify similarities and differences in the response to ethanol by each mutant when compared with the wild-type strain.

The experimental approach comprised two sets of microarray experiments, the first a three-way reference experiment (18 arrays) and the second a direct comparison experiment (3 arrays). This is illustrated in Figure 4.5.

In the **three-way reference experiment**, shown by thin arrows in Figure 4.5, each condition was compared to a reference pool of RNA. This pool was prepared by combining RNA from 3 separate growth curves from the wild-type W303-1A in the absence of added ethanol (0%). For each experimental condition, replicate growth experiments were performed on different days using separate glycerol stock (-80 °C) of the appropriate strain to ensure that each replicate experiment in a biological replicate set was distinct.

This approach permitted use of a number of statistical tools available for microarray analysis, such as 2-way ANOVA. Fold changes were calculated indirectly based on the relative gene expression levels in the stressed and unstressed samples with reference to the common control.

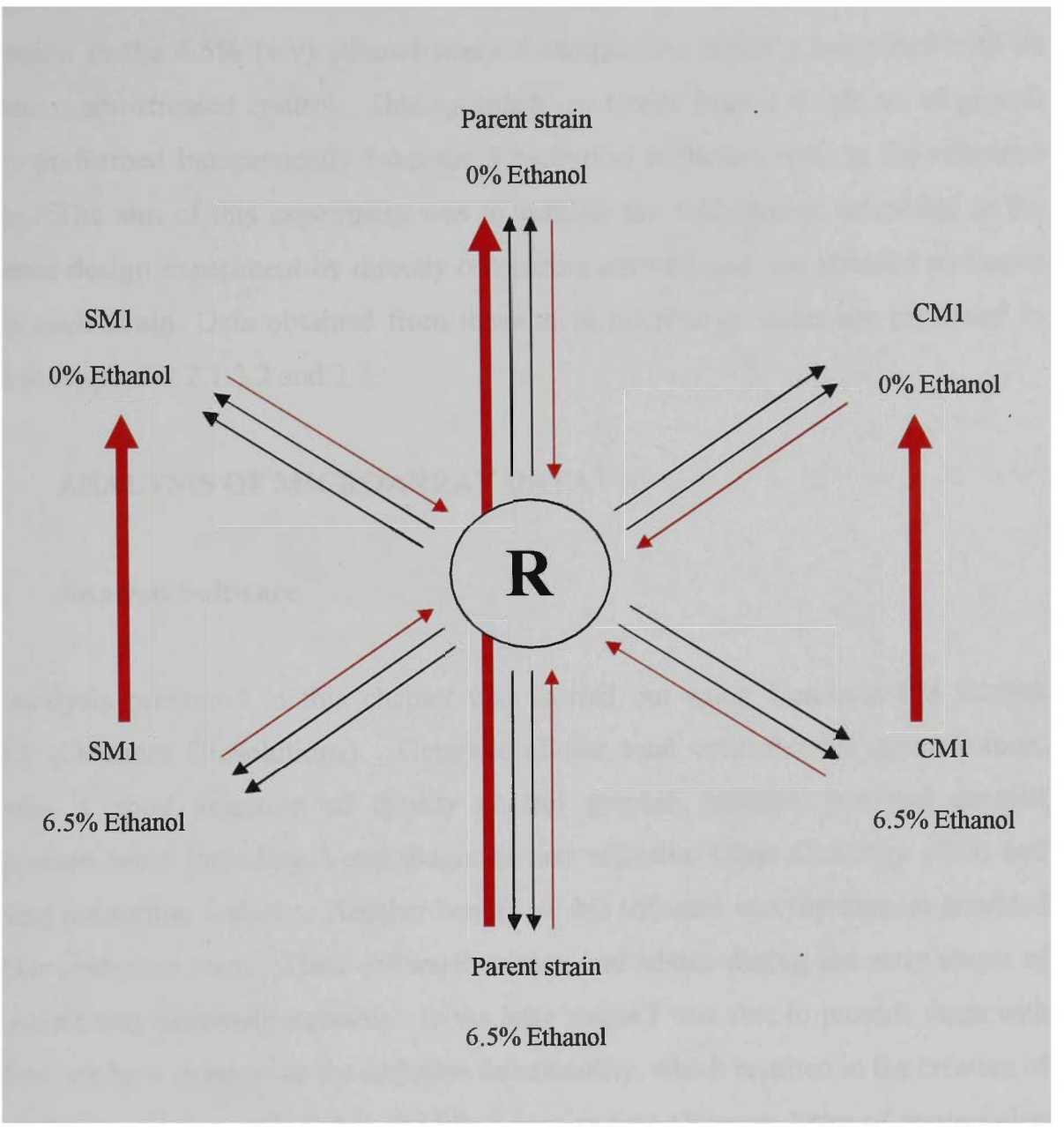

Figure 4.5: Microarray experimental design. Thin arrows represent the reference design experiments and thick arrows represent the direct comparison. Each arrow points from the sample designated as the control to the experimental sample cDNA. The reference design uses cDNA from three biological replicate growth curves for each experimental condition (wild-type, SMI and CM1 at 0 and 6.5% v/v ethanol) hybridised against a reference pool of cDNA (R) from the unstressed wild-type strain; the red colour in thin arrows indicates a dye swap experiment. The direct design comparisons were performed using cDNA from an independent set of growth curves representing the same experimental condition.

In the **direct comparison**, shown in Figure 4.5 by thick arrows, the same approach as Alexandre *et al.*, (2001) and Chandler *et al.*, (2004) was adopted for each strain: gene expression in the 6.5% (v/v) ethanol-stressed sample was directly compared with its respective non-stressed control. This approach used cells from a single set of growth curves performed independently from the 3 biological replicates used in the reference design. The aim of this experiment was to validate the fold change calculated in the reference design experiment by directly comparing stressed and non-stressed replicates within each strain. Data obtained from these three microarray slides are presented in detail in Appendix 2.1.3.2 and 2.2.

4.4 ANALYSIS OF MICROARRAY DATA

4.4.1 Analysis Software

The analysis presented in this chapter was carried out using Genowiz TM version 4.0.2.1 (Ocimum Biosolutions). Genowiz allows total control over normalisation, provides a good selection of quality control graphic features, practical genelist comparison tools including Venn diagrams and effective Gene Ontology (GO) and pathway inspection features. Another benefit of this software was the support provided by their analytical team. Their software training and advice during the early stages of the project was extremely valuable. In the later stages I was able to provide them with feedback on how to improve the software functionality, which resulted in the creation of four software patches included in the latest version (see Ocimum letter of appreciation in the CD pocket).

4.4.2 Normalisation

Normalisation was found to be a particularly important step in the microarray data analysis. The method chosen for all experiments was Lowess. This widely accepted method, first published by Yang *et al.*, (2001), is now finding application in several fields of biological analysis. Lately there have been a number of publications warning that optimisation of this step has been greatly neglected, with a number of authors using arbitrary (default) settings regardless of suitability to the particular dataset (Berger *et al.*, 2004). This can reduce the efficiency of the normalisation and introduce bias into

the data. In the current study, Lowess parameters were optimised using the triplicates of wild-type W303-1A (red) at 0% ethanol versus the reference pool (green) or vice versa. As this is technically a "self versus self" hybridisation, successful normalisation should result in equal values for the red channel and green channel. Figure 4.6 supports the selected normalisation parameters and indicates good labelling efficiency for each dye. In addition, the normalisation parameters were inspected and approved by Ocimum Biosolutions Genowiz analytical support team.

To summarise, the current analysis was performed with Genowiz in the following order: Lowess normalisation (print-tip Lowess, smoothing parameter of 0.1 and degree of polynomial of 1 and 2 robust interactions); genes with missing values were excluded from the analysis.

4.5 ANALYSIS OF VARIANCE: 2-WAY ANOVA

4.5.1 Introduction

In order to select genes participating in the ethanol stress response, two-way ANOVA was applied to gene expression data from the **three-way reference design** experiment. This approach removes uninteresting sources of variance from the dataset by identifying genes that show a significant difference in expression due to ethanol and genes that differ in expression between the different strains.

Following Lowess normalisation, data were analysed using two-way ANOVA ($p \leq 0.001$). The two factors of interest were **ethanol** at two levels: 0 % (v/v) and 6.5 % (v/v) and **strain** with three levels: wild-type W303-1A, SM1 and CM1. Ethanol significant genes (ESG) show a statistically significant difference in expression ($p \leq 0.001$) between 0% and 6.5% (v/v) ethanol. Strain significant genes (SSG) show a significant difference in expression between the strains. **Interaction** significant genes (ISG) are influenced in a more complex way by the combination of both factors; this category includes genes are affected differently by ethanol across the three strains.

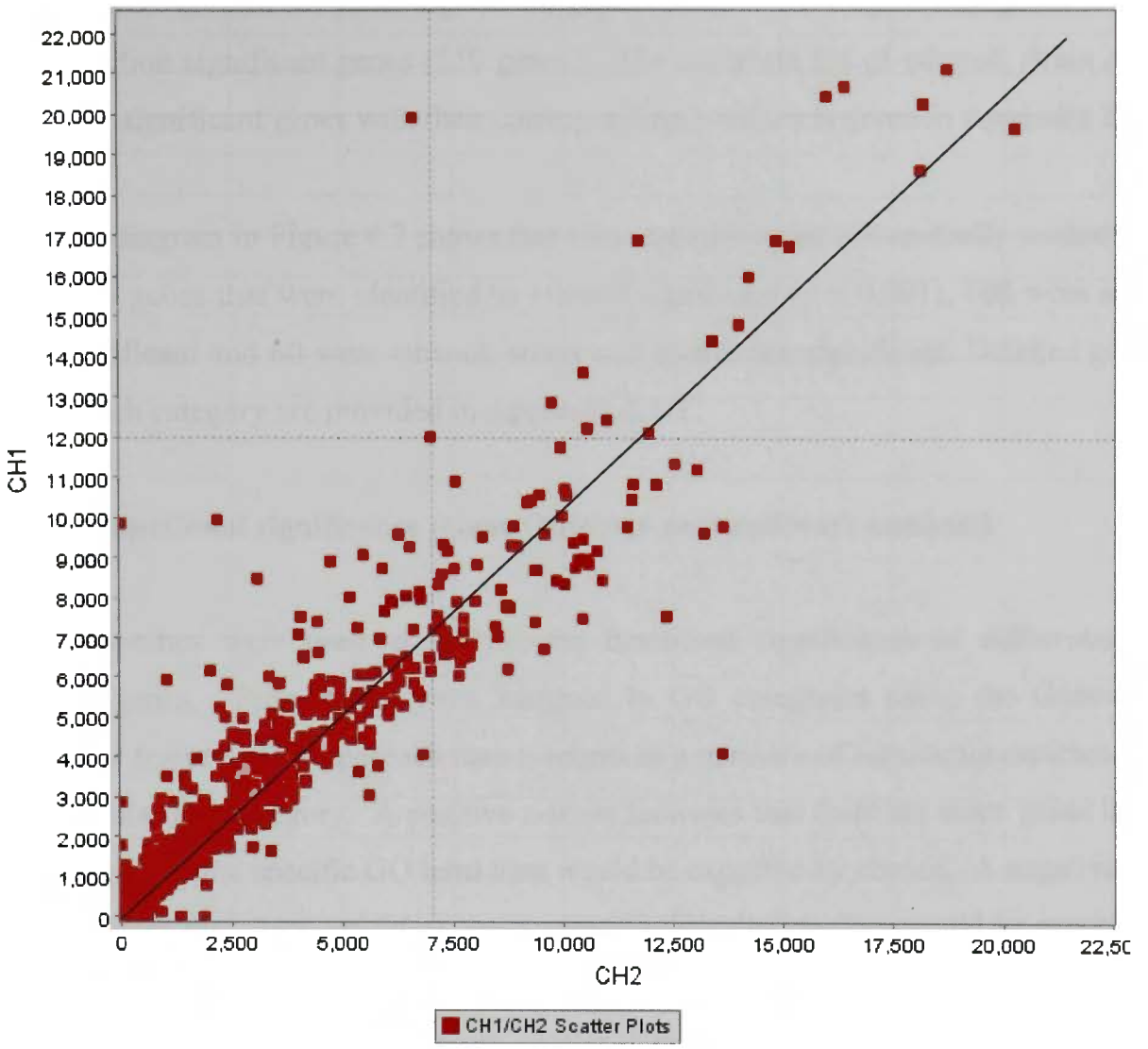

Figure 4.6: Channel plot of a self vs. self microarray slide after the Lowess normalisation step. The graph presents intensities of green (x axis) vs red (y-axes) signal of normalised genes presented as points. The normalisation was performed using smoothing parameter of 0.1, degree of polynomial of 1 and 2 robust interactions. A single biological replicate of W303-1A at 0% ethanol (red channel) was hybridised vs. reference pool of three biological replicates of W303-1A at 0% ethanol (green channel); one of them being the sample used as red channel. This is considered to be very close to self vs. self hybridisation, ideal for optimising normalisation parameters. The red and green signal intensities are approximately equal and located around y = x line of best fit as expected.

Genelists were created for strain significant genes (280), ethanol significant genes (576) and interaction significant genes (150 genes). The complete list of ethanol, strain and interaction significant genes with their corresponding p-values is given in Appendix 2.2.

The Venn diagram in Figure 4.7 shows that these categories are not mutually exclusive. Of the 576 genes that were identified as ethanol significant ($p < 0.001$), 168 were also strain significant and 60 were ethanol, strain and interaction significant. Detailed gene lists for each category are provided in Appendix 2.1.1.

4.5.2 Functional significance (Gene Ontology and pathways analysis)

Two approaches were used to analyse the functional significance of differentially regulated genes. First, genes were assigned to GO categories using the Genowiz bioanalysis feature. This approach uses z-scores as a measure of significant enrichment in a particular GO category. A positive z-score indicates that there are more genes in a list belonging to the specific GO term than would be expected by chance. A negative z-score indicates that there are fewer genes meeting the criterion than would be expected by chance (Doniger *et al.*, 2003). A z-score of 1.96 (or -1.96) correlates with a p value of 0.05. In the current study a z-score greater than 3 was considered significant. The higher the z-score the more significant the representation of that GO category within a list of genes. In some cases the GO terms were more simply inspected using Funspec (Robinson *et al.*, 2002) as this analysis provides lists of genes belonging to the specific GO category.

Genowiz provides very good pathway inspection features. Nevertheless, for some purposes, the graphical pathway displays of GenMAPP and MAPPFinder were found more informative (Doniger *et al.*, 2003).

4.5.3 K-means clustering

Due to the complexity of the experimental design and the limitations of ANOVA as a statistical tool, further partitioning of the ethanol, strain or interaction significant genes into smaller groups exhibiting closely matching profiles across the experimental conditions was required. K-means clustering maximises variance between clusters while

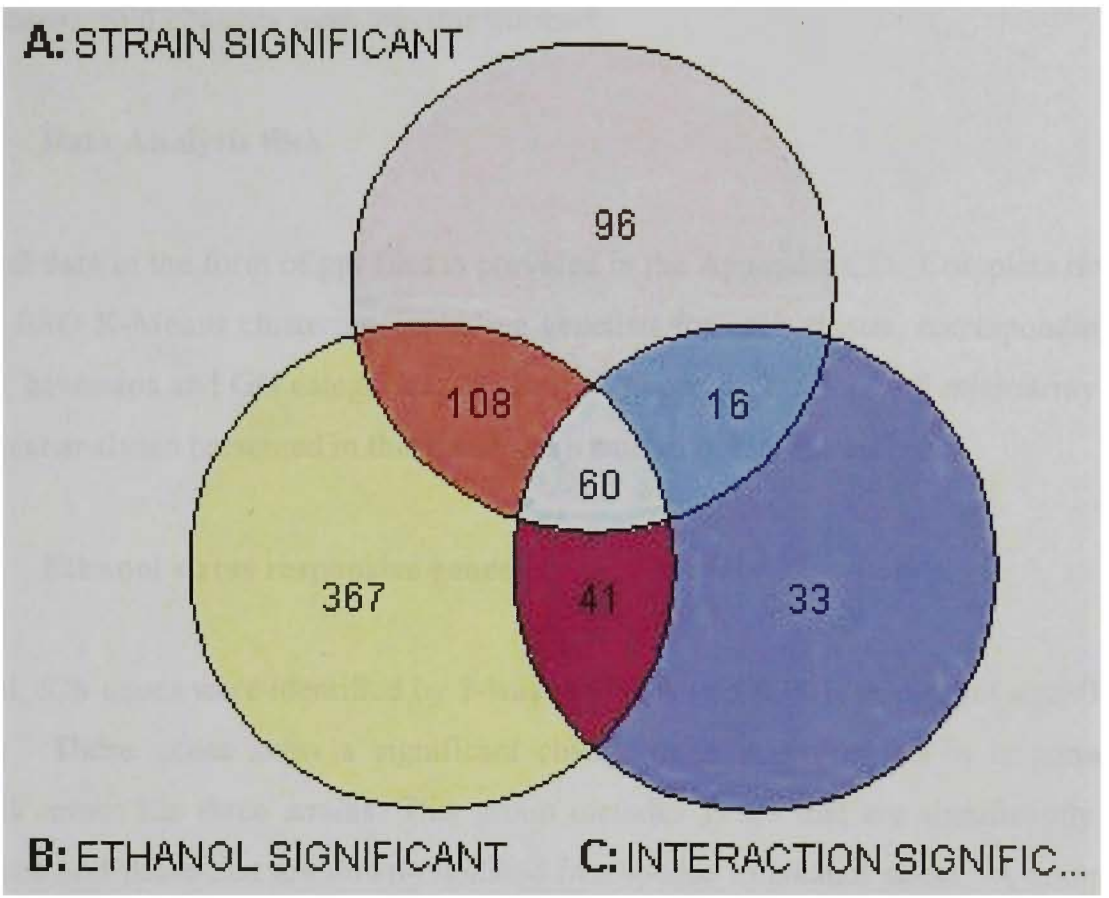

Figure 4.7: A Venn diagram showing the relationship between ethanol, strain and interaction significant genelists (2-way ANOVA ($p \leq 0.001$). Detailed gene-lists for interacting categories are given in Appendix 2.1.1.

minimising variance within the clusters thereby allowing genes that follow the same pattern of gene expression to be assigned into the same cluster. This approach was applied separately to the ethanol significant genes (ESG), strain significant genes (SSG) and the interaction significant genes (ISG). For all graphical displays of expression data (heat maps), fold changes were $\log_2$ transformed.

4.5.4 Data Analysis files

Original data in the form of gpr files is provided in the Appendix CD. Complete results for all ESG K-Means clustering, including genelists for each cluster, corresponding p-values, heatmaps and GO categories are given in Appendix 2.1.5.1. All microarray and statistical analyses presented in this thesis were carried out by the author.

4.5.5 Ethanol stress responsive genes

In total, 576 genes were identified by 2-way ANOVA ($p \leq 0.001$) as ethanol significant (ESG). These genes show a significant change in gene expression in response to ethanol across the three strains. This group includes genes that are significantly up-regulated and those that are down-regulated in response to ethanol stress. A complete list of ESG and associated heatmaps can be found in Appendix 2.1.1.

Further analysis of ESG was performed by K-Means clustering (100 interactions using the average linkage clustering method with Euclidean distance) to segregate the data into 10 clusters (Appendix 2.1.5.1). The expression profile of individual genes comprising each cluster and the mean expression profile for each group are shown in Figures 4.8 and Figure 4.9, respectively. These figures demonstrate the reproducibility of expression data across the three biological replicates representing each experimental condition.

As expected this approach separated the ESG into those that were enhanced or repressed by ethanol stress. Clusters 1, 3, 4, 5, 6 and 9 comprised genes that were up-regulated in response to ethanol across all three strains. This list of "ESG up-regulated genes" is considered in more detail in the following section. In contrast, genes in clusters 7, 8 and 10 were repressed by ethanol in all three strains. Again these genes were merged to

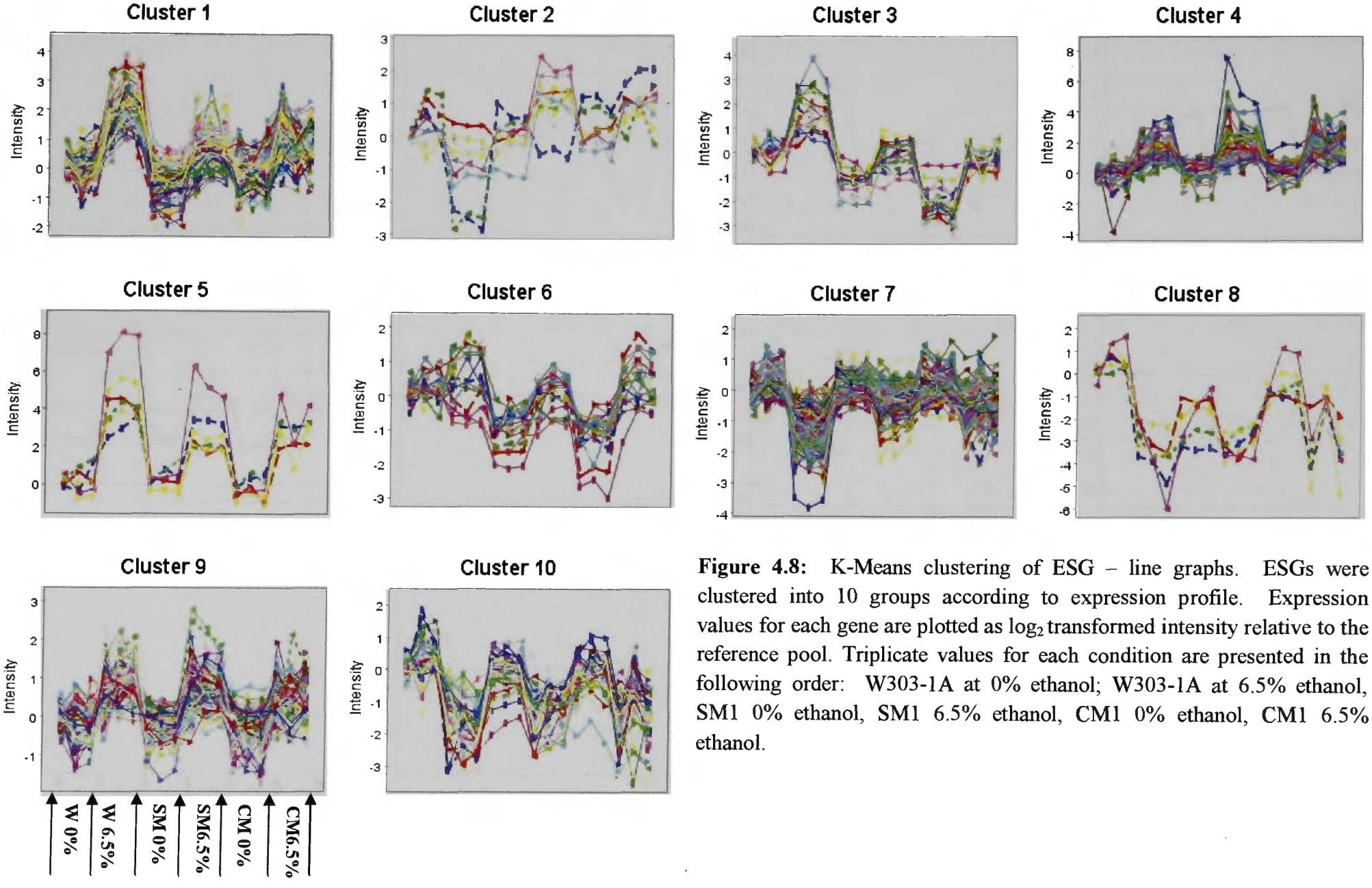

Figure 4.8: K-Means clustering of ESG – line graphs. ESGs were clustered into 10 groups according to expression profile. Expression values for each gene are plotted as $\log_2$ transformed intensity relative to the reference pool. Triplicate values for each condition are presented in the following order: W303-1A at 0% ethanol; W303-1A at 6.5% ethanol, SM1 0% ethanol, SM1 6.5% ethanol, CM1 0% ethanol, CM1 6.5% ethanol.

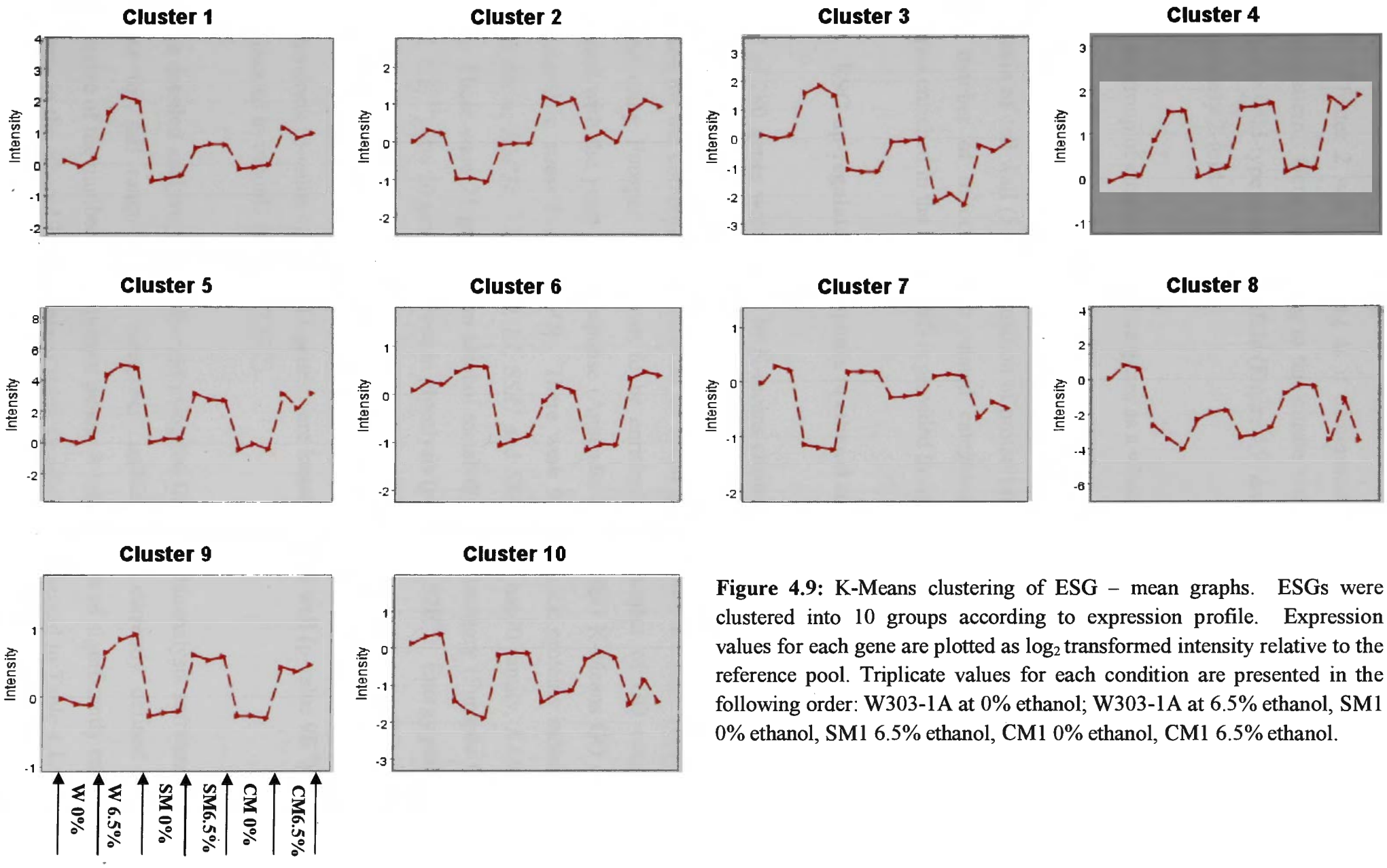

Figure 4.9: K-Means clustering of ESG – mean graphs. ESGs were clustered into 10 groups according to expression profile. Expression values for each gene are plotted as $\log_2$ transformed intensity relative to the reference pool. Triplicate values for each condition are presented in the following order: W303-1A at 0% ethanol; W303-1A at 6.5% ethanol, SM1 0% ethanol, SM1 6.5% ethanol, CM1 0% ethanol, CM1 6.5% ethanol.

form a list "ESG down-regulated genes" which is considered in more detail in Section 4.5.5.2. Cluster 2 was interesting as it demonstrated a distinctly different ethanol response pattern. Genes belonging to this cluster were down-regulated during ethanol stress in the wild-type to around 2-fold (Figure 4.9) and up-regulated in both mutants by approximately 2-fold.

When the group of ethanol significant genes as a whole was inspected for GO terms, the highest z-score was found in structural constituents of the ribosome (z-score 11.73), translation (11.00), glycolysis (9.22), unfolded protein binding (5.79), structural constituent of cell wall (5.65), regulation of proteolysis (4.49), response to stress (4.20) and a number of mitochondrion related categories. A complete analysis of GO categories enriched in the list of ESG is provided in Appendix 2.1.2.

4.5.5.1 ESG up-regulated in response to ethanol stress

A total of 280 genes were shown by K-means clustering to be up-regulated across all three strains in the presence of ethanol. A full list of ESG up-regulated genes and heatmaps for the component clusters can be found in Appendix 2.1.5.1. These were analysed using Funspec and shown to be enriched in a number of GO categories associated with the yeast stress response (Appendix 2.1.5.1/ESG K-Means GO / ESG up-regulated in stress Funspec GO). There were 9 heat shock proteins induced by ethanol stress: *HSP26, 12, 78, 60, 82, SSA1* and *SSA4* from hsp70 family, *KAR2* and *MDJ1*. There were 21 genes from alcohol metabolism GO category (Funspec GO p-value $1.31E^{-11}$) and 10 genes involved in glycolysis (p-value $4.92E^{-1}$). Energy pathways were also represented with 26 genes (p-value $3.9E^{-10}$) and response to stress GO with 27 genes (p-value $1.23E^{-8}$). There were 37 genes enhanced by ethanol stress, located to mitochondrion (p-value $1.07E^{-5}$), 11 genes were located in cell wall (p-value $9E^{-4}$) and 8 genes located to vacuole (p-value $7.6E^{-3}$).

A more detailed analysis was performed using the Genowiz bioanalysis software. This assesses the full range of GO categories, including all currently defined nodes irrespective of the number of component genes. A full analysis of significantly enriched GO terms in the ethanol-induced genes using Genowiz is presented in Table 4.1. From these results it was evident that a number of GO categories related to

Table 4.1: GO categories enriched among ESG up-regulated in ethanol stress in all 3 strains.

Gene Ontology Term	Z-scores
glycolysis	12.5955
gluconeogenesis	11.7999
glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity	8.0935
thiol-disulfide exchange intermediate activity	8.0365
ribonuclease activity	7.9902
RNA-directed DNA polymerase activity	7.9902
response to stress	7.7562
DNA-directed DNA polymerase activity	7.5554
peptidase activity	7.5554
D-xylose catabolic process	6.9027
arabinose catabolic process	6.9027
aldehyde reductase activity	6.9027
regulation of cell redox homeostasis	6.6614
phosphopyruvate hydratase complex	6.6078
age-dependent response to reactive oxygen species during chronological cell aging	6.6078
regulation of vacuole fusion, non-autophagic	6.6078
regulation of proteolysis	6.6078
retrotransposon nucleocapsid	6.4123
transposition, RNA-mediated	6.3346
protein folding	6.2605
oxygen and reactive oxygen species metabolic process	6.0787
SRP-dependent cotranslational protein targeting to membrane, translocation	5.8764
cytosol	5.5412
aldo-keto reductase activity	5.4621
glutathione peroxidase activity	5.4621
unfolded protein binding	5.3777
chaperone activator activity	5.2721
mitochondrial proton-transporting ATP synthase complex	5.2721
mitochondrial intermembrane space	5.0838
response to oxidative stress	5.0223
NAD biosynthetic process	4.9764
glutathione transferase activity	4.9764
lumen of vacuole with cell cycle-correlated morphology	4.9764
chitin- and beta-glucan-containing cell wall	4.8463
copper, zinc superoxide dismutase activity	4.6721
nucleoside diphosphate kinase activity	4.6721
pyrophosphatase activity	4.6721
biotin transport	4.6721
glycerophosphodiester transport	4.6721
kynureninase activity	4.6721
manganese superoxide dismutase activity	4.6721
regulation of arginine metabolic process	4.6721
glucokinase activity	4.6721
glutathione gamma-glutamylcysteinyltransferase activity	4.6721

Gene Ontology Term	Z-scores
argininosuccinate lyase activity	4.6721
nucleoside triphosphate biosynthetic process	4.6721
phosphoglycerate kinase activity	4.6721
phosphatidylethanolamine N-methyltransferase activity	4.6721
aspartate kinase activity	4.6721
S-formylglutathione hydrolase activity	4.6721
acyl carrier activity	4.6721
CDP-diacylglycerol-serine O-phosphatidyltransferase activity	4.6721
glycerophosphodiester transmembrane transporter activity	4.6721
2-hexaprenyl-6-methoxy-1,4-benzoquinone methyltransferase activity	4.6721
oligosaccharide catabolic process	4.6721
inositol polyphosphate multikinase activity	4.6721
glycerol-3-phosphate dehydrogenase activity	4.6721
carbonate dehydratase activity	4.6721
fructose-bisphosphate aldolase activity	4.6721
superoxide metabolic process	4.6721
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	4.6721
sulfite transmembrane transporter activity	4.6721
intracellular transport	4.6721
inositol trisphosphate 3-kinase activity	4.6721
protease inhibitor activity	4.6721
carbon utilization by utilization of organic compounds	4.6721
histidinol-phosphate transaminase activity	4.6721
alpha-mannosidase activity	4.6721
riboflavin synthase activity	4.6721
nicotinate-nucleotide diphosphorylase (carboxylating) activity	4.6721
phosphatidylserine biosynthetic process	4.6721
actin filament severing	4.6721
triose-phosphate isomerase activity	4.6721
nucleoside diphosphate phosphorylation	4.6721
3-hydroxyanthranilate 3,4-dioxygenase activity	4.6721
phosphoinositide 3-kinase activity	4.6721
aminopeptidase I activity	4.6721
phosphatidyl-N-methylethanolamine N-methyltransferase activity	4.6721
heat shock protein binding	4.6721
ATP-dependent protein binding	4.6721
protein import into nucleus, translocation	4.6721
3,4-dihydroxy-2-butanone-4-phosphate synthase activity	4.6721
biotin transporter activity	4.6721
high-affinity zinc ion transport	4.6721
saccharopepsin activity	4.6721
high affinity zinc uptake transmembrane transporter activity	4.6721
methylglyoxal reductase (NADH-dependent) activity	4.6721
structural constituent of cell wall	4.6533
mitochondrion	4.5995
ethanol metabolic process	4.4591
m7G(5')pppN diphosphatase activity	4.4591
vacuole, cell cycle-correlated morphology	4.3306

Gene Ontology Term	Z-scores
thioredoxin peroxidase activity	4.2466
response to copper ion	3.8929
alcohol dehydrogenase activity	3.8929
phosphopyruvate hydratase activity	3.8929
actin polymerization and/or depolymerization	3.8929
NADH oxidation	3.7127
vacuolar protein catabolic process	3.493
fermentation	3.4665
protein targeting to mitochondrion	3.4665
electron carrier activity	3.4665
regulation of transcription, mating-type specific	3.4665
riboflavin biosynthetic process	3.4665
ATP synthesis coupled proton transport	3.4181
mitochondrial matrix	3.4042
chaperone binding	3.2968
protein binding	3.1934
protein import into mitochondrial matrix	3.1682
formaldehyde catabolic process	3.1525
protein thiol-disulfide exchange	3.1525
actin monomer binding	3.1525
sulfite transport	3.1525
isocitrate dehydrogenase (NAD+) activity	3.1525
lactate metabolic process	3.1525
lipoic acid biosynthetic process	3.1525
protein autoubiquitination	3.1525
actin lateral binding	3.1525
glycogen (starch) synthase activity	3.1525
glycerone-phosphate O-acyltransferase activity	3.1525
carboxypeptidase C activity	3.1525
NADPH dehydrogenase activity	3.1525
orotate phosphoribosyltransferase activity	3.1525
pyruvate carboxylase activity	3.1525
positive regulation of telomere maintenance via telomerase	3.1525
ion transport	3.1525
inositol phosphate dephosphorylation	3.1525
peroxiredoxin activity	3.1525
homocitrate synthase activity	3.1525
pseudouridine synthesis	3.1525
glucose 6-phosphate utilization	3.1525
pyruvate kinase activity	3.1525
phosphoglucomutase activity	3.1525
phytochelatin biosynthetic process	3.1525
voltage-gated ion-selective channel activity	3.1525
glycerol-3-phosphate O-acyltransferase activity	3.1525
ethanol biosynthetic process during fermentation	3.1525
glucose 1-phosphate utilization	3.1525
carbamoyl-phosphate synthase complex	3.1525
actin filament depolymerization	3.1525
2-isopropylmalate synthase activity	3.1525

Gene Ontology Term	Z-scores
2-deoxyglucose-6-phosphatase activity	3.1525
cystathionine beta-lyase activity	3.1525
inositol-1(or 4)-monophosphatase activity	3.1525
dodecenoyl-CoA delta-isomerase activity	3.1525
mitochondrial isocitrate dehydrogenase complex (NAD+)	3.1525
glycerone kinase activity	3.1525
cellular bud scar	3.1525
RNA metabolic process	3.1287
proteasome core complex, alpha-subunit complex (sensu Eukaryota)	3.1287
translation regulator activity	3.1287
cytoplasm	3.12
protein ubiquitination during ubiquitin-dependent protein catabolic process	3.1199
integral to mitochondrial outer membrane	3.1199

NAD^+/NADH, glycerol and glycogen metabolism were also enhanced by exposure to non-lethal ethanol stress. It was also apparent that the GO categories, retrotransposition nucleocapsid (z-score 6.41) and transposition RNA mediated (z-score 6.33), were significantly represented in this category, this was revisited in Section 4.6, Figures 4.24 and 4.25.

4.5.5.2 ESG down-regulated in response to ethanol stress

A total of 287 ethanol significant genes were found to be down-regulated across all three strains using K-means clustering. A significant number of these genes encoded constituents of the ribosome; 29 genes representing components of the small and 36 representing components the large ribosomal subunit. There were also 9 genes involved in ergosterol metabolism and 9 regulators of translation (Appendix 2.1.5.1/ ESG K-Means GO/ESG down-regulated in stress Funspec GO). Table 4.2 presents Genowiz bioanalysis result for genes repressed by ethanol stress.

4.5.5.3 Mutant-specific ESG

Genes in ESG K-Means cluster 2 showed an interesting expression pattern. This group of 9 genes (Figure 4.10, Table 4.3 and for genlists with p-values see Appendix 2.1.5.1) were repressed in response to stress in wild-type by approximately 2-fold, but

Table 4.2: GO categories enriched in ESG down-regulated by ethanol stress, separated by K-means.

Gene Ontology Term	Z-scores
structural constituent of ribosome	18.1497
translation	16.778
cytosolic small ribosomal subunit (sensu Eukaryota)	16.7713
cytosolic large ribosomal subunit (sensu Eukaryota)	16.7284
regulation of translational fidelity	10.9373
ribosomal large subunit assembly and maintenance	10.7117
small subunit processome	9.9564
ribosome biogenesis and assembly	8.7161
ribosomal small subunit assembly and maintenance	8.2312
ergosterol biosynthetic process	7.6223
rRNA processing	6.947
G-protein alpha-subunit binding	6.523
GMP metabolic process	6.523
phenylalanine-tRNA ligase complex	6.523
cysteine metabolic process	6.523
tRNA aminoacylation for protein translation	6.523
nucleolus	6.0413
ribosome	6.0185
'de novo' cotranslational protein folding	5.9957
maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	5.2612
phenylalanyl-tRNA aminoacylation	5.2012
IMP dehydrogenase activity	5.2012
phenylalanine-tRNA ligase activity	5.2012
homoserine metabolic process	5.2012
translational elongation	5.1749
ribosomal large subunit biogenesis and assembly	4.7195
sterol 24-C-methyltransferase activity	4.6121
L-methionine secondary active transmembrane transporter activity	4.6121
aromatic amino acid transport	4.6121
FFAT motif binding	4.6121
adenylylsulfate kinase activity	4.6121
peptide-methionine-(S)-S-oxide reductase activity	4.6121
ribulose-phosphate 3-epimerase activity	4.6121
cytosine deaminase activity	4.6121
isoleucine metabolic process	4.6121
cysteinyl-tRNA aminoacylation	4.6121
asparaginyl-tRNA aminoacylation	4.6121
box C/D snoRNA 3'-end processing	4.6121
RNA strand annealing activity	4.6121
adenosylhomocysteinase activity	4.6121
organic acid transport	4.6121
anti-apoptosis	4.6121
positive regulation of ligase activity	4.6121
GDP-dissociation inhibitor activity	4.6121

Gene Ontology Term	Z-scores
ATPase activity, uncoupled	4.6121
mating-type alpha-factor pheromone receptor activity	4.6121
cysteine-tRNA ligase activity	4.6121
guanylate kinase activity	4.6121
Golgi vesicle transport	4.6121
squalene monooxygenase activity	4.6121
base-excision repair, base-free sugar-phosphate removal	4.6121
S-methylmethionine transport	4.6121
adenosine kinase activity	4.6121
C-8 sterol isomerase activity	4.6121
protection from non-homologous end joining at telomere	4.6121
D-serine ammonia-lyase activity	4.6121
cystathionine beta-synthase activity	4.6121
cell projection biogenesis	4.6121
drug binding	4.6121
3-isopropylmalate dehydratase activity	4.6121
cytosine metabolic process	4.6121
regulation of intracellular lipid transport	4.6121
deoxyhypusine monooxygenase activity	4.6121
aromatic amino acid transmembrane transporter activity	4.6121
regulation of transcription by glucose	4.6121
stearoyl-CoA 9-desaturase activity	4.6121
chorismate synthase activity	4.6121
fructose-bisphosphatase activity	4.6121
COPI coating of Golgi vesicle	4.6121
uracil phosphoribosyltransferase activity	4.6121
cytidine metabolic process	4.6121
tRNA (cytosine-5-)-methyltransferase activity	4.6121
delta24(24-1) sterol reductase activity	4.6121
S-methylmethionine transmembrane transporter activity	4.6121
unsaturated fatty acid biosynthetic process	4.6121
Ran GTPase activator activity	4.6121
transsulfuration	4.6121
conversion of met-tRNAf to fmet-tRNA	4.6121
negative regulation of transcription factor import into nucleus	4.6121
high-affinity tryptophan transmembrane transporter activity	4.6121
acetyl-CoA C-acetyltransferase activity	4.6121
transcription termination	4.6121
chitin catabolic process	4.6121
proline-tRNA ligase activity	4.6121
lipid glycosylation	4.6121
hypusine biosynthetic process	4.6121
sulfur amino acid transport	4.6121
homoserine dehydrogenase activity	4.6121
alanyl-tRNA aminoacylation	4.6121
phosphatidylserine catabolic process	4.6121
protein tyrosine/serine/threonine phosphatase activity	4.6121
GMP synthase (glutamine-hydrolyzing) activity	4.6121
7S RNA binding	4.6121

Gene Ontology Term	Z-scores
C-5 sterol desaturase activity	4.6121
alanine-tRNA ligase activity	4.6121
sterol 14-demethylase activity	4.6121
5-aminolevulinate synthase activity	4.6121
homoserine kinase activity	4.6121
NAD+ diphosphatase activity	4.6121
flavin reductase activity	4.6121
NADH metabolic process	4.6121
selenocysteine metabolic process	4.6121
glucosamine 6-phosphate N-acetyltransferase activity	4.6121
phosphoinositide metabolic process	4.6121
farnesyl diphosphate biosynthetic process, mevalonate pathway	4.6121
dihydroorotate dehydrogenase activity	4.6121
gene conversion at mating-type locus, DNA repair synthesis	4.6121
nicotinate phosphoribosyltransferase activity	4.6121
RNA methylation	4.6121
endochitinase activity	4.6121
ribosome assembly	4.511
ribosomal small subunit export from nucleus	4.511
telomere maintenance	4.3585
methionine metabolic process	4.3555
translation initiation factor activity	4.3373
small nucleolar ribonucleoprotein complex	4.2819
nucleoplasm	4.1375
translational initiation	3.9048
threonine metabolic process	3.8355
pyrimidine salvage	3.8355
eukaryotic translation initiation factor 4F complex	3.8355
glucose catabolic process to ethanol	3.8355
leucine biosynthetic process	3.8355
rRNA modification	3.6517
translation elongation factor activity	3.6517
nuclear nucleosome	3.6517
ATP-dependent RNA helicase activity	3.5272
RNA binding	3.4921
extracellular region	3.4911
snRNA pseudouridine synthesis	3.413
pseudouridine synthase activity	3.413
chromatin assembly or disassembly	3.3544
maturation of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	3.2392
snoRNA binding	3.2265
tryptophan transport	3.1081
UDP-N-acetylglucosamine biosynthetic process	3.1081
box H/ACA snoRNP complex	3.1081
tyrosyl-tRNA aminoacylation	3.1081
GTP biosynthetic process	3.1081
leucyl-tRNA aminoacylation	3.1081
maturation of 5.8S rRNA	3.1081

Gene Ontology Term	Z-scores
high affinity iron permease complex	3.1081
RNA import into nucleus	3.1081
ferroxidase activity	3.1081
nuclear membrane	3.1081
sulfite reductase (NADPH) activity	3.1081
methylenetetrahydrofolate dehydrogenase (NADP+) activity	3.1081
asparagine-tRNA ligase activity	3.1081
branched chain family amino acid catabolic process	3.1081
hydroxymethylglutaryl-CoA reductase (NADPH) activity	3.1081
intrinsic to vacuolar membrane	3.1081
tyrosine-tRNA ligase activity	3.1081
sulfite reductase complex (NADPH)	3.1081
5S rRNA binding	3.1081
cysteine biosynthetic process	3.1081
folic acid biosynthetic process	3.1081
telomere clustering	3.1081
NADH dehydrogenase activity	3.1081
pseudouridine synthesis	3.1081
uridylate kinase activity	3.1081
formate-tetrahydrofolate ligase activity	3.1081
triplex DNA binding	3.1081
ER-Golgi intermediate compartment	3.1081
fructose import	3.1081
nucleotide-sugar transport	3.1081
calmodulin-dependent protein kinase activity	3.1081
deadenylation-independent decapping	3.1081
phosphate transmembrane transporter activity	3.1081
iron assimilation by reduction and transport	3.1081
methenyltetrahydrofolate cyclohydrolase activity	3.1081
DSIF complex	3.1081
protein homooligomerization	3.1081
2-isopropylmalate synthase activity	3.1081
glycyl-tRNA aminoacylation	3.1081
glycine-tRNA ligase activity	3.1081
phosphogluconate dehydrogenase (decarboxylating) activity	3.1081
cystathionine gamma-lyase activity	3.1081
G-protein coupled receptor protein signaling pathway	3.1081
leucine-tRNA ligase activity	3.1081
anaphase-promoting complex activation during mitotic cell cycle	3.1081
hexokinase activity	3.1081
nucleotide-sugar transmembrane transporter activity	3.1081
methyltransferase activity	3.1081
branched-chain-amino-acid transaminase activity	3.1081
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3.0781
maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	3.0636
polysome	3.0636

Table 4.3: GO categories enriched in ESG induced in mutants and repressed in the wild-type in response to ethanol (K-Means cluster 2).

Gene Ontology Term	Z-scores
biotin carboxylase activity	26.6312
acetyl-CoA carboxylase activity	18.806
proline catabolic process	15.3345
regulation of pH	13.2624
serine hydrolase activity	9.9851
nuclear membrane organization and biogenesis	9.9851
fatty acid biosynthetic process	9.3277
structural constituent of cell wall	7.268
protein import into nucleus	4.8514
mitochondrial large ribosomal subunit	3.7841
transcription activator activity	3.7365
specific RNA polymerase II transcription factor activity	3.5603

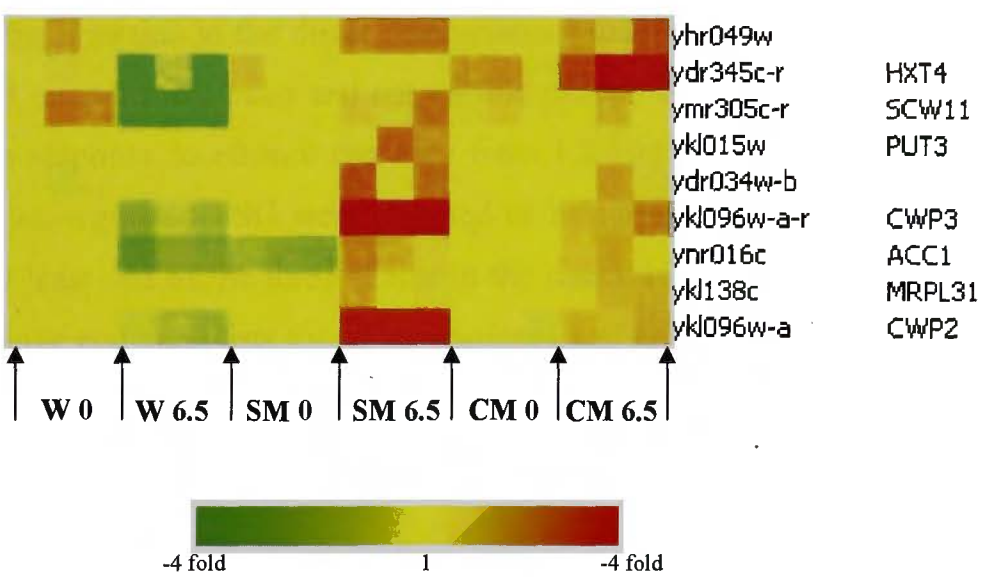

Figure 4.10: Heatmap representing gene expression from ESG K-Means cluster 2.

induced by a similar amount in stressed mutants. On average these genes were expressed more than 4-fold higher in both mutants at 6.5% (v/v) ethanol when compared to the wild-type at 6.5% (v/v) ethanol. *HXT4* differed slightly from this pattern; it was repressed in the presence of ethanol in both the wild-type and SM1 but induced in CM1. This group of genes appears to contain significant differences in ethanol stress response between the mutants and wild-type and significantly overlaps with SSG cluster 7, as 5 of 9 genes from this cluster are also SSG (see Section 4.5.6).

4.5.5.4 Comparison with the direct design microarray analysis

The direct design approach allowed direct hybridisation of 0% versus 6.5% (v/v) ethanol within each strain. For the reference design experiment, this difference in expression was calculated based on the relative gene expression levels in the stressed and unstressed samples relative to a common reference pool. Overall, there was a high level of correlation between the results obtained in the direct and reference design. 90% of the ESG up-regulated in ethanol stress were up-regulated by more than 1.5 fold in at least one of the 3 strains in the direct comparisons (note that ESG in reference design were selected on statistical basis and not on fold change difference). Their change in expression in response to ethanol can vary from 1.2-fold to over 100-fold. Similarly 82.6% of down-regulated ESG were detected as being down-regulated by more than 1.5-fold in at least one of the three strains in the direct design experiment. Heatmaps with fold change and gene lists for all up-regulated and down-regulated genes in direct microarray comparisons are provided in Appendix 2.2.1. These show high correlation between the reference and direct design data.

4.5.6 Strain significant genes

Using 2-way ANOVA, 280 genes were identified as being strain significant ($p \leq 0.001$). These genes are particularly interesting as they demonstrate differences in expression between the three strains. Whereas ethanol significant genes (ESG) provide insight primarily into the ethanol stress response, strain significance highlights differences in the gene expression profiles of the mutants when compared with the wild-type. A complete list of SSG and the heat map profiling expression of this group of genes is given in Appendix 2.1.5.3.

The strain significant genes (SSG) were clustered using K-Means into 10 clusters with 100 interactions using average linkage clustering with Euclidean distance. Expression profiles for genes in each cluster and the mean expression profile are presented in Figures 4.11 and 4.12. Detailed results are presented in Appendix 2.1.5.3. Clusters 2, 4, and 7 included genes that were expressed at a higher level in 6.5% (v/v) ethanol than the wild-type at 6.5% (v/v) ethanol in either one or both mutants.

SSG cluster 2 comprised 65 genes (Figure 4.13, view A) that were strongly down-regulated in the wild-type by stress (mean -3 fold) whereas the effect in the mutants was negligible (1.14-fold in SM1 and 1.31-fold in CM1). The mean expression of genes in this cluster in the presence of 6.5% (v/v) ethanol stress was 2.69-fold higher in SM1 and 2.27-fold higher in CM1 than in wild-type W303-1A. The Funspec cluster GO interpreter (Appendix 2.1.5.3) showed that 20 of these genes (30.7%) are structural constituents of the ribosome, 11 are involved in RNA binding and 23 are involved in protein biosynthesis. Overall 53 genes (81.5%) were involved in cell growth (Table 4.4). This analysis highlights differences between the wild-type and mutant strains in ribosomal synthesis.

Cluster 4 included genes that were highly up-regulated but to different extents across all three strains. The mean fold difference between 0% and 6.5% (v/v) ethanol was 6.84 for the wild-type, 5.13 for SM1 and 4.27 for CM1. Five of the 10 genes in this cluster (Figure 4.13 view B) are known stress response genes (*CUP1, AHP1, HOR7, DDR2* and *HSP31*) and YNL054W-B is retrotransposon related and is listed as a pseudogene in SGD. There were also several uncharacterised genes assigned to this category including YNL134C in which it was shown by BlastP analysis (Balakrishnan *et al.*, 2005) to display a striking level of homology to genes belonging to the alcohol dehydrogenase family of *S .cerevisiae* including *ADH2* ($5.5E^{-8}$), *ADH3* ($1.8E^{-7}$), *ADH1* ($1.1E^{-6}$), and *ADH5* ($7.3E^{-6}$). Interestingly, both *ADH1* and *ADH2* were found to be ethanol and strain significant in the current study. When the search for homologies was extended to other species (PSI-BLAST), YNL134C was found to be 97.9% identical to *Geobacillus stearothermophilus* alcohol dehydrogenase with e-value of $4E^{-76}$.

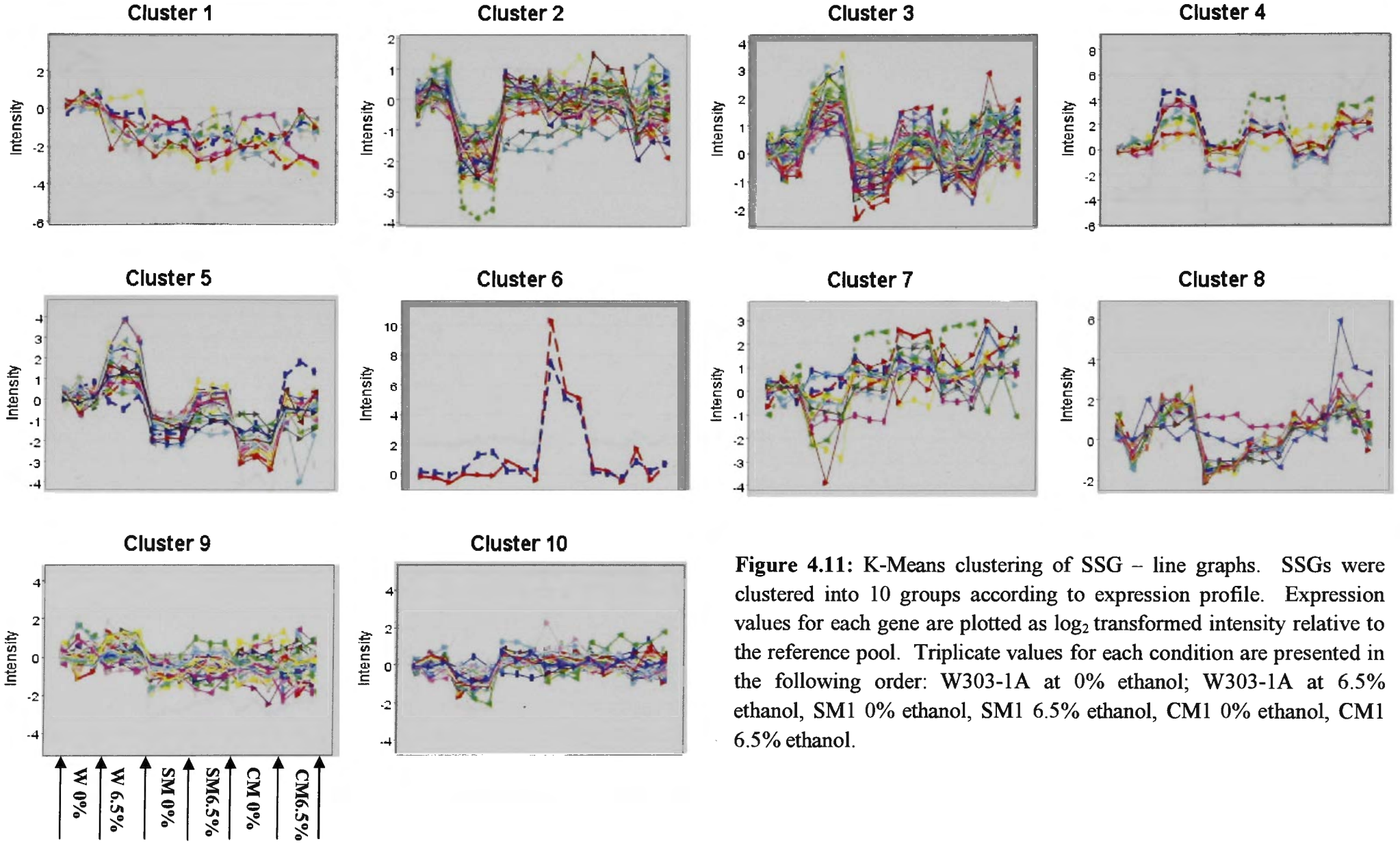

Figure 4.11: K-Means clustering of SSG – line graphs. SSGs were clustered into 10 groups according to expression profile. Expression values for each gene are plotted as $\log_2$ transformed intensity relative to the reference pool. Triplicate values for each condition are presented in the following order: W303-1A at 0% ethanol; W303-1A at 6.5% ethanol, SM1 0% ethanol, SM1 6.5% ethanol, CM1 0% ethanol, CM1 6.5% ethanol.

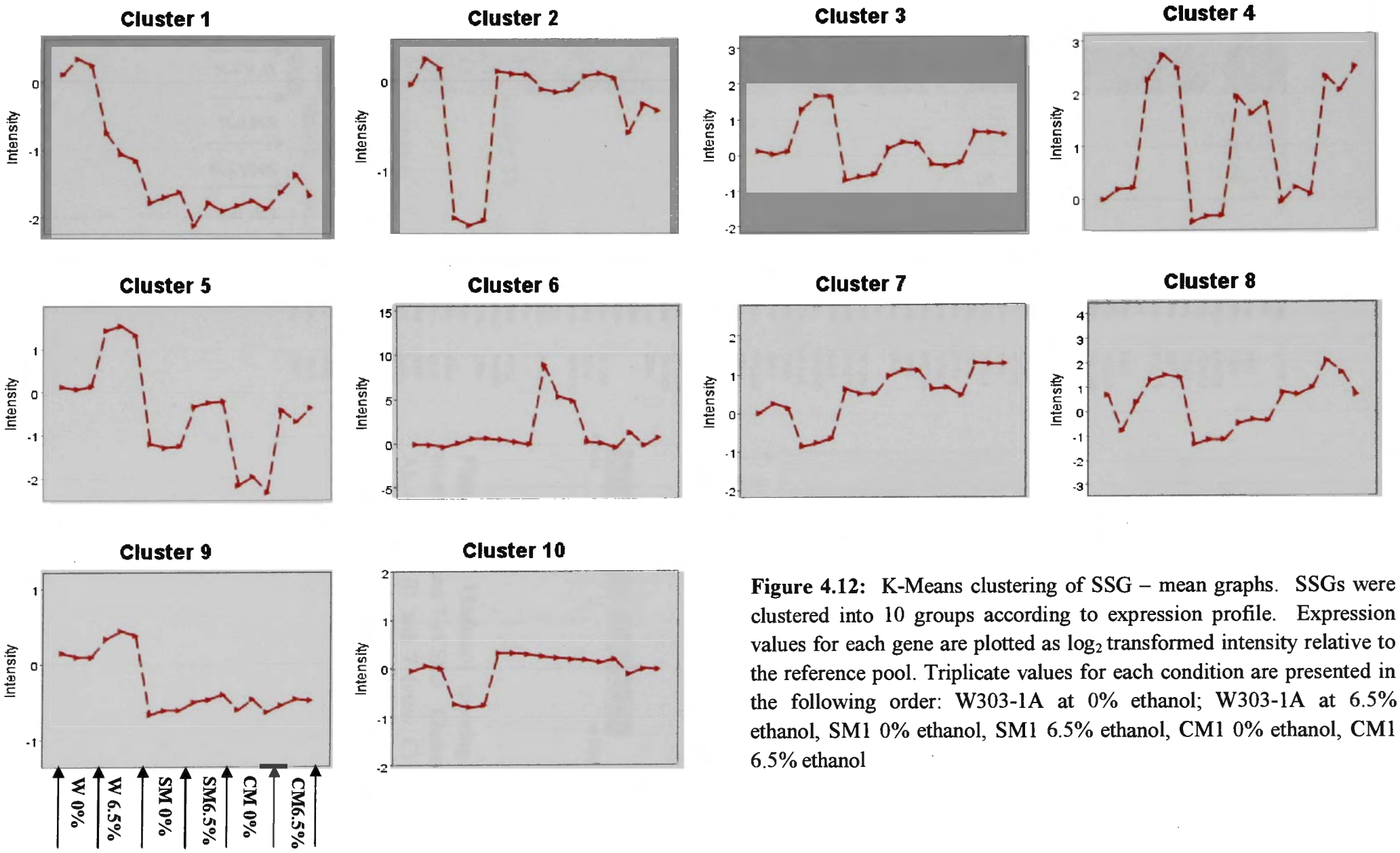

Figure 4.12: K-Means clustering of SSG – mean graphs. SSGs were clustered into 10 groups according to expression profile. Expression values for each gene are plotted as $\log_2$ transformed intensity relative to the reference pool. Triplicate values for each condition are presented in the following order: W303-1A at 0% ethanol; W303-1A at 6.5% ethanol, SM1 0% ethanol, SM1 6.5% ethanol, CM1 0% ethanol, CM1 6.5% ethanol

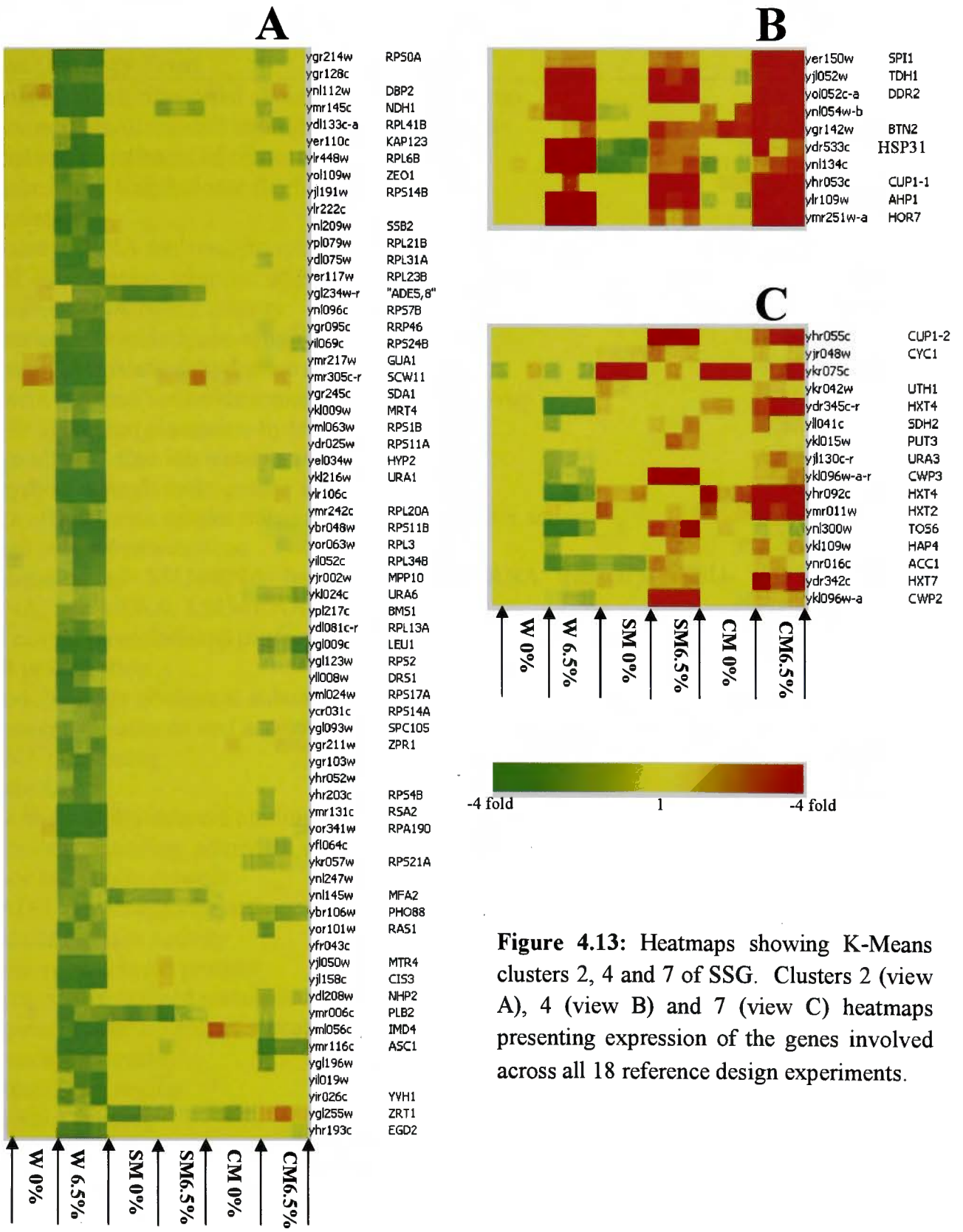

Figure 4.13: Heatmaps showing K-Means clusters 2, 4 and 7 of SSG. Clusters 2 (view A), 4 (view B) and 7 (view C) heatmaps presenting expression of the genes involved across all 18 reference design experiments.

Table 4.4: GO terms enriched in SSG genes strongly repressed in response to ethanol in wild-type only (K-Means cluster 2).

Gene Ontology Term	Z-scores
cytosolic small ribosomal subunit (sensu Eukaryota)	15.7333
ribosomal small subunit assembly and maintenance	15.62
structural constituent of ribosome	12.2826
regulation of translational fidelity	11.6941
translation	11.3136
cysteinyl-tRNA aminoacylation	9.866
GDP-dissociation inhibitor activity	9.866
cysteine-tRNA ligase activity	9.866
D-serine ammonia-lyase activity	9.866
3-isopropylmalate dehydratase activity	9.866
protein tyrosine/serine/threonine phosphatase activity	9.866
GMP synthase (glutamine-hydrolyzing) activity	9.866
high-affinity zinc ion transport	9.866
dihydroorotate dehydrogenase activity	9.866
high affinity zinc uptake transmembrane transporter activity	9.866
small subunit processome	9.6338
maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	9.5345
'de novo' cotranslational protein folding	8.6911
cell proliferation	8.6911
cytosolic large ribosomal subunit (sensu Eukaryota)	7.6552
ribosome biogenesis and assembly	7.1607
rRNA processing	7.1455
nucleolus	7.0988
G-protein alpha-subunit binding	6.9052
G-protein signaling, adenylate cyclase activating pathway	6.9052
GMP metabolic process	6.9052
NADH dehydrogenase activity	6.9052
uridylate kinase activity	6.9052
cysteine metabolic process	6.9052
glycerophospholipid metabolic process	6.9052
G-protein coupled receptor protein signaling pathway	6.9052
ribosome assembly	6.765
extracellular region	6.4272
snoRNA binding	6.0702
ribosomal large subunit assembly and maintenance	5.5951
nascent polypeptide-associated complex	5.5799
IMP dehydrogenase activity	5.5799
rRNA pseudouridine synthesis	5.5799
ribosomal large subunit biogenesis and assembly	5.4839
'de novo' pyrimidine base biosynthetic process	5.4082
telomere maintenance	4.8432
mating pheromone activity	4.782
TRAMP complex	4.782
90S preribosome	4.782

Gene Ontology Term	Z-scores
polyadenylation-dependent ncRNA catabolic process	4.782
lysophospholipase activity	4.782
ncRNA polyadenylation during polyadenylation-dependent ncRNA catabolic process	4.2322
tRNA catabolic process	4.2322
glucose mediated signaling	4.2322
cellular response to starvation	4.2322
rRNA catabolic process	4.2322
glucose catabolic process to ethanol	4.2322
leucine biosynthetic process	4.2322
traversing start control point of mitotic cell cycle	3.8223
rDNA heterochromatin	3.8223
snRNA pseudouridine synthesis	3.8223
nucleolar preribosome, large subunit precursor	3.8223
pseudouridine synthase activity	3.8223
chronological cell aging	3.5007
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3.5007
extrinsic to plasma membrane	3.5007
ATP-dependent RNA helicase activity	3.3165
negative regulation of translation	3.0202
protein transmembrane transporter activity	3.0202
mRNA catabolic process, nonsense-mediated decay	3.0202

InterPro Scan inspection of domains indicates the presence of an alcohol dehydrogenase domain with a striking e-value of $3.2E^{-145}$. Detailed results related to the analysis of YNL134C are provided in Appendix 2.1.5.3. The above suggests possible role of YNL134C as *ADH* isoenzyme.

In cluster 7, 16 genes were down-regulated in response to ethanol stress in the wild-type strain (mean fold down-regulation - 1.54) but slightly up-regulated by stress in SM1 and CM1 (1.46- and 1.52-fold respectively). Nevertheless, at 6.5% (v/v) ethanol, these genes were more highly expressed in both mutants than in the wild-type strain; on average 3.3-fold higher in SM1 and 3.78-fold higher in CM1 (Figure 4.13, view C) . This cluster included 4 hexose transporter genes, *HXT2, 3, 4*, and *7*, all of which were more highly expressed in CM1 than in the wild-type (approximately 3-fold) at 6.5% (v/v) ethanol and two of them, *HXT3* and *HXT4,* were more than 3-fold higher in SM1 than in the wild-type at 6.5% (v/v) ethanol. GO categories associated with genes from this cluster are provided in Table 4.5.

Table 4.5: GO terms associated with SSG repressed in response to ethanol in the wild-type and induced in both mutants (K-Means cluster 7)

Gene Ontology Term	Z-scores
pentose transmembrane transporter activity	29.9256
biotin carboxylase activity	19.9624
mannose transmembrane transporter activity	15.3244
fructose transmembrane transporter activity	15.3244
acetyl-CoA carboxylase activity	14.0812
glucose transmembrane transporter activity	13.957
hexose transport	13.957
proline catabolic process	11.4692
mitochondrial respiratory chain complex II	9.9084
mitochondrial electron transport, succinate to ubiquinone	9.9084
regulation of pH	9.9084
CCAAT-binding factor complex	9.9084
response to copper ion	8.8406
succinate dehydrogenase (ubiquinone) activity	8.8406
electron carrier activity	8.0505
nuclear membrane organization and biogenesis	7.4349
regulation of carbohydrate metabolic process	6.9376
loss of chromatin silencing during replicative cell aging	6.9376
electron transport	6.9376
copper ion binding	6.9376
fatty acid biosynthetic process	6.9376
chitin- and beta-glucan-containing cell wall	6.01
membrane fraction	5.8571
transcription activator activity	5.65
structural constituent of cell wall	5.3749
tricarboxylic acid cycle	4.9786
transcription	3.5984
protein import into nucleus	3.5243
plasma membrane	3.4289
mitochondrial intermembrane space	3.387

4.5.7 Interaction significant genes

Identifying ethanol and strain significance provides information on the influence of each factor separately on gene expression. In the case of interaction significant genes (ISG) the combination of both factors plays a role in defining the overall level of gene expression. This group includes genes where strain significantly influences the ethanol stress response.

In total 150 genes were determined by 2–way ANOVA ($p \leq 0.001$) to be interaction significant (ISG). Many of these genes have been considered in the preceding sections describing ESG or SSG. Nevertheless it was considered important to look separately at this group. Once again, the K-Means clustering method was used to inspect ISG. This separated the genes into 10 clusters based on commonality of the expression profile. (Figures 4.14 and 4.15). Detailed gene-lists with corresponding p-values and heat-maps for each cluster, together with gene ontology results are provided in Appendix 2.1.5.2.

K-means clustering successfully identified genes that were expressed at particularly high levels in SM1 only, or CM1 only. Cluster 1 comprised 11 genes that were stress-induced only in SM1 whereas the same genes were down-regulated in the wild-type strain in response to 6.5% (v/v) ethanol. (Figures 4.14, 4.15 and 4.16 view A) Expression was not affected by ethanol in CM1.

The two genes in cluster 2, *DDR2* and *SIP18,* were very highly up-regulated in ethanol stress in all three strains. Interestingly both the degree of up-regulation and the overall level of expression of *SIP18* was significantly higher in SM1 than in either of the other strains. This was confirmed in the direct design comparison of SM1 at 0% vs. 6.5% (v/v) ethanol which showed that *SIP18* was 71.9-fold stress-induced in SM1 only (Appendix 2.4). GO categories enriched in this SM1 specific cluster are provided in Table 4.6.

The ethanol response in cluster 8 (Figure 4.16, view B; Table 4.7) might be considered specific for mutant CM1. Visual inspection of the mean and line graphs indicated that 17 genes from this cluster were slightly repressed in response to ethanol stress in the wild-type, slightly stress-induced in SM1 (1.4-fold) and stress-induced with a mean of 2.8-fold in CM1. *YGP1, SHM2* and *3, ACP1, IDH2, ADH3, MRPL31* and *ZRT1* displayed the highest CM1 specificity. Three genes from this cluster, *CRH1, DSE2* and *YGP1,* are located to cell wall. Similarly *THI6* (cluster 5) showed no significant change in expression in response to ethanol in either the wild-type or SM1, but highly stress-induced (25-fold) in CM1 and therefore expressed at a level more than 20-fold higher than in the wild-type strain at 6.5% (v/v) ethanol. Up-regulation of *THI6* can therefore be considered a CM1-specific ethanol stress-response.

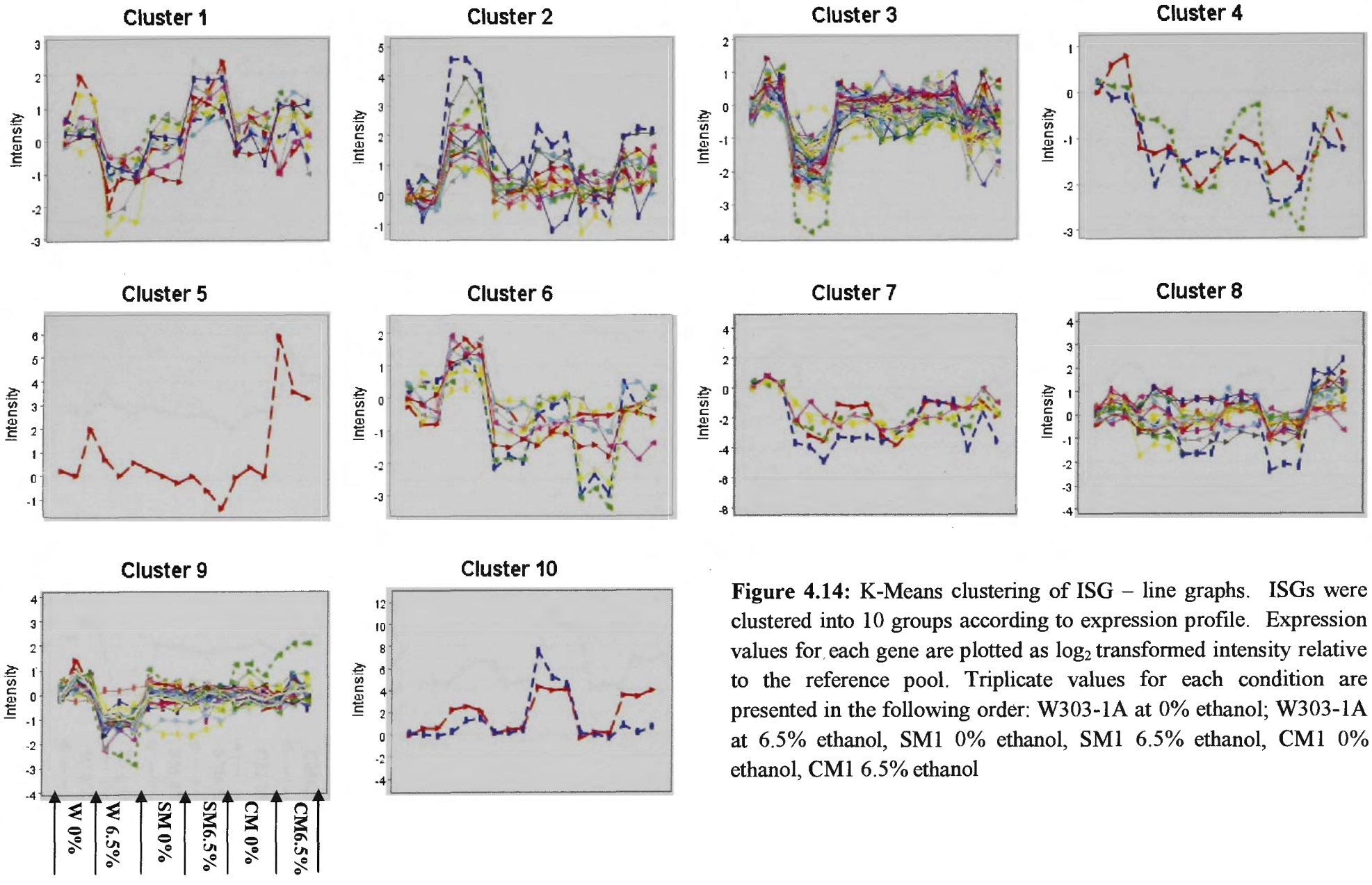

Figure 4.14: K-Means clustering of ISG – line graphs. ISGs were clustered into 10 groups according to expression profile. Expression values for each gene are plotted as $\log_2$ transformed intensity relative to the reference pool. Triplicate values for each condition are presented in the following order: W303-1A at 0% ethanol; W303-1A at 6.5% ethanol, SM1 0% ethanol, SM1 6.5% ethanol, CM1 0% ethanol, CM1 6.5% ethanol

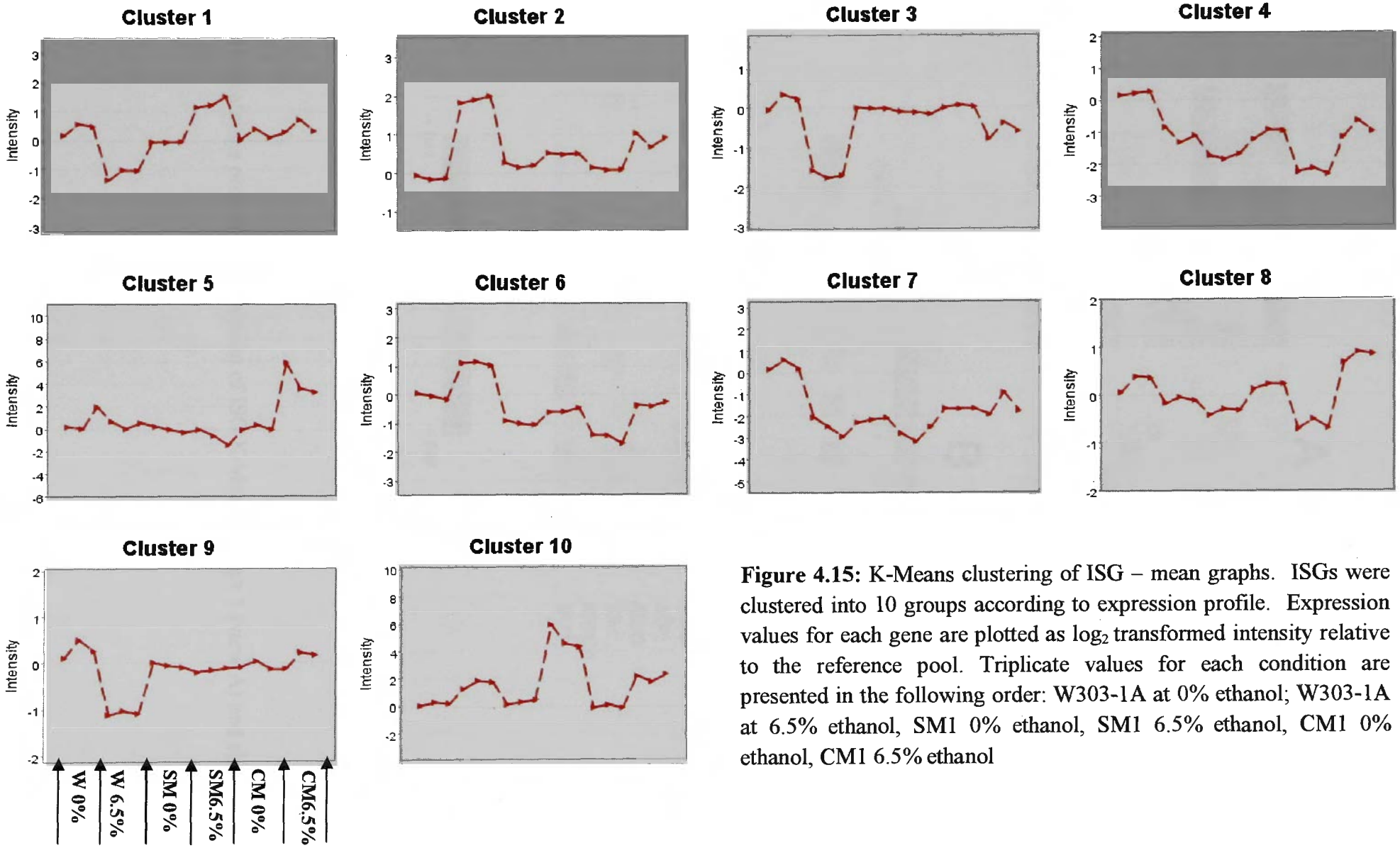

Figure 4.15: K-Means clustering of ISG – mean graphs. ISGs were clustered into 10 groups according to expression profile. Expression values for each gene are plotted as $\log_2$ transformed intensity relative to the reference pool. Triplicate values for each condition are presented in the following order: W303-1A at 0% ethanol; W303-1A at 6.5% ethanol, SM1 0% ethanol, SM1 6.5% ethanol, CM1 0% ethanol, CM1 6.5% ethanol

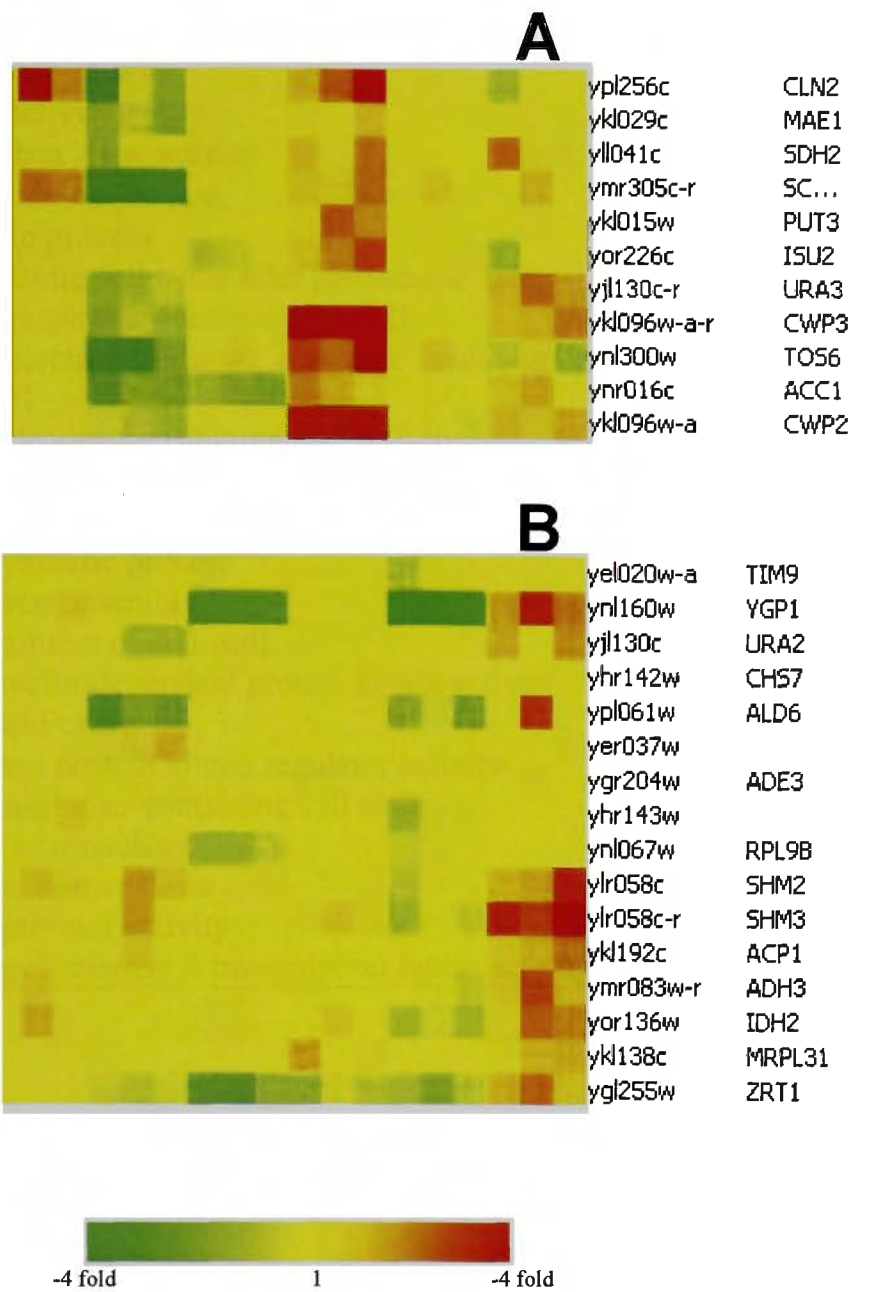

Figure 4.16: Heatmaps presenting expression of ISG K-Means cluster 1 (view A) and cluster 8 (view B).

Table 4.6: GO categories associated with K-Means SM1-specific cluster 1 of ISG

Gene Ontology Term	Z-scores
biotin carboxylase activity	24.085
malic enzyme activity	24.085
acetyl-CoA carboxylase activity	17.0026
amino acid metabolic process	17.0026
proline catabolic process	13.8597
re-entry into mitotic cell cycle after pheromone arrest	13.8597
mitochondrial respiratory chain complex II	11.983
mitochondrial electron transport, succinate to ubiquinone	11.983
regulation of pH	11.983
succinate dehydrogenase (ubiquinone) activity	10.7002
nuclear membrane organization and biogenesis	9.0133
pyruvate metabolic process	8.4172
fatty acid biosynthetic process	8.4172
iron-sulfur cluster assembly	7.1423
structural constituent of cell wall	6.5479
regulation of cyclin-dependent protein kinase activity	6.2991
tricarboxylic acid cycle	6.0753
cyclin-dependent protein kinase regulator activity	4.9572
chitin- and beta-glucan-containing cell wall	4.8185
protein import into nucleus	4.3489
cellular iron ion homeostasis	4.2659
transcription activator activity	3.3295
specific RNA polymerase II transcription factor activity	3.1679

Table 4.7: GO categories associated with K-Means CM1-specific cluster 8 of ISG

Gene Ontology Term	Z-scores
acyl carrier activity	19.3649
glutamine metabolic process	19.3649
aspartate carbamoyltransferase activity	19.3649
high-affinity zinc ion transport	19.3649
high affinity zinc uptake transmembrane transporter activity	19.3649
isocitrate dehydrogenase (NAD+) activity	13.6576
lipoic acid biosynthetic process	13.6576
methylenetetrahydrofolate dehydrogenase (NADP+) activity	13.6576
folic acid and derivative metabolic process	13.6576
formate-tetrahydrofolate ligase activity	13.6576
methenyltetrahydrofolate cyclohydrolase activity	13.6576
acetate biosynthetic process	13.6576
glycine hydroxymethyltransferase activity	13.6576
mitochondrial isocitrate dehydrogenase complex (NAD+)	13.6576

Gene Ontology Term	Z-scores
response to nutrient	11.1224
aldehyde dehydrogenase [NAD(P)+] activity	11.1224
carbamoyl-phosphate synthase (glutamine-hydrolyzing) activity	11.1224
mitochondrial intermembrane space protein transporter complex	9.6072
pyrimidine base biosynthetic process	9.6072
purine base biosynthetic process	8.5705
isocitrate metabolic process	8.5705
one-carbon compound metabolic process	7.2055
glucosidase activity	6.7224
protein import into mitochondrial inner membrane	6.7224
chitin- and beta-glucan-containing cell wall	5.8025
cytokinesis, completion of separation	5.6874
'de novo' pyrimidine base biosynthetic process	5.4308
cell wall chitin biosynthetic process	5.4308
glutamate biosynthetic process	5.2038
tricarboxylic acid cycle	4.8186
extracellular region	4.2353
integral to plasma membrane	3.4033
mitochondrial intermembrane space	3.2695

Clusters 3 and 9 displayed similar expression patterns (Table 4.8). Genes in these clusters were down-regulated under ethanol stress in the wild-type but hardly changed in response to stress in either mutant. These genes are therefore more highly expressed in both mutants than in wild-type in the presence of 6.5% ethanol. GO categories enhanced in clusters 3 and 9 (83 genes) are presented in Table 4.8 while heatmaps and genelists are provided in Appendix 2.1.5.2. Genes were mostly associated with ribosome (cytosolic small ribosomal subunit z-score 11.49 and ribosome assembly 9.04). Genes involved in lipid, particularly sterol metabolism were also significantly represented in this group.

Table 4.8: GO categories associated with ISG repressed in response to ethanol stress in wild-type only (K-Means merged clusters 3 and 9).

Gene Ontology Term	z-score
cytosolic small ribosomal subunit (sensu Eukaryota)	11.4918
ribosome assembly	9.0495
ribosomal small subunit assembly and maintenance	9.023
myosin II complex	8.7184
ADP biosynthetic process	8.7184
aromatic amino acid transport	8.7184
glutamate metabolic process	8.7184
saccharopine dehydrogenase (NADP+, L-glutamate-forming) activity	8.7184
cysteinyl-tRNA aminoacylation	8.7184
dimethylallyltranstransferase activity	8.7184
RNA strand annealing activity	8.7184
adenosylhomocysteinase activity	8.7184
GDP-dissociation inhibitor activity	8.7184
cysteine-tRNA ligase activity	8.7184
geranyltranstransferase activity	8.7184
adenosine kinase activity	8.7184
posttranslational protein targeting to membrane	8.7184
3-isopropylmalate dehydratase activity	8.7184
adenylosuccinate lyase activity	8.7184
aromatic amino acid transmembrane transporter activity	8.7184
farnesyl diphosphate biosynthetic process	8.7184
cytosol to ER transport	8.7184
C-4 methylsterol oxidase activity	8.7184
high-affinity tryptophan transmembrane transporter activity	8.7184
lipid glycosylation	8.7184
GMP synthase (glutamine-hydrolyzing) activity	8.7184
C-5 sterol desaturase activity	8.7184
sterol 14-demethylase activity	8.7184
C-22 sterol desaturase activity	8.7184
selenocysteine metabolic process	8.7184
dihydroorotate dehydrogenase activity	8.7184
nicotinate phosphoribosyltransferase activity	8.7184
structural constituent of ribosome	8.6788
ergosterol biosynthetic process	8.2752
translation	7.9609
ribosome biogenesis and assembly	7.8977
rRNA processing	7.6853
nucleolus	7.321
cytosolic large ribosomal subunit (sensu Eukaryota)	6.5505
tryptophan transport	6.0842
aspartate catabolic process	6.0842
G-protein alpha-subunit binding	6.0842
aspartate transaminase activity	6.0842
branched chain family amino acid catabolic process	6.0842
GMP metabolic process	6.0842

Gene Ontology Term	z-score
NADH dehydrogenase activity	6.0842
pseudouridine synthesis	6.0842
nucleotide-sugar transport	6.0842
acetyl-CoA biosynthetic process	6.0842
cysteine metabolic process	6.0842
aspartate biosynthetic process	6.0842
G-protein coupled receptor protein signaling pathway	6.0842
asparagine biosynthetic process from oxaloacetate	6.0842
acetate-CoA ligase activity	6.0842
nucleotide-sugar transmembrane transporter activity	6.0842
aconitate hydratase activity	6.0842
branched-chain-amino-acid transaminase activity	6.0842
ribosomal small subunit export from nucleus	5.9248
regulation of translational fidelity	4.95
nuclear nucleosome	4.95
nascent polypeptide-associated complex	4.9019
1,3-beta-glucan synthase activity	4.9019
1,3-beta-glucan synthase complex	4.9019
SRP-dependent cotranslational protein targeting to membrane	4.9019
isoprenoid biosynthetic process	4.9019
pseudouridylate synthase activity	4.9019
homoserine metabolic process	4.9019
cytokinesis, contractile ring formation	4.9019
ribosomal large subunit assembly and maintenance	4.7986
ribosomal large subunit biogenesis and assembly	4.7353
ribosomal small subunit biogenesis and assembly	4.7065
telomere maintenance	4.4666
small subunit processome	4.4472
DNA-directed RNA polymerase I complex	4.2967
osmosensor activity	4.1881
adenylate kinase activity	4.1881
nicotinate nucleotide salvage	4.1881
citrate metabolic process	4.1881
mating pheromone activity	4.1881
purine base metabolic process	4.1881
TRAMP complex	4.1881
N-glycan processing	4.1881
contractile ring contraction involved in cytokinesis	4.1881
Sec62/Sec63 complex	4.1881
polyadenylation-dependent ncRNA catabolic process	4.1881
1,3-beta-glucan biosynthetic process	4.1881
RNA-dependent ATPase activity	4.1881
ncRNA polyadenylation during polyadenylation-dependent ncRNA catabolic process	3.695
tRNA catabolic process	3.695
glucose mediated signaling	3.695
cellular response to starvation	3.695
osmosensory signaling pathway via Sho1 osmosensor	3.695
rRNA catabolic process	3.695

Gene Ontology Term	z-score
propionate metabolic process	3.695
'de novo' cotranslational protein folding	3.695
microfilament motor activity	3.695
glucose catabolic process to ethanol	3.695
nucleolar preribosome, small subunit precursor	3.695
late endosome membrane	3.695
leucine biosynthetic process	3.695
rRNA primary transcript binding	3.695
extracellular region	3.5579
chromatin assembly or disassembly	3.4424
traversing start control point of mitotic cell cycle	3.3264
nucleotide metabolic process	3.3264
Elongator holoenzyme complex	3.3264
alpha-1,2-mannosyltransferase activity	3.3264
nucleoplasm	3.2338
establishment and/or maintenance of chromatin architecture	3.0497
transcription from RNA polymerase I promoter	3.0497
chronological cell aging	3.0365
posttranslational protein targeting to membrane, translocation	3.0365
extrinsic to plasma membrane	3.0365
branched chain family amino acid biosynthetic process	3.0365
signal transduction during filamentous growth	3.0365

4.6 UNRAVELLING THE ETHANOL STRESS RESPONSE

The above analysis identified a number of GO categories that are strongly up-regulated during ethanol stress and a number of categories affected differently in the wild-type and mutant strains. A number of significant categories were selected for further consideration.

To compare gene expression profiles in selected pathways, the fold differences in gene expression between the selected conditions (for example, fold change differences between triplicates of the wild-type and mutant strain at 6.5% (v/v) ethanol) were imported into GenMAPP and MAPPFinder (Doniger *et al.*, 2003) which in turn allowed inspection of all pathways significantly affected by change in expression. To visualise as many steps in the selected pathway as possible, all genes that passed 2-way ANOVA as either ESG, SSG or ISG with a p-value ≤ 0.05 were included in this analysis, as 2-way ANOVA with p-value ≤ 0.001 did not provide enough genes to perform pathway inspection.

Glycolysis and carbohydrate metabolism: Significant changes were observed across all three strains in the expression of genes encoding enzymes involved in glycolysis and carbohydrate metabolism, as a result of ethanol stress. Genes associated with glycolysis were significantly induced in all three strains (Figures 4.17 - 4.19) in response to ethanol. Figures 4.20 and 4.21 compare glycolytic genes in SM1 and CM1 respectively to the wild-type strain at 6.5% (v/v) ethanol and demonstrate clearly that, despite the overall up-regulation of genes in this pathway across all three strains in response to ethanol stress, genes involved in glycolysis were ultimately expressed at lower levels in the mutants than in the wild-type strain.

Conversion of acetaldehyde to acetate: In all three strains genes associated with the conversion of acetaldehyde to acetate and ultimately Acetyl CoA, were affected by ethanol stress (Figures 4.17 - 4.19). Although the *ALD4* gene was up-regulated by stress in both SM1 (2.2-fold) and CM1 (7.9-fold) (Figures 4.18 and 4.19), *ALD4* was most strongly stress induced in the wild-type by 9-fold (Figure 4.17). Overall, *ALD4* was expressed at a much higher level in the wild-type than in either mutant at 6.5% (v/v) ethanol (green in Figures 4.20 and 4.21). In contrast, *ALD6* and *ASC2* were more highly expressed in both mutants compared to the wild-type (Figures 4.20 and 4.21). Similarly, both *ADH1* and *ADH5* were up-regulated during stress in both mutants and *ADH2* was up-regulated during stress in CM1, however, the final expression levels at 6.5% (v/v) ethanol for these genes are higher in the wild-type.

Ergosterol metabolism: A significant difference in the expression of genes associated with ergosterol biosynthesis is evident from GenMAPP pathway map presented in Figure 4.22. This pathway is significantly enhanced in CM1 when compared to the wild-type at 6.5% (v/v) ethanol. This enhancement is not evident in SM1 as shown in Appendix 2.3.1.

Ribosome: Distinct differences were observed in the expression of ribosomal genes in the wild-type and mutant strains. Gene ontology analysis originally showed that ribosomal genes as a group were down-regulated due to the presence of ethanol however the degree of down-regulation was much greater in the wild-type than in the

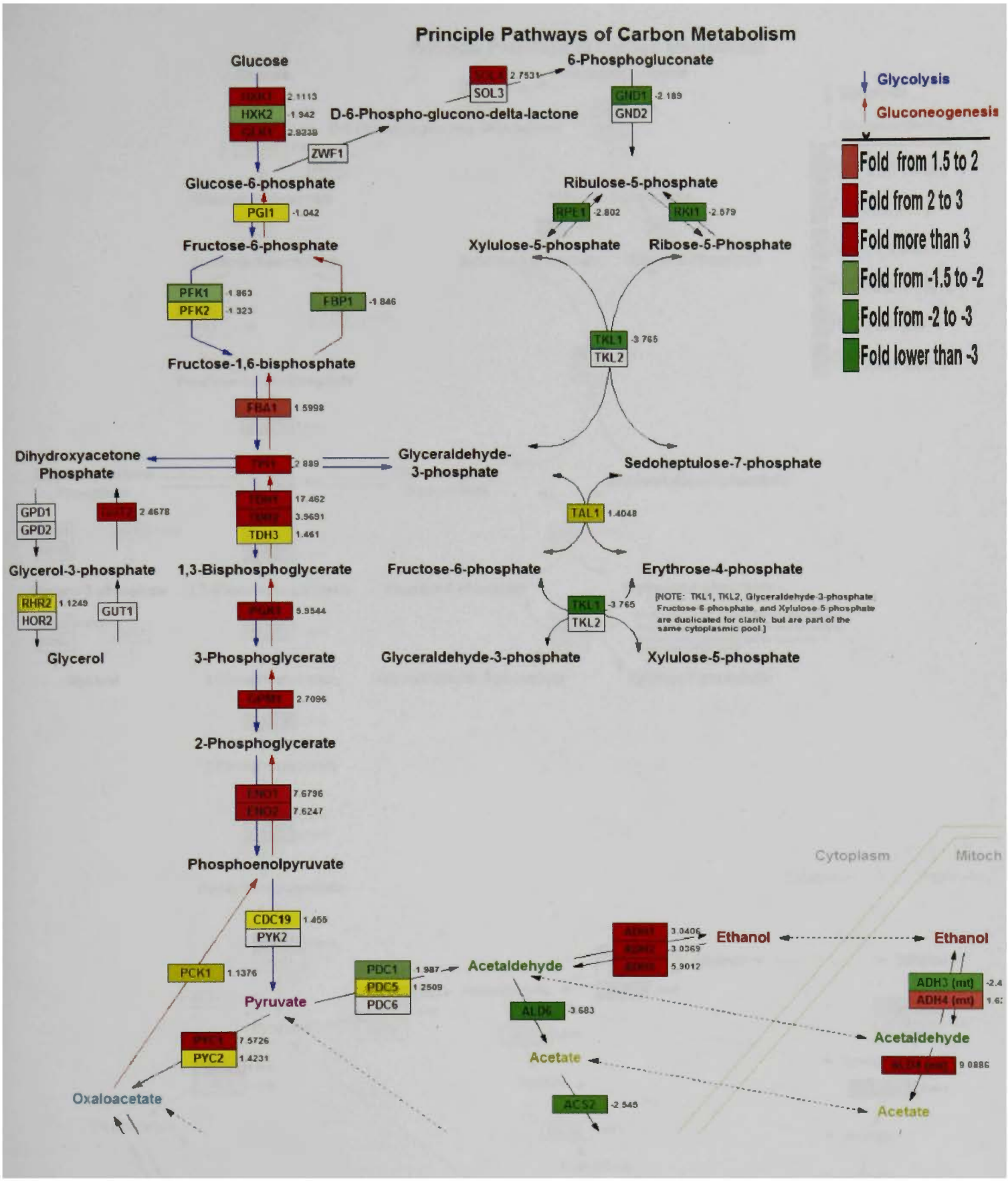

Figure 4.17: Effect of ethanol stress on carbohydrate metabolism in wild-type W303-1A. Genes more highly expressed in the wild-type strain in the presence of 6.5% ethanol compared to 0% ethanol are coloured in shades of red according to the legend provided; genes down-regulated during ethanol stress are represented in green with fold change indicated on the map. Genes shown in yellow had no change in expression and white coloured genes did not pass 2-way ANOVA (0.05). A complete map is available in Appendix 2.3.1.

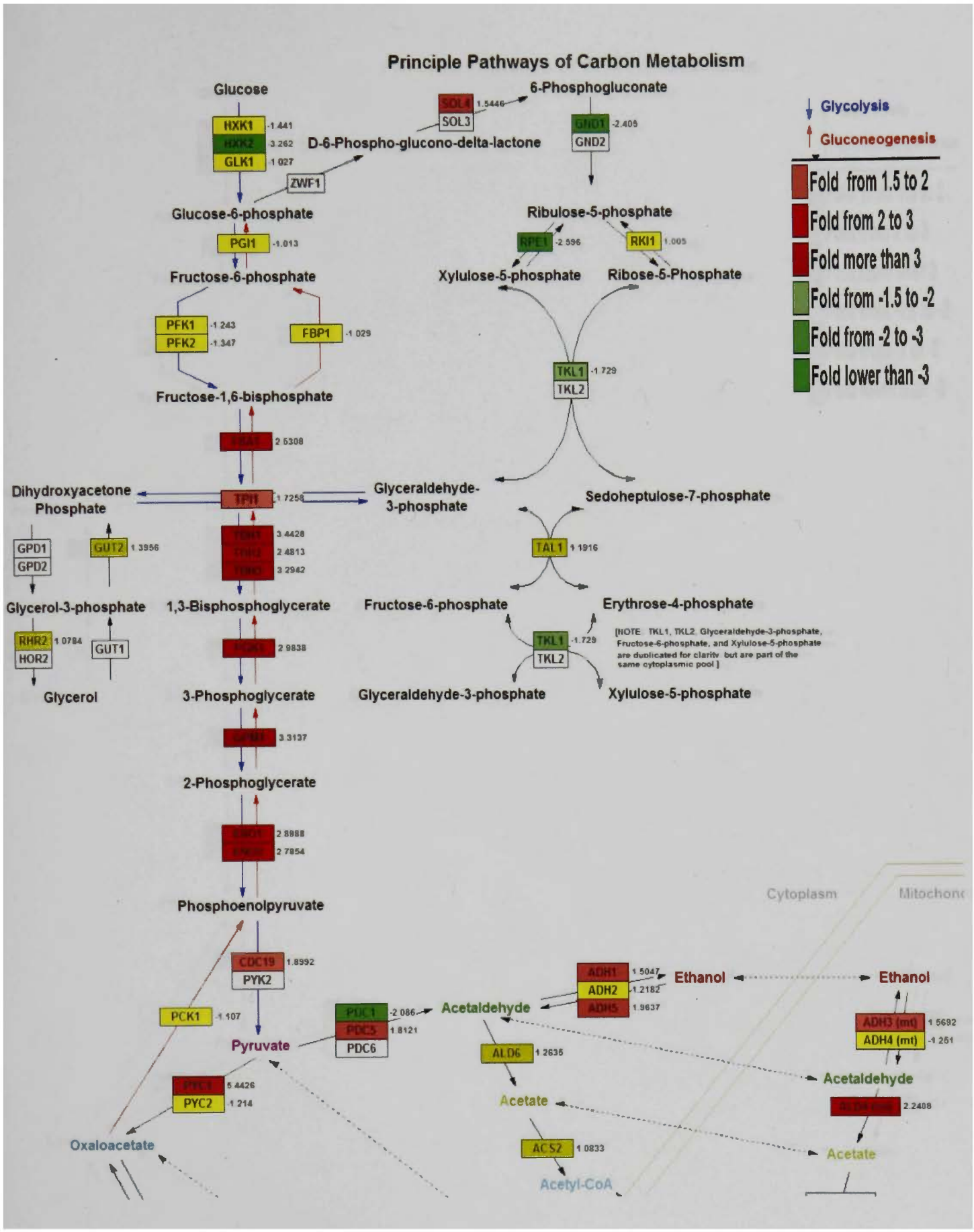

Figure 4.18: The effect of ethanol stress on carbohydrate metabolism in SM1. Genes more highly expressed in SM1 in the presence of 6.5% ethanol compared to 0% ethanol are coloured in shades of red according to the legend provided. Genes down-regulated in stress in SM1 are represented in green with fold change indicated on the map. Genes shown in yellow had no change in expression and white coloured genes did not pass 2-way ANOVA (0.05). A complete map is available in Appendix 2.3.1.

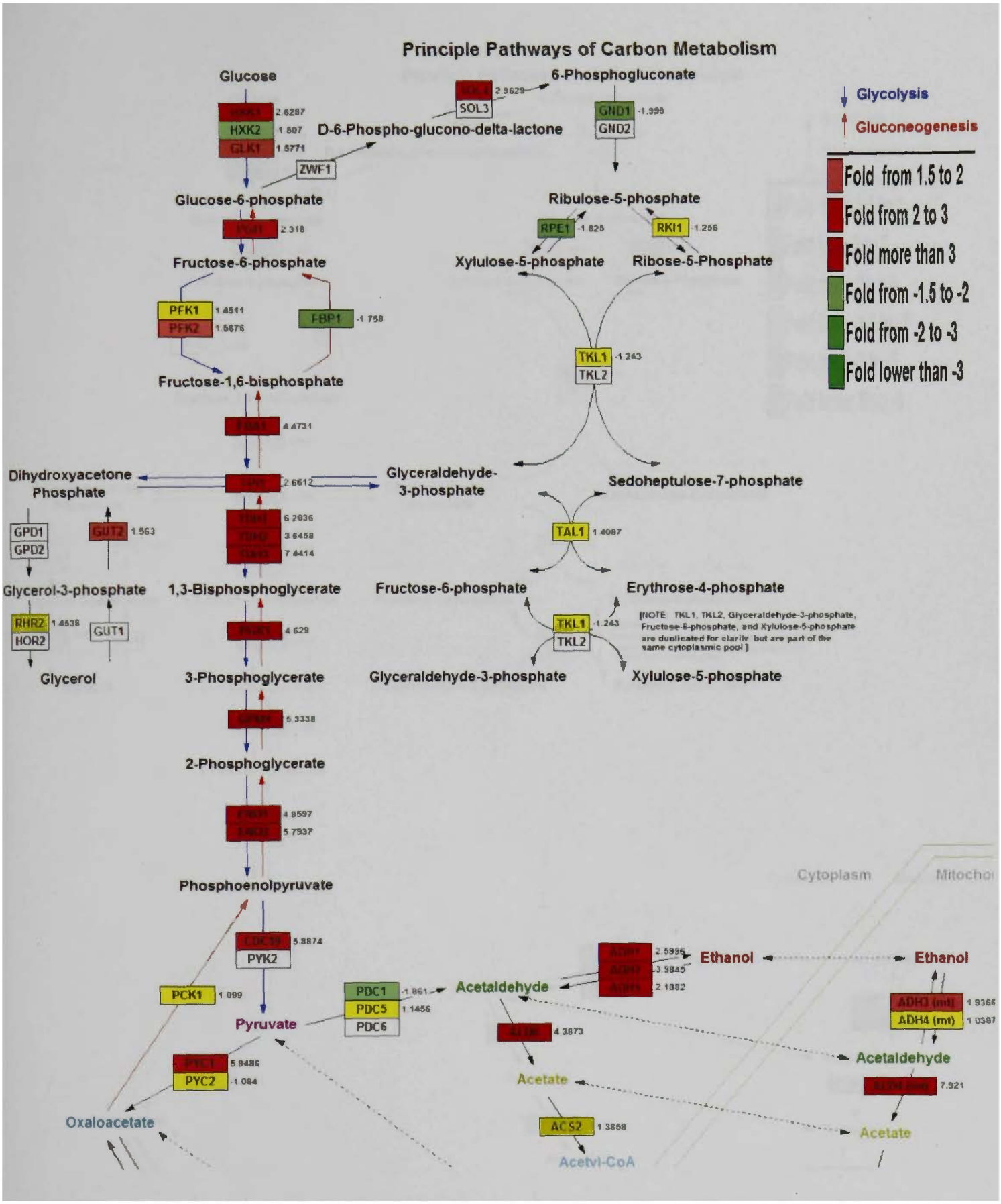

Figure 4.19: The effect of ethanol stress on carbohydrate metabolism in CM1. Genes more highly expressed in CM1 in the presence of 6.5% ethanol compared to 0% ethanol are coloured in shades of red according to the legend provided. Genes down-regulated in stress in CM1 are represented in green with fold change indicated on the map. Genes shown in yellow had no change in expression and white coloured genes did not pass 2-way ANOVA (0.05). A complete map is available in Appendix 2.3.1.

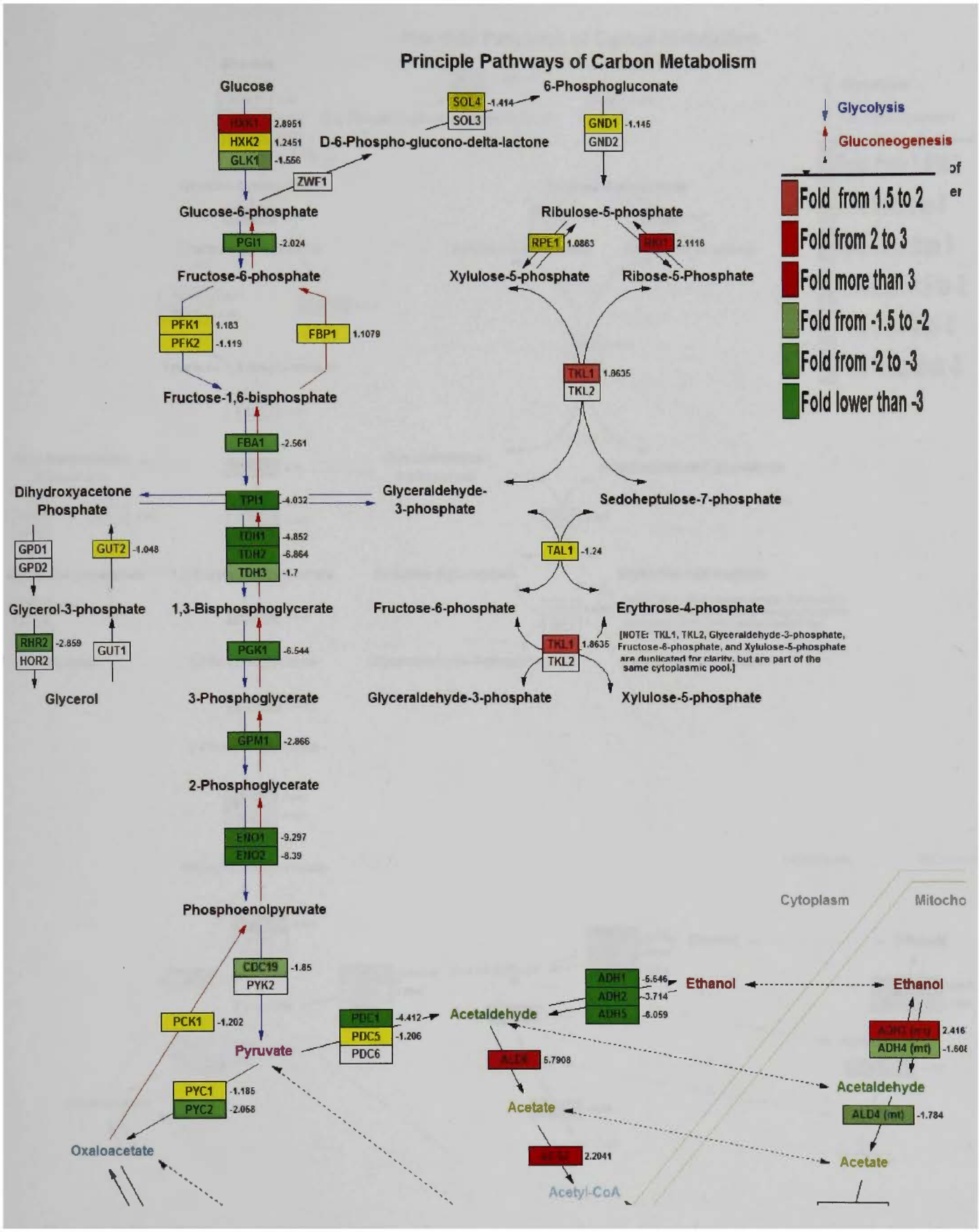

Figure 4.20: Differences in expression of genes associated with Glycolysis between W303-1A and CM1 at 6.5% ethanol. Genes more highly expressed in CM1 than in W303-1A in the presence of 6.5% ethanol are coloured in shades of red according to the legend provided, and genes with higher expression levels in the wild-type compared to CM1 are represented green with fold change indicated on the map. Genes shown in yellow had no change in expression and white coloured genes did not pass 2-way ANOVA (0.05).

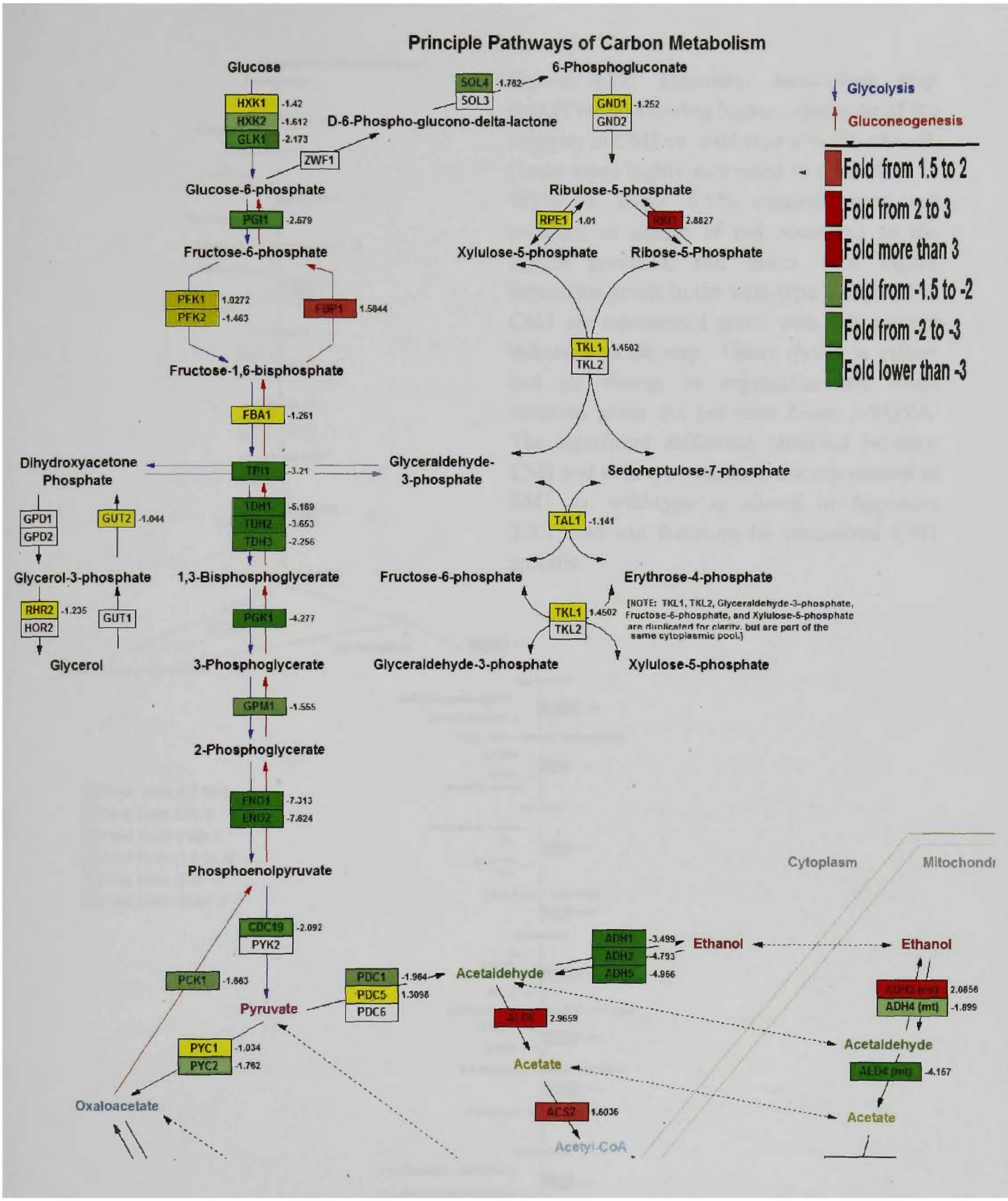

Figure 4.21: Differences in expression of genes associated with Glycolysis between W303-1A and SM1 at 6.5% ethanol. Genes more highly expressed in SM1 compared to W303-1A in the presence of 6.5% ethanol are coloured in shades of red according to the legend provided, and genes with higher expression levels in the wild-type compared to SM1 are represented green with fold change indicated on the map. Genes shown in yellow had no change in expression and white coloured genes did not pass 2-way ANOVA (0.05). A complete map is available in Appendix 2.3.1.

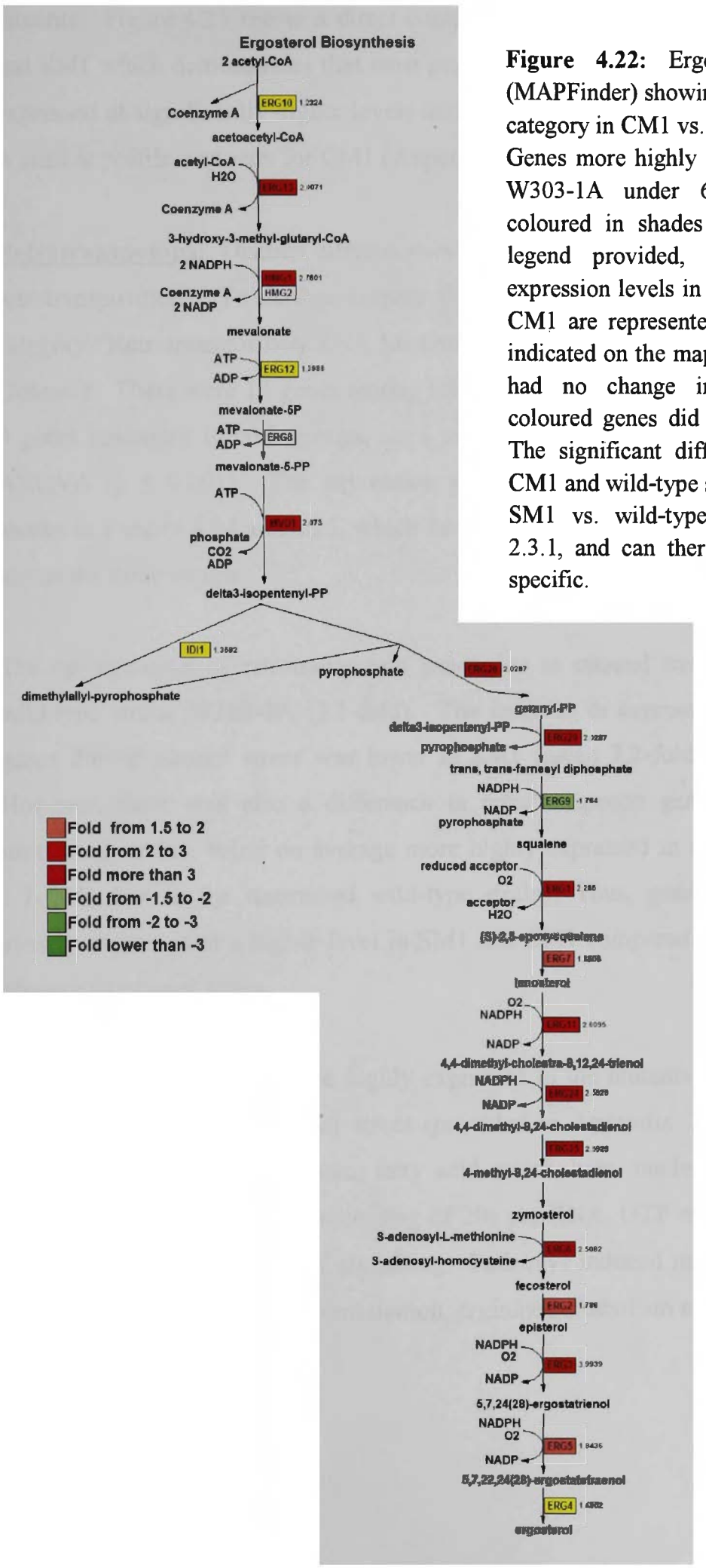

Figure 4.22: Ergosterol metabolism map (MAPFinder) showing higher expression of the category in CM1 vs. wild-type at 6.5% ethanol. Genes more highly expressed in CM1 than in W303-1A under 6.5% ethanol stress are coloured in shades of red according to the legend provided, and genes with higher expression levels in the wild-type compared to CM1 are represented green with fold change indicated on the map. Genes shown in yellow had no change in expression and white coloured genes did not pass 2-way ANOVA. The significant difference observed between CM1 and wild-type strains is not reproduced in SM1 vs. wild-type as shown in Appendix 2.3.1, and can therefore be considered CM1 specific.

mutants. Figure 4.23 shows a direct comparison of expression levels in the wild-type and SM1 which demonstrates that most genes contributing to ribosome biogenesis were expressed at significantly higher levels under ethanol stress than in the wild-type strain. A similar profile was seen for CM1 (Appendix 2.3.1).

Retrotransposons: Distinct differences were seen in the expression of genes related to retrotransposition. To further inspect this GO category, a list of genes from GO category "Retrotransposition RNA Mediated" (95 genes) was imported from SGD into Genowiz. There were 13 genes among ESG and 26 genes among SSG categories, with 9 genes belonging to both groups, *i.e.* a total of 30 genes out of 95 that passed 2–way ANOVA ($p \leq 0.001$). The expression profile of these 30 retrotransposon genes is shown in Figures 4.24 and 4.25, which demonstrate the differences in expression levels across the three strains.

The up-regulation of retrotransposon genes due to ethanol stress was highest in the wild-type strain, W303-1A (3.1-fold). The increase in expression of retrotransposon genes during ethanol stress was lower in SM1 (mean 2.2-fold) and CM1 (1.3-fold). However, there was also a difference in retrotransposon gene expression between unstressed strains, being on average more highly expressed in unstressed CM1 (mean 1.7-fold) than in the unstressed wild-type strain. Thus, genes in this category are already expressed at a higher level in SM1 and CM1 compared to the wild-type in the absence of ethanol stress.

Other categories that are more highly expressed in the mutants compared to the wild-type under 6.5% (v/v) ethanol stress (provided in Appendix 2.3.1) are: Acetyl CoA metabolism, steroid metabolism, fatty acid metabolism, nucleolus, ribosome (Figure 4.23), protein biosynthesis, processing of 20s pre-RNA, GTP metabolism, TCA cycle, translation factors and MAPK signalling. Pathways induced in ethanol stress for each strain presented are: DNA recombination, arginine metabolism and vitamin metabolism.

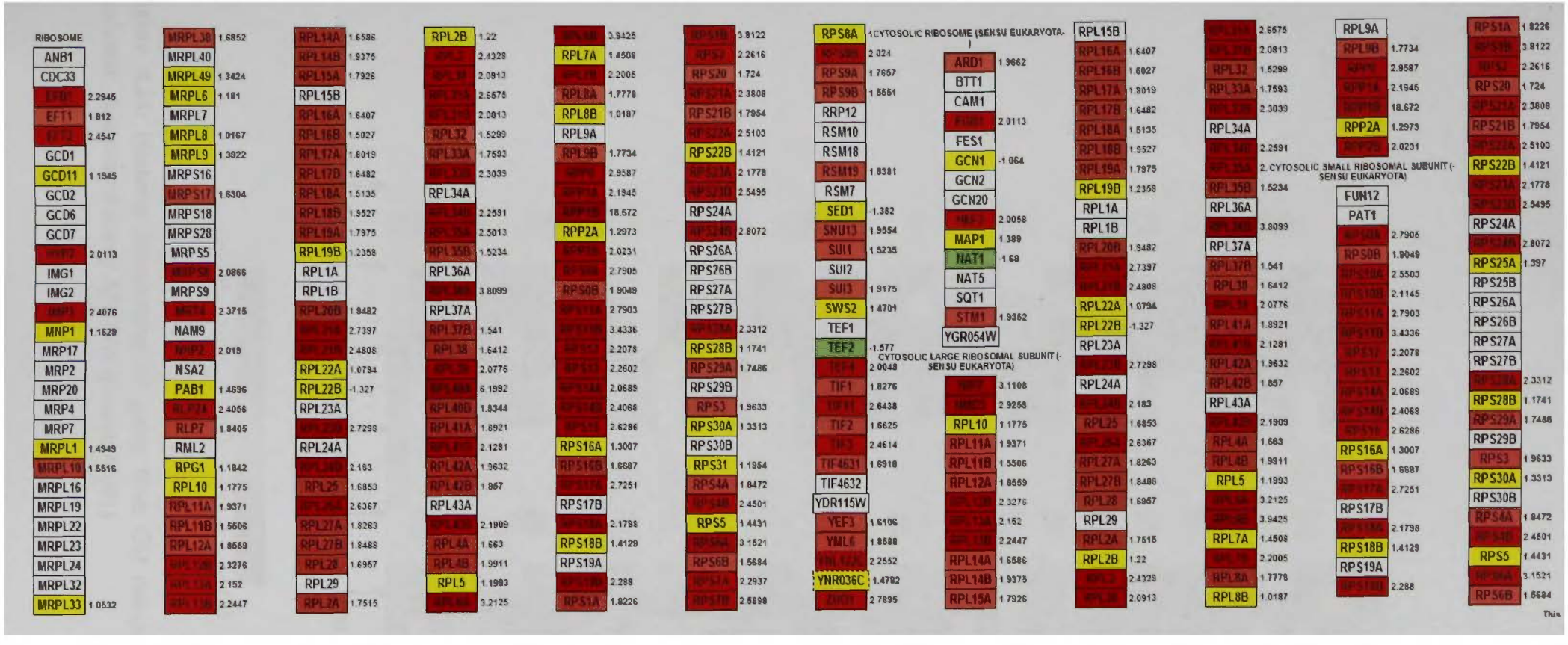

Figure 4.23: Difference in expression of genes associated with GO category 'Ribosome' between the wild-type and SM1 at 6.5% (v/v) ethanol. Genes more highly expressed in SM1 compared to the wild-type are coloured in shades of red as per legend provided in Figure 4.22. Genes less highly expressed in SM1 are coloured green. The map shows relatively high expression levels of this category in SM1 compared to the wild-type. A similar level of increased gene expression in this category was also observed in CM1 compared to the wild-type under ethanol stress. Complete maps for both mutants are available in Appendix 2.3.1.

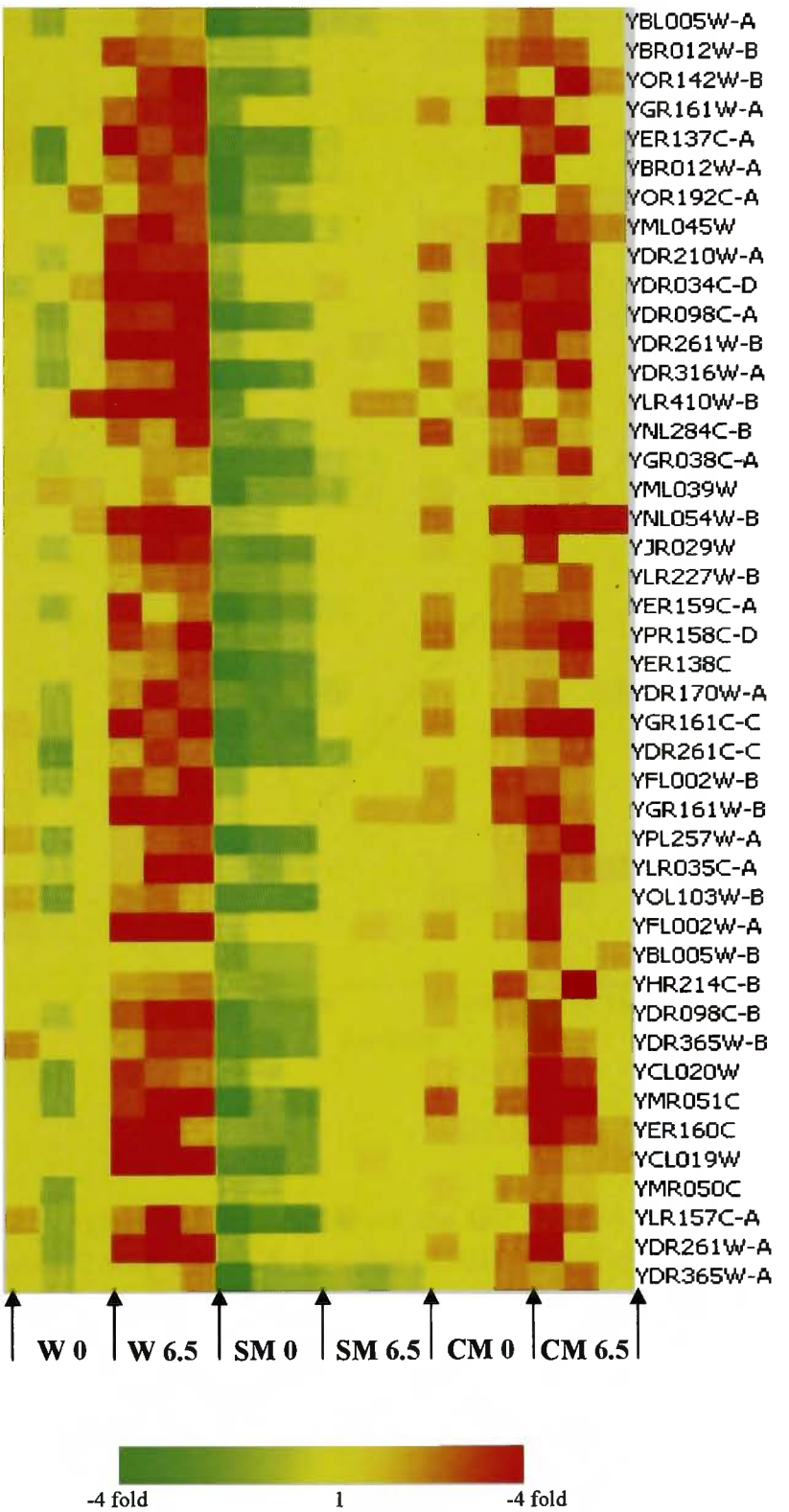

Figure 4.24: Heatmap representing 30 genes from GO category 'Retrotransposition RNA mediated', that passed 2-way ANOVA (p-value 0.001).

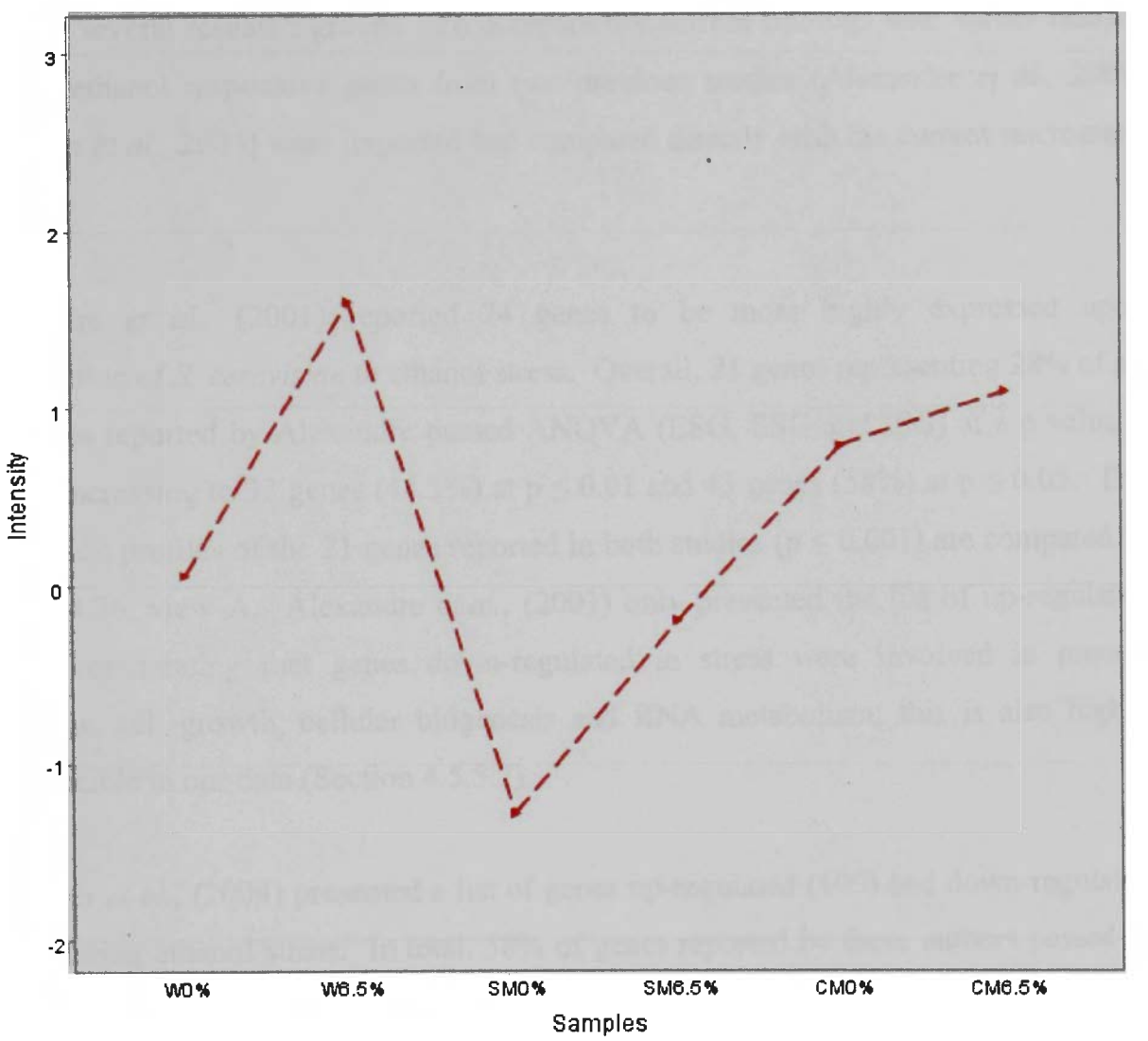

Figure 4.25: Mean expression of 30 genes from the GO category 'Retrotransposition RNA mediated', that passed 2-way ANOVA (p-value 0.001). Intensity is defined as the mean expression value (n=3) for each experimental condition (W303-1A, SM1 and CM1 at 0% and 6.5%) relative to the reference control pool for each strain. Intensity values are $\log_2$ transformed *i.e.* an intensity of 1 represents a 2-fold difference in expression.

4.7 COMPARISONS WITH PREVIOUS RESEARCH

The response of *S. cerevisiae* to ethanol has previously been investigated at a molecular level by several research groups. To compare the current findings with earlier results, lists of ethanol responsive genes from two previous studies (Alexandre *et al.*, 2001; Chandler *et al.*, 2004) were imported and compared directly with the current microarray results.

Alexandre *et al.*, (2001) reported 74 genes to be more highly expressed upon introduction of *S. cerevisiae* to ethanol stress. Overall, 21 genes representing 28% of all the genes reported by Alexandre passed ANOVA (ESG, SSG and ISG) at a p-value $\leq$ 0.001; increasing to 33 genes (44.5%) at $p \leq 0.01$ and 43 genes (58%) at $p \leq 0.05$. The expression profiles of the 21 genes reported in both studies ($p \leq 0.001$) are compared in Figure 4.26, view A. Alexandre *et.al.*, (2001) only presented the list of up-regulated genes, commenting that genes down-regulated in stress were involved in protein synthesis, cell growth, cellular biogenesis and RNA metabolism; this is also highly reproducible in our data (Section 4.5.5.2).

Chandler *et al.*, (2004) presented a list of genes up-regulated (100) and down-regulated (269) during ethanol stress. In total, 38% of genes reported by these authors passed 2-way ANOVA with a p-value ≤ 0.001 in the current study (Figure 4.26, view B); rising to 51 % ($p \leq 0.01$) and 66% ($p \leq 0.05$). This level of correlation was also observed with the down-regulated genes where a total of 99 (36.8%) genes reported as down-regulated in stress by the authors also passed ANOVA ($p \leq 0.001$) in the current study. A comparison of the genes down-regulated in stress in the two studies is shown in Figure 4.27.

Hirasawa *et. al.*, (2007) investigated gene expression in response to 5% (v/v) ethanol stress in two strains of sake yeast. The authors did not present a full list of ethanol responsive genes but found that tryptophan supplementation in the culture medium and over expression of the genes for tryptophan biosynthesis increased ethanol tolerance. In the current study there was no difference in expression of tryptophan biosynthesis genes in response to ethanol stress across any of the three strains and overall levels of expression were similar in the wild-type and ethanol-tolerant mutants.

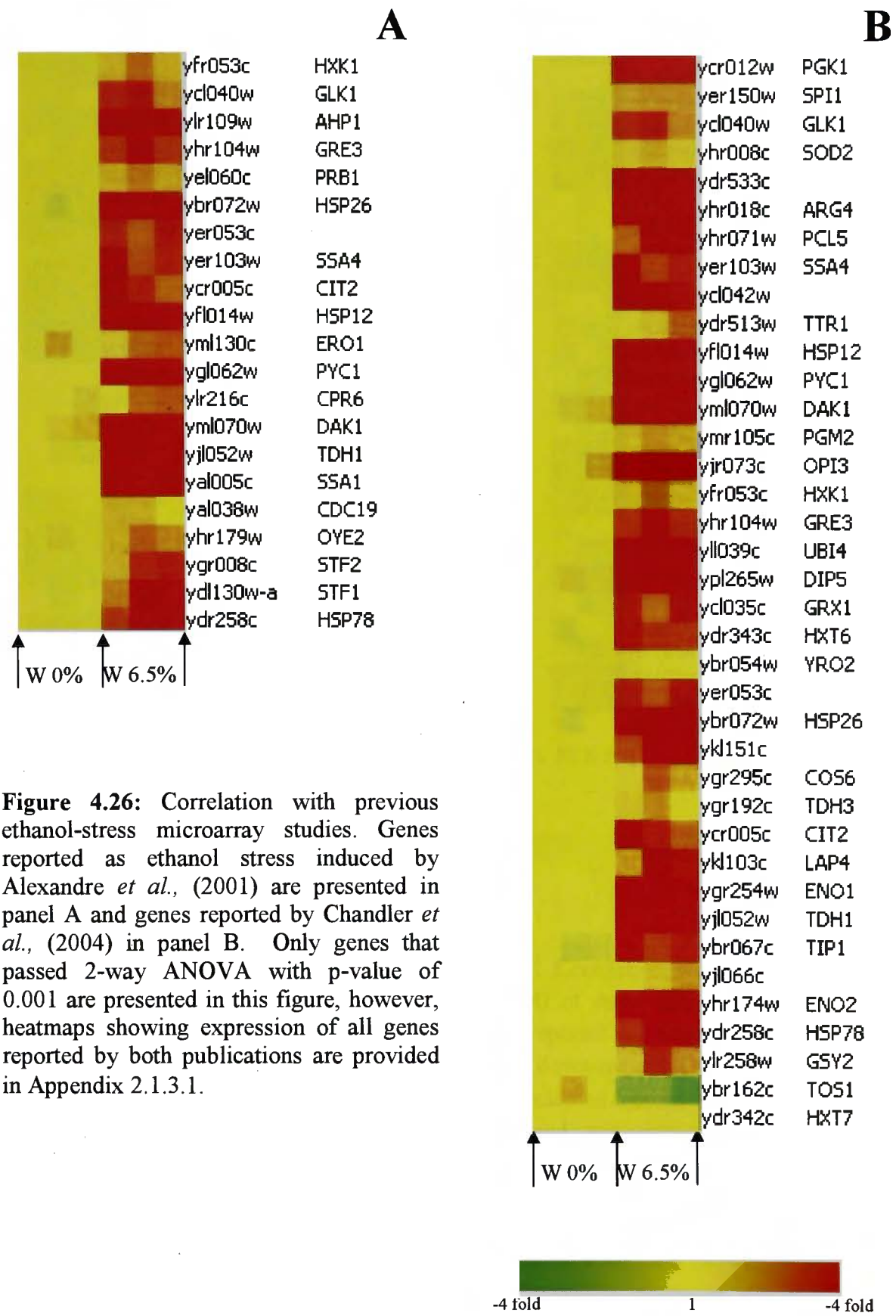

Figure 4.26: Correlation with previous ethanol-stress microarray studies. Genes reported as ethanol stress induced by Alexandre *et al.*, (2001) are presented in panel A and genes reported by Chandler *et al.*, (2004) in panel B. Only genes that passed 2-way ANOVA with p-value of 0.001 are presented in this figure, however, heatmaps showing expression of all genes reported by both publications are provided in Appendix 2.1.3.1.

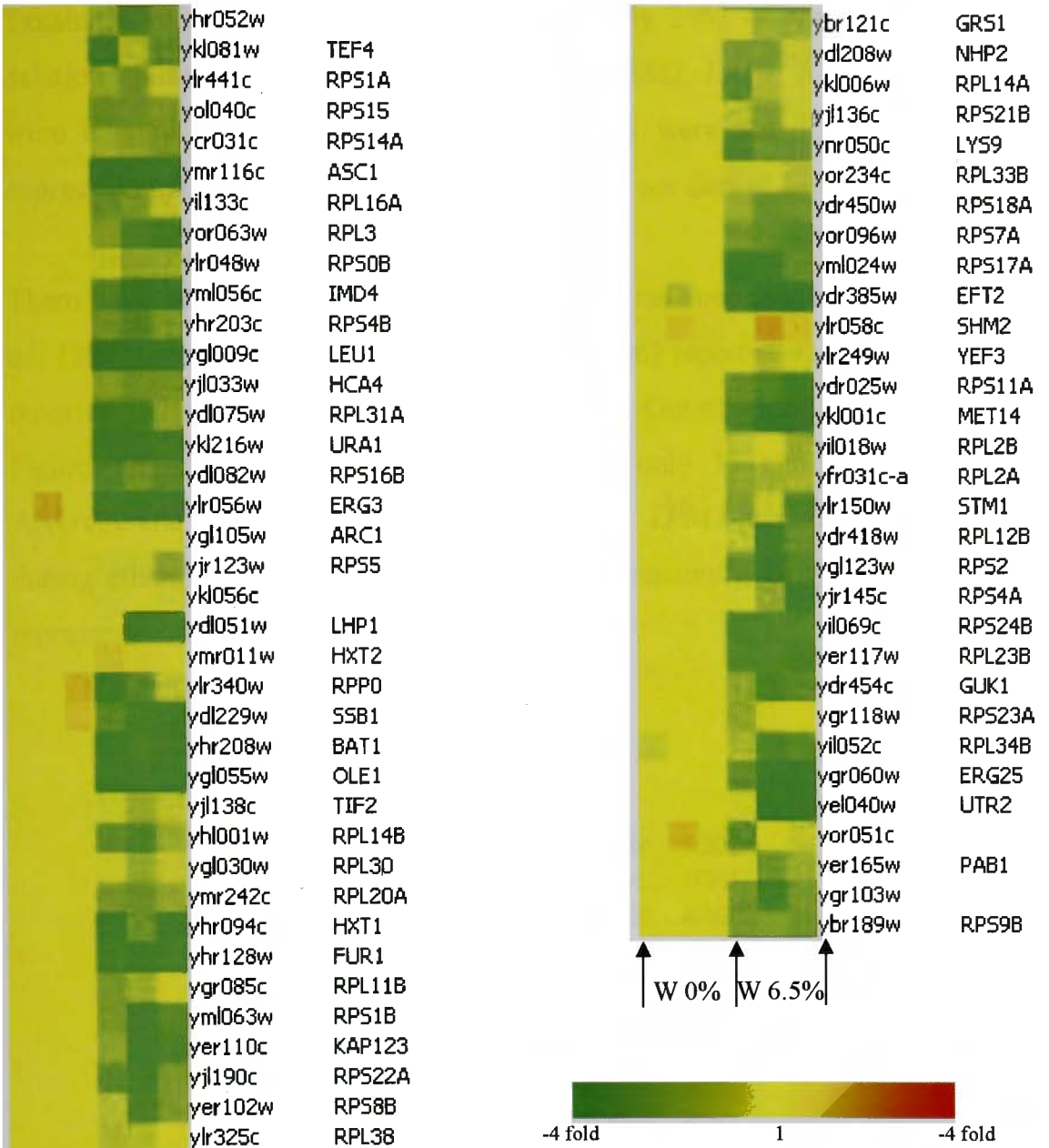

Figure 4.27: Correlation with Chandler *et al.*, (2004) of down-regulated genes. The genes reported by Chandler *et al.*, (2004) as down-regulated in ethanol stress are presented against triplicates of unstressed and triplicates of stressed wild-type W303-1A strain all hybridised vs. unstressed W303-1A reference pool. Only genes that passed 2-way ANOVA with p-value of 0.001 are presented in this figure, however, heatmaps showing all genes reported are provided in Appendix 2.1.3.1.

Takahashi *et al.*, (2001) screened approximately 20% of non-essential *S. cerevisiae* deletion mutants and found that deletions of *BEM2, PAT1, ROM2, VPS34* and *ADA2* were ethanol sensitive. Neither of the genes were found to significantly change expression upon introduction of ethanol stress in our data in any of the three strains.

There have been 3 major studies covering all yeast nonessential deletions. Kubota *et al.*, (2004) reported 256, van Voorst *et al.*, (2006) reported 46 and Fujita *et al.*, (2006) reported 137 ethanol sensitive deletion mutants. Out of a total of 350 genes (Chapter 1, Figure 1.1) reported by these three studies, only 13 genes (3.7%) passed 2-way ANOVA and only 3 of the 13, namely *SOD2, TPM1* and *ARG82* were up-regulated during ethanol stress (Figure 4.28) while the remaining 10 genes were ethanol stress repressed.

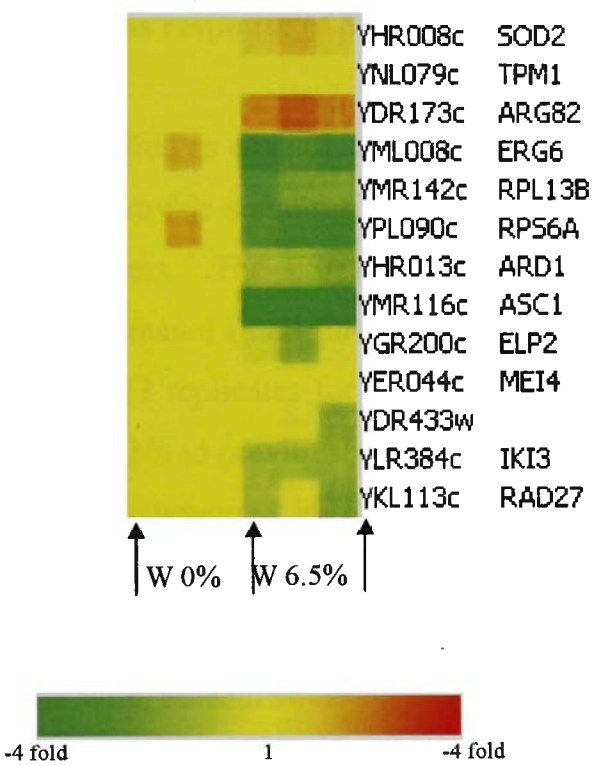

Figure 4.28: Correlation with deletion library studies on ethanol sensitive strains. The genes reported by Kubota *et al.*, (2004), Fujita *et al.*, (2006) or van Voorst *et al.*, (2006) as represented in 2-way ANOVA (p-value 0.001) in the current study. Only 3 out of 13 genes reported to have a role in survival under non-lethal ethanol stress were up-regulated in response to ethanol.

4.8 DISCUSSION

The main objectives of this chapter were to identify changes in the expression of genes that occur in all three *S. cerevisiae* strains (W303-1A, SM1 and CM1) in response to ethanol stress and to investigate the differences in the response between the wild-type and mutant strains; the purpose being to identify how the mutants improved their ethanol stress response. The various ESGs were grouped according to their GO categories so that the relative importance of various cell functions could be determined. Although the limitations of such analysis are recognised, *i.e.* changes in gene expression do not necessarily reflect changes that occur at the proteome or phenome level, the results presented in this chapter provide evidence that the overall activity of some cell functions appear to be of high priority for change in ethanol-stressed stressed cells. The cell functions that fall into this category and their proposed role in the ethanol stress response are presented in the following discussion.

4.8.1 The ethanol stress response of *S. cerevisiae*

The group of genes identified as ethanol significant using 2-way ANOVA with p-value < 0.001 are expected to provide some insight into the transcriptional events stimulated by non-lethal ethanol stress. The ESGs were identified using K-Means for genes induced or repressed by ethanol in all three strains, across 9 cultures for each stressed and unstressed condition (3 replicates for each of the 3 strains). Although the results were found to be comparable to previously published work on gene expression changes caused by ethanol stress (supported by independent direct microarray comparison of stressed vs. unstressed samples of each strain), the use of three closely related, but phenotypically different, strains provided significant insights on how *S. cerevisiae* is affected by, and copes with, ethanol stress.

The list of ESGs induced by ethanol exposure in all three strains (*i.e.* W303-1A, SM1 and CM1) suggests that the cell is struggling to maintain a sustainable glycolytic flux during ethanol stress (Section 4.5.5.1; Table 4.1). This is evident from the substantial number of glycolysis-associated genes with increased expression during ethanol stress, suggesting that the cell is attempting to stimulate glycolytic activity. A GO analysis of all three strains during ethanol stress assigned a z-score of 12.59 for glycolysis, noting

that a z-score of 2 is considered to be significant. Of the total of 16 genes associated with glycolysis in *S. cerevisiae*, 10 genes were ESG and up-regulated in all three strains with stringent p-value less than 0.001 (Appendix 2.1.5.1/ESG K-Means/ ESG upregulated in stress Funspec GO.xls). Increasing the p-value to 0.05 (as in Figures 4.17 - 4.21) increased this ratio to 15 of 16 glycolysis genes being ESG in this set of data. Previous studies have also observed increased expression in glycolysis-associated genes in *S. cerevisiae* during ethanol stress (Alexandre *et al.*, 2001; Chandler *et. al.*, 2004; Fujita *et al.*, 2004). Although these workers observed glycolysis-associated genes to be up regulated by ethanol, the number of affected genes in such studies was considerably less than that observed in the current investigation. Chandler *et al.*, (2004) reported 6 glycolysis genes, namely *PGK1, TDH3, ENO1, ENO2,* and *TDH1,* and Alexandre *et al.*, (2001) reported 3 genes, namely *CDC19, GPM2,* and *TDH1*. Chandler *et al.*, (2004) proposed that ethanol-stressed cells could be experiencing a pseudo-starvation state, whereby the negative impact of ethanol on glycolytic activity combined with the energy demands associated with the cell stress response leads to a starvation state, despite ample substrate being available in the medium. The results of the current study support this concept.

Of the glycolytic genes affected by ethanol stress, it is particularly notable that *TDH1,* which encodes glyceraldehyde-3-phosphate dehydrogenase, is upregulated in all 3 strains, but is most highly expressed in the wild-type (Table 4.1; z-score 8.09 and Figures 4.17 - 4.19). *TDH1, TDH2* and *TDH3* have a key position in glycolysis and are the only glycolytic enzymes that use NAD^+ as a cofactor, which may not be coincidental since the importance of maintaining cellular redox balance during various types of stress has been noted previously (Moraitis and Curran 2007; Stanley *et al.*, 1997; Ueom *et al.*, 2003).

It was recently found that reductive stress in *S. cerevisiae* (resulting from the accumulation of NADH) caused considerable elevation in *TDH1* expression, leading to the conclusion that its expression is regulated by reductive stress caused by cytoplasmic accumulation of NADH (Valadi *et al.*, 2004). With this in mind, the results presented in this chapter suggest that ethanol-stressed *S. cerevisiae* experience reductive stress, most likely due to NADH accumulation. Furthermore, the current study identified in all three strains a number of redox-related, up-regulated ESGs in the GO categories of

redox homeostasis (z-score 6.66), NAD biosynthetic process (z-score 4.97) and NADH oxidation (z-score 3.71) (Table 4.1).

The influence of redox balance on cell metabolism was discussed in Section 1.4 and, in particular, the influence of acetaldehyde in stimulating the acclimatization of *S. cerevisiae* to non-lethal ethanol stress (Stanley *et al.*, 1993; Stanley *et al.*, 1997; Vriesekoop and Pamment 2005). These authors speculated that the acetaldehyde effect was related to NADH oxidation; NADH is a cofactor in the reduction of acetaldehyde to ethanol by alcohol dehydrogenase. It was suggested that ethanol stress causes a loss of intracellular acetaldehyde, due to a loss in membrane integrity, leading to an intracellular shortage of NAD^+ which reduces *TDH* activity and, therefore, glycolytic flux; the addition of acetaldehyde to ethanol-stressed cells increases NAD^+ supply, stimulating *TDH* activity. The results of the current work show for the first time that restoring *TDH* activity appears to be a top priority for an ethanol-stressed cell, supporting the concept that an NAD^+ shortage is restricting glycolytic activity.

One way the cell can improve NAD^+ supply is to increase mitochondrial-based NADH oxidation; respiration oxidises reduced electron carrier NADH to NAD^+, thereby contributing to redox balance in the cell (Belenky *et al.*, 2007; Panozzo *et al.*, 2002). Aerobic cultures were used in the ethanol stress experiments described in this thesis so functional mitochondria would have been present in the stressed cells. Indeed, there is evidence that all three strains were attempting to increase mitochondrial activity by the up-regulation of ESGs in the GO categories of "mitochondrion" category (Z-score 4.60), "mitochondrial proton-transporting ATP synthase complex" (Z-score 5.27), "mitochondrial intermembrane space" (Z-score 5.08), "mitochondrial outer membrane" (Z-score 3.12) and "mitochondrial matrix" (Z-score 3.40) (Table 4.1). Overall, there are 37 ethanol significant mitochondrial genes induced during ethanol stress in all three strains (Appendix 2.1.5.1/ESG up-regulated in stress Funspec GO). Furthermore, pathway maps (GenMAPP) with corresponding fold change disclose the induction (in all three ethanol-stressed strains) of ten genes involved in respiration, namely *STF1, ATP17, CYC7, COX13, STF2, QCR10, COX5B, QCR8, COX17* and *ATP18* (Appendix 2.3.1, mitochondrial electron transport chain.bmp; mitochondrial inner membrane.bmp).

This scale of increase in the expression of respiration-associated genes in ethanol-stressed cells has not been reported in previous studies. Alexandre *et al.,* (2001) and Chandler *et al.,* (2004) both reported the up-regulation of mitochondrial genes *ALD4*, *HSP78* and *CIT2* in *S. cerevisiae* during ethanol stress, with the former also reporting *CIT1* and the latter also reporting *PDX1* as up-regulated; this number of ethanol stress-responsive mitochondrion-located genes is quite low compared to present study. *S. cerevisiae* deletion library screens report 21 mitochondrial genes to be ethanol stress related (Fujita *et al.,* 2006; Kubota *et al.,* 2004; van Voorst *et al.,* 2006), however, none of these genes were observed to be ethanol stress-related in previous gene expression studies or in the current study.

Connections between mitochondrial activity and cell stress have been identified in previous studies. Mitochondria were found to be targets for ethanol-caused cell damage (Aguilera and Benitez 1985) and heat pre-treatment of *S. cerevisiae* is known to induce hyperpolarisation of the inner mitochondrial membrane which activates *HSP104* synthesis, and possibly the synthesis of other HSPs (Rachenko *et al.,* 2004; Rikhvanov *et al.,* 2005); *HSP104* was previously shown to be critical for ethanol tolerance (Sanchez *et al.,* 1992). Rikhvanov *et al.,* (2005) suggested that the mitochondrion could be a regulator of the heat shock response in *S. cerevisiae* which, given the considerable overlap in the response of *S. cerevisiae* to both heat stress and ethanol stress, supports the strong association between mitochondrial activity and ethanol stress in the current study. It is also well-known that *S. cerevisiae* petites (respiratory-deficient mutants) are more sensitive to ethanol stress than the wild-type strain (Aguilera and Benitez 1985; Ibeas and Jimenez 1997).

A number of GO categories indicate the importance of protein folding/refolding and proteolysis in the ethanol stress response (Table 4.1). GO categories such as regulation of proteolysis, protein folding, unfolded protein binding, chaperone activator activity, all with z-scores >5, suggest the need by ethanol-stressed cells to undertake changes in protein profile in response to the stress. Proteolysis is known to play a significant role in stressed cells as ethanol exposure increases the number of aberrant proteins. It has been suggested that the accumulation of misfolded proteins could be a mediator and sensor of cellular stress and provoke at least part of the stress response (Trotter *et al.,* 2001). Indeed there are 10 chaperones up-regulated among ESG up-regulated in all 3

strains (p-value <0.001); 9 of them (*SSA1, HSP26, HSP78, SSA4, HSP12, MDJ1, KAR2, HSP60* and *HSP82*) heat shock proteins (Appendix 2.1.5.1/ESG up-regulated in stress Funspec GO) which is consistent with previous studies.

Although the role of thiamine in ethanol detoxification in humans is well known, there is no evidence for, or suggestion on, its role in the yeast ethanol stress response reported. There are 9 vitamin-associated genes in the list of up-regulated ESG in all three strains (Appendix 2.1.5.1/ESG up-regulated in stress Funspec GO), namely *RIB3* and *5*, *BNA 1,5* and *6, PYC1, RPI1, SNZ1* and *THI2*. Furthermore, fold stress-induction of a number of genes associated with vitamin metabolism, in all three strains, is presented in the pathway (GenMAPP) section (Appendix 2.3.1). This data suggests an increased need by the stressed cell for vitamins, which is surprising given that the medium contains excess amounts of vitamins and may reflect an ethanol-facilitated failure in membrane-associated nutrient transport activity.

In contrast to yeast ethanol stress, the relationship between ethanol and thiamine has been well investigated in mammalian-based research. It has been established that increased alcohol consumption causes thiamine deficiency due to ethanol-caused alteration of thiamine metabolism through the reduction of the vitamin conversion to its metabolically active form-thiamine pyrophosphate (TPP) (Ba *et al.*, 1996; Zimatkin and Zimatkina 1996). Thiamine has been routinely administered to patients suffering alcohol-abuse related symptoms (Agabio 2005; Day *et al.*, 2004; Frankenburg 2008). Thiamine is a precursor of TPP which is a prosthetic group of a number of enzymes and is known to control expression of PDC5 (Muller *et al.*, 1999). Coenzyme and anticoenzyme derivates of vitamins are able to increase or reduce enzyme activity upon binding to the enzyme (Schellenberger 1998). An anti-vitamin derivate of thiamine, oxithiamine, after phosphorylation, shows high affinity for thiamine-dependant enzymes and significantly reduces their activity (Strumilo *et al.*, 1984). In wine fermentations, early thiamine depletion is known to cause slow or stuck fermentations (Bataillon *et al.*, 1996). The potential role of vitamins, especially thiamine, in ethanol stress is yet to be established.

Similarly, for amino acids, arginine biosynthesis genes reported to be ethanol stress induced by Fujita *et al.,* (2004), were found to be ethanol-stress induced in all three strains used in the current work despite a surplus of arginine in the medium (Appendix 2.3.1). The expression of arginine biosynthesis genes is subject to the general control of amino acid biosynthesis; conditions of starvation for any of a number of amino acids elicits the synthesis of Gcn4, which activates amino acid biosynthesis genes by interacting with upstream activation sites (Crabeel *et al.,* 1990). It may be that the cell, starved of external amino acid supply due to ethanol-mediated inhibition of membrane-associated transporters, needs to synthesise its own amino acids.

Figures 4.24 and 4.25, together with Table 4.1, demonstrate retrotransposition GO categories in the ethanol stress response. Retrotransposons, evidently induced during ethanol stress in all 3 strains, but most strongly in the wild-type, are mobile genetic elements similar to retroviruses. They propagate by reverse transcription of single stranded RNA into double stranded DNA which integrates into the host genome and are not infectious for neighbouring yeast cells. There are five distinct retrotransposons families in *S.cerevisiae*, Ty 1-5; only Ty1-3 are known to be transcriptionally active (Sandmeyer 1992).

Transposition rates are low in yeast and the number of retrotransposons remains relatively constant by cell balancing transposition and excision events (Fink 1986). The relationship between host and retrotransposons has been well investigated and a number of host transcription factors and events are documented to be involved in transcriptional control of these genes. Ty1 element expression, normally kept under control, is reported to be induced under various stressful conditions such as DNA damage (Bradshaw and McEntee 1989), uv-light exposure (Rolfe 1985), ionizing radiation (Sacerdot *et al.,* 2005), adenine starvation (Todeschini *et al.,* 2005), nitrogen starvation (Morillon *et al.,* 2000) and cell response to mycotoxin citrinin (Iwahashi *et al.,* 2007), whereas Ty3 transposition was shown to be inhibited by different cellular stressors including heat and ethanol (Menees and Sandmeyer 1996). It has been suggested that activation of the Ty1 transposition could play a role in adaptive mutagenesis in response to stress (Morillon *et al.,* 2000), however, the connection between retrotransposition and ethanol stress in yeast has not been researched. Surprisingly, no previous studies have reported any of the 95 genes belonging to GO category 'Retrotransposition RNA

mediated' (Figure 4.24) in the ethanol stress response. It has been suggested that other environmental stresses may activate Ty1 transposition via Gcn4p, master regulator under a range of environmental stresses (Natarajan *et al.*, 2001).

Although the overall level of genes associated with retrotransposition increases in stress for all 3 strains (Figure 4.24 and 4.25) the biggest impact was observed in the wild-type W303-1A. CM1 showed negligible stress induction of the genes in this category, but also had the highest level of transposition genes in the absence of stress. SM1 had the lowest level of retrotransposition under ethanol stress, approximately 4-fold lower than the wild-type. Regardless of differences in the level of up-regulation of these genes, Figures 4.24 and 4.25 demonstrate that retrotransposition is stress-induced and may have a role in the ethanol stress response of *S. cerevisiae*.

Ethanol stress resulted in the down-regulation of genes associated with ribosomal synthesis in all three *S. cerevisiae* strains (Table 4.2), which is consistent with the observations of other studies on transcription events during ethanol stress (Alexandre *et al.*, 2001; Chandler *et al.*, 2004). GO categories related to transcription and translation events in the cell also appear to be highly repressed during ethanol stress (Table 4.2). This is consistent with the cell entering growth arrest when first exposed to ethanol stress, as observed in the work of Chapter 3 (Gasch *et al.*, 2000). Ribosomal synthesis requires substantial energy input from the cell and is believed to be repressed during ethanol stress to conserve energy (Warner 1999).

An unexpected result was the down-regulation of genes associated with the GO categories of sterol metabolism, and in particular ergosterol biosynthesis, in ethanol-stressed *S. cerevisiae* (Table 4.2); pathway maps show that sterol metabolism is repressed in all three strains during stress (Appendix 2.3.1). Although this is consistent with the observation that the sterol content of cells is generally lowered during ethanol stress, it is inconsistent with previous findings that ethanol-stressed *S. cerevisiae* generally increase their content of ergosterol and other unsaturated membrane sterols (Walker-Caprioglio *et al.*, 1990). Also, it has been demonstrated that supplementation of growth medium with ergosterol improves the ethanol tolerance of *S. cerevisiae* (Thomas *et al.*, 1978). Sterol biosynthesis is an energy-intensive pathway (Smith *et al.*,

1996) and a reduction in metabolic activity of this pathway may again reflect energy conservation measures in ethanol-stressed cells.

ESG lists did not correlate with deletion library studies (Figure 4.28). This lack of correlation extends to previously published ethanol stress expression studies and is also a factor between different deletion library screenings as discussed in Chapter 1, Section 1.3.2.2. It is possible that the genes correlating with ethanol sensitive deletions could be induced early in the ethanol stress response, returning to 'normal' expression levels within 1 hour; therefore escaping detection in the microarray studies. On the other hand, it may be that the genes identified as important in the genome-wide studies may not need to respond to ethanol stress by increasing expression levels, rather, the constitutive levels of the gene product may be sufficient to address ethanol stress.

4.8.2 Why are SM1 and CM1 more ethanol stress tolerant than W303-1A?

The results in this chapter provide evidence suggesting that glycolytic flux in ethanol-stressed *S. cerevisiae* cells is problematic and a redox imbalance might be responsible for a reduction in glycolytic activity (Section 4.8.1). Assuming this to be the case, it may be argued that SM1 and CM1 acquired their higher ethanol stress tolerance by inheriting ways of improving their NADH oxidation rate during ethanol stress. The most convincing evidence of this is provided in Chapter 3 (Section 3.7.3) where it is shown that both mutants produce significantly more glycerol than the wild-type strain, either in the presence or absence of ethanol stress. Higher glycerol production rates in CM1 and SM1 must also result in higher NADH oxidation rates and therefore increase in NAD^+ production in the cell. Given that higher glycerol production in both mutants appears to be constitutive, glycerol biosynthesis genes in the mutants may not be ESGs, which appears to be the case. If the increased glycerol production was a consequence of transcriptional change, glycerol biosynthesis genes could be expected to belong to the SSG list. This was not the case, since of all the genes associated with glycerol biosynthesis, only *GPP1* (*RHR2*) was identified by 2-way ANOVA as SSG (p-value of 1.99 E^{-4}), even at a p-value as high as 0.05. Given these outcomes, it is most probable that the increased glycerol levels in the mutants are due to mutation(s) affecting the specific activity of enzymes associated with the glycerol pathway, and/or glycerol transport, rather than a result of transcriptional change.

A predicted higher NAD^+ production rate in the mutants (due to higher glycerol production) could be expected to stimulate *TDH* activity at the enzyme level over and above that observed in the wild-type strain. This is supported by the considerably lower *TDH* expression rates in the mutants during ethanol stress compared to the wild-type strain, suggesting that *TDH* activity is higher in the ethanol-stressed mutant cells and there is less need for increased production of *TDH* enzymes (Figures 4.20 - 4.21). This is supported by previous studies that show high *TDH1* expression to be indicative of redox stress in the cell, with reduced *TDH1* expression reflecting lower redox stress levels *ie.* less NADH accumulation (Valadi *et al.,* 2004). Furthermore, all of the glycolysis-associated genes are less highly expressed in the mutants compared to the wild-type during ethanol stress, suggesting that glycolytic activity overall is higher in the mutants than the wild-type during stress. This may be a direct result of the higher *TDH1* activity in ethanol-stressed mutant cells (due to improved NAD^+ supply), which is conceivably a major bottleneck in glycolysis during ethanol stress.

Consistent with the notion of the mutants having increased glycolytic flux, is the increased expression levels of genes associated with sugar transport categories, such as mannose transmembrane transport (z-score 15.32), glucose transmembrane transport (z-score 13.96) and hexose transport (z-score 13.96) (Tables 4.3 and 4.5). The genes with increased expression levels in the sugar transport-related GO categories in the mutants are primarily high affinity glucose transporters *HXT2, 4* and *7,* and low affinity *HXT3.* Improved glucose import in the mutants is supported by the results presented in Chapter 3 demonstrating that in medium containing 20 g l^{-1} glucose, glucose utilization by both mutants is superior to the wild-type during ethanol stress (Figure 3.19).

Higher glycolytic activity in the mutants would improve cellular energetics, which is likely to stimulate functions and metabolic pathways that are energy demanding, but required if the inhibitory effects of ethanol are to be addressed. Two particular cell functions that meet this requirement are protein production and synthesis of components comprising the cell wall and membrane. It may, therefore, not be a coincidence that these two categories of cell function also are the most highly up-regulated of all categories in the mutants relative to the wild-type during ethanol stress. Protein production appears to be stimulated in the mutants compared to the wild-type, with higher expression levels of genes associated with categories such as transcription

activator activity (Table 4.3; z-score 3.73), cytosolic small ribosomal subunits (z-score 15.73), translation (z-score 11.31), rRNA processing (z-score 7.14) and nucleolus (z-score 7.09) (Tables 4.4 and also 4.8, Figure 4.23 and pathways presented in Appendix 2.3.1).

Another well-known response to ethanol stress is the alteration of membrane composition to restore membrane function that has been compromised by ethanol exposure (see Section 1.3.1.2). This activity also appears to be stimulated to a higher extent in the mutants compared to the wild-type with gene expression being higher in a number of GO categories related to membrane fraction, lipid biosynthesis, sterol biosynthesis (especially that related to ergosterol) (Tables 4.5-4.8), fatty acid biosynthesis (Table 4.3; z-score 9.32), nuclear membrane organization and biogenesis (z-score 7.43), and plasma membrane (z-score 3.43) (Table 4.5). The fact that the mutants do not repress the expression levels of genes in these energy-expensive categories during ethanol stress to the same extent as the wild-type strain supports the speculation that the mutants are more energy efficient under such stress.

Another consequence of increased glycolytic flux in the mutants during ethanol stress may be realised in receiving pathways, such as the fermentation pathway and TCA cycle. Both mutants produced more acetic acid than the wild-type during ethanol stress and these mutants also had higher expression levels of *ALD6* (aldehyde dehydrogenase) and *ACS2* (acetyl CoA sythetase) (Figures 4.20-4.21). The products of these genes oxidise acetaldehyde to acetate, and acetate to acetyl CoA, respectively. This suggests that during ethanol stress, the mutants, with their higher glycolytic flux, are able to increase acetyl CoA production, which is used as a substrate in the TCA cycle. This would be advantageous for an ethanol-stressed cell that needs TCA cycle intermediates to supply the demands of anabolic pathways such as those used for the synthesis of amino acids, sterols, fatty acids, purines and pyrimidines. In this respect, both mutants had higher *PUT3* expression levels compared to the wild-type in response to ethanol stress (Figure 4.13). *PUT3* is a transcriptional activator of proline utilisation genes (des Etages *et al.*, 1996; Marczak and Brandriss 1991) which, combined with an increase in proline catabolism genes (z-score 15.33; Tables 4.3 and 4.5), suggests that faster proline utilisation may occur in the mutants during ethanol stress. It has been suggested that proline has a stress protective role since recombinant sake strains designed to

accumulate proline showed increased ethanol tolerance (Takagi *et al.,* 2005) and in a separate study, proline enhanced freezing, oxidative and ethanol tolerance compared to the wild-type (Sekine *et al.,* 2007). The role of proline in ethanol stress has not been investigated but it may be related to its role in protein synthesis and as a nitrogen source when other sources are not available, such as occurs during ethanol stress (Brandriss and Magasanik 1981).

K-Means clustering of ISG allowed the selection of an additional two clusters of ISG specific for the SM1 (cluster 1) and CM1 (cluster 8) ethanol stress response. The SM1-specific cluster 1 is made-up of an additional 11 genes that are more highly expressed, by more than 4–fold, in SM1 than in the wild-type during ethanol stress (Table 4.6). Most of the SM1-specific genes however belong to the same categories that were observed to be commonly important for both strains, as described above. This supports the important roles of the processes and categories that were observed to respond to ethanol stress in both mutants (Tables 4.3, 4.5 and 4.6). This correlation of genes specific in individual mutants with genes specific for both mutants extends to CM1 specific cluster 8 of ISG (Table 4.7) with the exception of a few categories that are present only in this mutant, such as the up-regulation of high affinity zinc transport. The role of zinc as cofactor for a number of transcription factors may explain its role during ethanol stress.

Overall, this study has demonstrated that the ethanol tolerance of wild-type *S. cerevisiae* strains can be improved through an evolutionary engineering approach. The similarities in physiological, metabolic and transcriptional changes that occurred in the ethanol-tolerant mutants, which were generated via different mutational approaches (*viz.* spontaneous evolution and chemical mutagenesis), suggests that improvement in the activity of some key functions in an ethanol-stressed cell is critical if the cell is to increase its ethanol stress tolerance. According to this study, one key function may be the restoration of the NAD^+/NADH redox balance. The mutants appear to have achieved this by increasing glycerol metabolism as a means of facilitating an increased turnover of NADH. There is evidence in this study to suggest that this may impact on cell metabolism by stimulating glycolytic activity and, as a consequence, improve cellular energetics which could in turn stimulate the activity of energy-demanding ethanol stress response processes, although this would require confirmation.

CHAPTER 5

CONCLUSIONS AND FURTHER DIRECTIONS

The main objective of the present study was to create ethanol-tolerant mutants for the purpose of using them as tools to further investigate the complex mechanisms behind increased ethanol tolerance. The mutants were generated using an adaptive evolution approach on mutagenised and non-mutagenised *S. cerevisiae* W303-1A. Selected mutants showing significant increases in ethanol tolerance were metabolically profiled to determine differences in their central carbon metabolism. Microarray analysis was then performed to compare the transcriptional profiles of the wild-type and ethanol-tolerant mutants in the presence and absence of non-lethal ethanol stress. Gene ontology data-mining was then performed to identify areas of cell function that may be important in the cellular response to ethanol stress.

5.1 CONCLUSIONS

- Adaptive evolution of chemically mutagenised and non-mutagenised *S. cerevisiae* W303-1A was performed to create the stable ethanol-tolerant mutants, SM1 and CM1; ethanol was used as the selection pressure. These mutants were shown to have acquired significant improvements in their tolerance of non-lethal and lethal ethanol concentrations.

- Metabolic analysis showed that both mutants were better able to utilise glucose at 20 g l^{-1} (the glucose concentration in which they were generated) than the wild-type in the presence of a non-lethal ethanol stress. When the glucose concentration was increased to 100 g l^{-1}, SM1 utilised glucose at similar rates in the presence or absence of added ethanol, significantly outperforming the wild-type and CM1. CM1 struggled to use glucose at concentrations of 100 g l^{-1}, both in the presence and absence of ethanol stress, which may be a legacy of the chemical mutagenesis used to create the strain.

- Both mutants produced significantly more glycerol than the wild-type in presence or absence of ethanol stress. This common feature in the mutants appears to be associated with their acquired ethanol tolerance; possibly by increasing the NADH oxidation rate and thereby facilitating improved maintenance of the NAD^+/NADH redox balance.

- SM1 and CM1 responded to ethanol stress by increasing acetic acid production compared to the wild-type; acetic acid production by any strain was not detectable in the absence of ethanol stress. Although SM1 produced considerably more acetic acid compared to the wild-type, the increase in acetic acid production by CM1 was only marginally higher. This is important to note since it is generally believed that gains in glycerol production generally coincide with increases in acetic acid production.

- The microarray data presented in this thesis suggests, for the first time, a positive role for the mitochondrion in the ethanol stress response of *S. cerevisiae*.

- Both mutants had lower *TDH1* expression levels than the wild-type during ethanol stress. Elevated *TDH1* expression levels have been linked to increased redox stress in the cell (Valadi *et al.*, 2004), which suggests that during ethanol stress both SM1 and CM1 have lower levels of redox stress compared to the wild-type.

- The wild-type had considerably higher expression levels of genes associated with glycolysis compared to either mutant during ethanol stress. This suggests that during ethanol stress the wild-type was attempting to increase glycolytic activity to a greater extent than either mutant. The potentially lower redox stress in the mutants may account for this outcome.

- The higher expression levels of glucose transporters in both mutants compared to the wild-type during ethanol stress is consistent with their higher glucose uptake rates (in 20 g l^{-1} glucose) in the presence of ethanol.

- During ethanol stress and compared to the wild-type strain, the mutants had increased expression levels of genes associated with the following areas of cell function: cell wall, membrane, mitochondrial respiration, pH maintenance, transcription and translation, fatty acid and sterol biosynthesis. Given the earlier conclusions, it may be that the predicted higher glycolytic activity of the mutants during ethanol stress may improve cellular energetics, thus potentially stimulating activity of the above energy-demanding cell functions.

- During ethanol stress, the mutants reduced the expression levels of genes associated with transcription and translation to a lesser degree than the wild-type, indicating that the need to conserve energy during stress was less critical in the mutants compared to the wild-type.

- It can be speculated from the transcriptional data during ethanol stress, that higher vitamin biosynthesis, especially B group vitamins, could be beneficial for improving ethanol stress tolerance.

- This study, for the first time, indicates increased expression of retrotransposon genes in yeast during ethanol stress. Compared to the wild-type, the mutants had reduced lower expression levels of retrotransposon genes during ethanol stress.

5.2 FURTHER DIRECTIONS

The present study provided insights into ethanol-tolerance, but also raised a number of questions. The following are some suggestions for further research based on the current findings.

- This project demonstrated that SM1 and CM1 respond to, and tolerate, ethanol stress considerably better than the wild-type. It is possible that the mechanisms responsible for the improved ethanol stress tolerance of SM1 and CM1 may also improve the tolerance of these strains to a range of other stressors. This could be further investigated by using physiology-based experiments to determine the

stress response of these strains to other environmental stressors such as temperature shock, freezing-thawing, extreme pH, oxidative and osmotic stress.

- There is evidence showing that the transcriptional response of yeast to ethanol stress changes over time (Chandler *et al.*, 2004; Kubota *et al.*, 2004). Thus, it would be of interest to follow and compare the transcriptional responses of the wild-type, SM1 and CM1 as they acclimatise to ethanol stress over a time-course.

- Although metabolic profiles of the three strains were performed during growth on 20 g l^{-1} and 100 g l^{-1} of glucose, the amount of ethanol produced from these glucose levels was insufficient to determine whether the mutants could produce higher ethanol yields compared to the wild-type. Cultivations on higher glucose concentrations *ca.* 300 g l^{-1}, should be performed to determine if, compared to the wild-type, SM1 and CM1 have higher ethanol productivities in later stages of fermentation and can produce higher ethanol yields.

- The adaptive evolution approach used in this study to generate ethanol-tolerant mutants should be applied to the generation of ethanol-tolerant industrial yeast strains, such as those used in wine and beer industries. This approach to increasing ethanol tolerance could also be applied to non-yeast ethanologenic strains, such as *Zymomonas mobilis* and the ethanol-producing *E. coli* KO11. If this approach improves the ethanol tolerance of the latter strains, their prokaryotic metabolism may provide some interesting insights on how other microorganisms can alter cell function to improve ethanol tolerance.

- The increased glycerol production and lower *TDH1* expression levels in the mutants during ethanol stress suggests that the NAD^+/NADH redox balance may have an important role in ethanol stress response and ethanol tolerance. This could be further investigated by determining the concentrations of key intermediates in, and cofactors of, central metabolism of the wild-type, CM1 and SM1 during ethanol stress. Some recommended intermediates and cofactors include glyceraldehyde-3-phosphate, 3-phosphoglycerate, dihydroxyacetone

phosphate, Fructose-1,6-diphosphate, pyruvate, acetaldehyde, NAD^+, NADH, ADP and ATP.

- With reference to the preceding comments, the importance of maintaining redox balance in ethanol-stressed yeast could also be explored by using a genetic engineering approach for increasing NADH oxidation. Such genetically modified strains have already been constructed for various studies, but not at this stage for investigations into ethanol-stress tolerance. The following strains, or similar constructs, could be tested to determine their ethanol stress tolerance compared to the wild-type: an *S. cerevisiae* overexpressing an NADH oxidase encoded by the *Streptococcus pneumoniae NOX* gene (Vemuri *et al.*, 2007) and and an *S. cerevisiae* overexpressing *GPD1* (De Barros Lopes *et al.*, 2000)

- On a slightly different note, reducing ethanol production in some wine strains is of particular interest to the wine industry. Attempts at achieving this by increasing glycerol production in wine strains have inevitably resulted in an undesirable increase in acetic acid production. The increased glycerol production by CM1 without a substantial increase in acetic acid production makes this strain a valuable tool for determining how increased glycerol production may be achieved without causing significant increases in acetic acid production.

- The role of vitamins, especially thiamine, in response to ethanol stress is another area worthy of further investigation. The role of thiamine in overcoming the detrimental effects of ethanol toxicity in humans is well known, however, there is no evidence, other than that in the current study, to suggest a role for thiamine and other vitamins in yeast ethanol stress tolerance. Interestingly, traditional winemakers have been adding thiamine to wine fermentations to prevent stuck or sluggish fermentations.

- Genetic and molecular analysis should be carried out to identify and characterise genes that confer ethanol tolerance in SM1 and CM1. Approaches to be used might include:

  a) Backcrossing mutants to the parent strain and following segregation of the ethanol-tolerance phenotype in meiotic progeny to determine the number of genes involved and whether or not they are dominant.

  b) Identifying 'ethanol-tolerance mutations' by:

     i) Performing a karyotype analysis of the mutants (using CHEF gels) to determine whether the mutants have chromosomal mutations

     ii) Screening expression libraries of the mutants in the parental strain background to isolate genes that confer ethanol tolerance

     iii) Performing SNP analysis (as described in Gresham *et al.,* 2006, Schacherer *et al.,* 2007 or Perkel, 2008) to identify mutations in CM1 and SM1. It is likely that there will be many incidental mutations, particularly in the chemically induced mutant, that do not contribute to an ethanol-tolerance phenotype. To identify those that do, all mutations would be systematically introduced into the parent strain, and tested for their capacity to confer ethanol-tolerance.

Depending on results from the above and availability of funding, the genomes CM1 and SM1 could be sequenced to identify mutations. Mutations in CM1 and SM1 could then be systematically introduced into the parent strain and tested for their ability to confer ethanol-tolerance. Whilst this approach might seem somewhat extravagant, costs of DNA sequencing will soon reach a level that will make whole genome sequencing a real option for many laboratories.

REFERENCES

Aarnio, T.H., Suihko, M.L. and Kauppinen, V.S. 1991, 'Isolation of acetic acid tolerant baker's yeast variants in a turbidostat', *Appl. Biochem. Biotechnol.*, vol. 27, pp. 55-63.

Agabio, R. 2005, 'Thiamine administration in alcohol-dependent patients', *Alcohol Alcohol*, vol. 40, no. 2, pp. 155-6.

Aguilera, A. and Benitez, T. 1985, 'Role of mitochondria in ethanol tolerance of *Saccharomyces cerevisiae*', *Arch Microbiol*, vol. 142, no. 4, pp. 389-92.

Aguilera, A. and Benitez, T. 1986, 'Ethanol-sensitive mutants of *Saccharomyces cerevisiae*', *Arch Microbiol*, vol. 143, no. 4, pp. 337-44.

Aguilera, F., Peinado, R.A., Millan, C., Ortega, J.M. and Mauricio, J.C. 2006, 'Relationship between ethanol tolerance, H+ -ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains', *Int J Food Microbiol*, vol. 110, no. 1, pp. 34-42.

Albers, E., Gustafsson, L., Niklasson, C. and Liden, G. 1998, 'Distribution of 14C-labelled carbon from glucose and glutamate during anaerobic growth of *Saccharomyces cerevisiae*', *Microbiology*, vol. 144 (Pt 6), pp. 1683-90.

Alexandre, H., Rousseaux, I. and Charpentier, C. 1994a, 'Ethanol adaptation mechanisms in *Saccharomyces cerevisiae*', *Biotechnol Appl Biochem*, vol. 20 (Pt 2), pp. 173-83.

Alexandre, H., Rousseaux, I. and Charpentier, C. 1994b, 'Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in *Saccharomyces cerevisiae* and Kloeckera apiculata', *FEMS Microbiol Lett*, vol. 124, no. 1, pp. 17-22.

Alexandre, H., Plourde, L., Charpentier, C. and Francois, J. 1998, 'Lack of correlation between trehalose accumulation, cell viability and intracellular acidification as induced by various stresses in *Saccharomyces cerevisiae*', *Microbiology*, vol. 144 (Pt 4), pp. 1103-11.

Alexandre, H., Ansanay-Galeote, V., Dequin, S. and Blondin, B. 2001, 'Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*', *FEBS Lett*, vol. 498, no. 1, pp. 98-103.

Allison, S.D., Chang, B., Randolph, T.W. and Carpenter, J.F. 1999, 'Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding', *Arch Biochem Biophys*, vol. 365, no. 2, pp. 289-98.

Alper, H., Moxley, J., Nevoigt, E., Fink, G.R. and Stephanopoulos, G. 2006, 'Engineering yeast transcription machinery for improved ethanol tolerance and production', *Science*, vol. 314, no. 5805, pp. 1565-8.

Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M. and Adler, L. 1997, 'The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation', *Embo J*, vol. 16, no. 9, pp. 2179-87.

Arami, S., Hada, M. and Tada, M. 1997, 'Reduction of ATPase activity accompanied by photodecomposition of ergosterol by near-UV irradiation in plasma membranes prepared from *Saccharomyces cerevisiae*', *Microbiology*, vol. 143 (Pt 7), pp. 2465-71.

Attfield, P.V. 1987, 'Trehalose accumulates in *Saccharomyces cerevisiae* during exposure to agents that induce heat shock response', *FEBS Lett*, vol. 225, no. 1-2, pp. 259-63.

Attfield, P.V. 1997, 'Stress tolerance: the key to effective strains of industrial baker's yeast', *Nat Biotechnol*, vol. 15, no. 13, pp. 1351-7.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1997, *Current Protocols in Molecular Biology*, John Wiley & Sons Inc.

Ba, A., Seri, B.V. and Han, S.H. 1996, 'Thiamine administration during chronic alcohol intake in pregnant and lactating rats: effects on the offspring neurobehavioural development', *Alcohol Alcohol*, vol. 31, no. 1, pp. 27-40.

Bakker, B.M., Bro, C., Kotter, P., Luttik, M.A., van Dijken, J.P. and Pronk, J.T. 2000, 'The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in *Saccharomyces cerevisiae*', *J Bacteriol*, vol. 182, no. 17, pp. 4730-7.

Bakker, B.M., Overkamp, K.M., van Maris, A.J., Kotter, P., Luttik, M.A., van Dijken, J.P. and Pronk, J.T. 2001, 'Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*', *FEMS Microbiol Rev*, vol. 25, no. 1, pp. 15-37.

Balakrishnan, R., Christie, K.R., Costanzo, M.C., Dolinski, K., Dwight, S.S., Engel, S.R., Fisk, D.G., Hirschman, J.E., Hong, E.L., Nash, R., Oughtred, R., Skrzypek, M., Theesfeld, C.L., Binkley, G., Dong, Q., Lane, C., Sethuraman, A., Weng, S., Botstein, D. and Cherry, J.M. 2005, 'Fungal BLAST and Model Organism BLASTP Best Hits: new comparison resources at the Saccharomyces Genome Database (SGD)', *Nucleic Acids Res*, vol. 33, no. Database issue, pp. D374-7.

Barber, A.R., Vrieskoop, F. and Pamment, N.B. 2002, 'Effect of acetaldehyde on *Saccharomyces cerevisiae* exposed to range of chemical and environmental stresses.' *Enzyme and Microbial Technology*, vol. 30, pp. 240-50.

Barbour, L., Hanna, M. and Xiao, W. 2006, ' Mutagenesis', in *Methods in Molecular Biology; Yeast Protocol, Second Edition*, Humana Press, vol. 313, pp. 121-7.

Bataillon, M., Rico, A., Sablayrolles, J.M., Salmon, J.M. and Barre, P. 1996, 'Early thiamin assimilation by yeast under enological conditions: Impact on alcoholic fermentation kinetics.' *Journal of Fermentation and Bioengineering*, vol. 82, no. 2, pp. 145-50.

Baxter, B.K., James, P., Evans, T. and Craig, E.A. 1996, 'SSI1 encodes a novel Hsp70 of the *Saccharomyces cerevisiae* endoplasmic reticulum', *Mol Cell Biol*, vol. 16, no. 11, pp. 6444-56.

Beaven, M., Charpentier, C. and Rose, A.H. 1982, 'Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. ' *J Gen Microbiol*, vol. 128, pp. 1447-55.

Becker, J. and Craig, E.A. 1994, 'Heat-shock proteins as molecular chaperones', *Eur J Biochem*, vol. 219, no. 1-2, pp. 11-23.

Belazzi, T., Wagner, A., Wieser, R., Schanz, M., Adam, G., Hartig, A. and Ruis, H. 1991, 'Negative regulation of transcription of the *Saccharomyces cerevisiae* catalase T (CTT1) gene by cAMP is mediated by a positive control element', *Embo J*, vol. 10, no. 3, pp. 585-92.

Belenky, P., Bogan, K.L. and Brenner, C. 2007, 'NAD+ metabolism in health and disease', *Trends Biochem Sci*, vol. 32, no. 1, pp. 12-9.

Berger, J.A., Hautaniemi, S., Jarvinen, A.K., Edgren, H., Mitra, S.K. and Astola, J. 2004, 'Optimized Lowess normalisation parameter selection for DNA microarray data.' *BMC Bioinformatics*, vol. 5.

Betz, A. and Becker, J.-U. 1975, 'Metabolite Concentrations and Phase Relations in d-Fructose Induced Glycolytic Oscillations.' *Physiologia Plantarum*, vol. 33, no. 4, pp. 285-9.

Birch, R.M. and Walker, G.M. 2000, 'Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*', *Enzyme Microb Technol*, vol. 26, no. 9-10, pp. 678-87.

Bjorkqvist, S., Ansell, R., Adler, L. and Liden, G. 1997, 'Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*', *Appl Environ Microbiol*, vol. 63, no. 1, pp. 128-32.

Bose, S., Dutko, J.A. and Zitomer, R.S. 2005, 'Genetic factors that regulate the attenuation of the general stress response of yeast', *Genetics*, vol. 169, no. 3, pp. 1215-26.

Bracken, A.P. and Bond, U. 1999, 'Reassembly and protection of small nuclear ribonucleoprotein particles by heat shock proteins in yeast cells', *Rna*, vol. 5, no. 12, pp. 1586-96.

Bradshaw, V.A. and McEntee, K. 1989, 'DNA damage activates transcription and transposition of yeast Ty retrotransposons', *Mol Gen Genet*, vol. 218, no. 3, pp. 465-74.

Brandriss, M.C. and Magasanik, B. 1981, 'Subcellular compartmentation in control of converging pathways for proline and arginine metabolism in *Saccharomyces cerevisiae*', *J Bacteriol*, vol. 145, no. 3, pp. 1359-64.

Brisson, D., Vohl, M.C., St-Pierre, J., Hudson, T.J. and Gaudet, D. 2001, 'Glycerol: a neglected variable in metabolic processes?' *Bioessays*, vol. 23, no. 6, pp. 534-42.

Brown, S.W. and Oliver, S.G. 1982, 'Isolation of ethanol-tolerant mutants of yeast by continuous selection', *Eur. J. Appl. Microbiol. Biotechnol.*, vol. 16, pp. 119-22.

Bukau, B. and Horwich, A.L. 1998, 'The Hsp70 and Hsp60 chaperone machines', *Cell*, vol. 92, no. 3, pp. 351-66.

Bulman, A.L. and Nelson, H.C. 2005, 'Role of trehalose and heat in the structure of the C-terminal activation domain of the heat shock transcription factor', *Proteins*, vol. 58, no. 4, pp. 826-35.

Bulman, A.L., Hubl, S.T. and Nelson, H.C. 2001, 'The DNA-binding domain of yeast heat shock transcription factor independently regulates both the N- and C-terminal activation domains', *J Biol Chem*, vol. 276, no. 43, pp. 40254-62.

Burnie, J.P., Carter, T.L., Hodgetts, S.J. and Matthews, R.C. 2006, 'Fungal heat-shock proteins in human disease', *FEMS Microbiol Rev*, vol. 30, no. 1, pp. 53-88.

Buttke, T.M., Jones, S.D. and Bloch, K. 1980, 'Effect of sterol side chains on growth and membrane fatty acid composition of *Saccharomyces cerevisiae*', *J Bacteriol*, vol. 144, no. 1, pp. 124-30.

Cakar, Z.P., Seker, U.O., Tamerler, C., Sonderegger, M. and Sauer, U. 2005, 'Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*', *FEMS Yeast Res*, vol. 5, no. 6-7, pp. 569-78.

Cartwright, C.P., Veazey, F.J. and Rose, A.H. 1987, 'Effect of ethanol on activity of the plasma-membrane ATPase in, and accumulation of glycine by, *Saccharomyces cerevisiae*', *J Gen Microbiol*, vol. 133, no. 4, pp. 857-65.

Carvalheiro, F., Roseiro, J.C. and Girio, F.M. 1999, 'Interactive effects of sodium chloride and heat shock on trehalose accumulation and glycerol production by *Saccharomyces cerevisiae*', *Food Microbiol*, vol. 16, pp. 543-50.

Cashikar, A.G., Duennwald, M. and Lindquist, S.L. 2005, 'A chaperone pathway in protein disaggregation. Hsp26 alters the nature of protein aggregates to facilitate reactivation by Hsp104', *J Biol Chem*, vol. 280, no. 25, pp. 23869-75.

Castillo Agudo, L. 1992, 'Lipid content of *Saccharomyces cerevisiae* strains with different degres of ethanol tolerance', *Appl Microbiol Biotechnol*, vol. 37, pp. 647-51.

Chandler, M., Stanley, G.A., Rogers, P. and Chambers, P. 2004, 'A genomic approach to defining the ethanol stress response in the yeast *Saccharomyces cerevisiae.* ' *Annals of Microbiology*, vol. 54, no. 4, pp. 427-54.

Chernoff, Y.O., Lindquist, S.L., Ono, B., Inge-Vechtomov, S.G. and Liebman, S.W. 1995, 'Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]', *Science*, vol. 268, no. 5212, pp. 880-4.

Cohen, B.A., Pilpel, Y., Mitra, R.D. and Church, G.M. 2002, 'Discrimination between paralogs using microarray analysis: application to the Yap1p and Yap2p transcriptional networks', *Mol Biol Cell*, vol. 13, no. 5, pp. 1608-14.

Collinson, L.P. and Dawes, I.W. 1992, 'Inducibility of the response of yeast cells to peroxide stress', *J Gen Microbiol*, vol. 138, no. 2, pp. 329-35.

Coote, P.J., Cole, M.B. and Jones, M.V. 1991, 'Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH', *J Gen Microbiol*, vol. 137, no. 7, pp. 1701-8.

Costa, V., Reis, E., Quintanilha, A. and Moradas-Ferreira, P. 1993, 'Acquisition of ethanol tolerance in *Saccharomyces cerevisiae*: the key role of the mitochondrial superoxide dismutase', *Arch Biochem Biophys*, vol. 300, no. 2, pp. 608-14.

Costa, V., Amorim, M.A., Reis, E., Quintanilha, A. and Moradas-Ferreira, P. 1997, 'Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase', *Microbiology*, vol. 143 (Pt 5), pp. 1649-56.

Crabeel, M., Lavalle, R. and Glansdorff, N. 1990, 'Arginine-specific repression in *Saccharomyces cerevisiae*: kinetic data on ARG1 and ARG3 mRNA transcription and stability support a transcriptional control mechanism', *Mol Cell Biol*, vol. 10, no. 3, pp. 1226-33.

Crowe, J.H., Crowe, L.M. and Chapman, D. 1984, 'Preservation of Membranes in Anhydrobiotic Organisms: The Role of Trehalose', *Science*, vol. 223, no. 4637, pp. 701-3.

Crowe, J.H., Carpenter, J.F. and Crowe, L.M. 1998, 'The role of vitrification in anhydrobiosis', *Annu Rev Physiol*, vol. 60, pp. 73-103.

Crowe, J.H., Whittam, M.A., Chapman, D. and Crowe, L.M. 1984, 'Interactions of phospholipid monolayers with carbohydrates', *Biochim Biophys Acta*, vol. 769, no. 1, pp. 151-9.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Wistrom, C.A., Spargo, B.J. and Anchordoguy, T.J. 1988, 'Interactions of sugars with membranes', *Biochim Biophys Acta*, vol. 947, no. 2, pp. 367-84.

Crowe, L.M. and Crowe, J.H. 1992, 'Anhydrobiosis: a strategy for survival', *Adv Space Res*, vol. 12, no. 4, pp. 239-47.

Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A. and Womersley, C. 1984, 'Effects of carbohydrates on membrane stability at low water activities', *Biochim Biophys Acta*, vol. 769, no. 1, pp. 141-50.

D'Amore, T., Crumplen, R. and Stewart, G. 1991, 'The involvement of trehalose in yeast stress tolerance. ' *J Ind Microbiol*, vol. 7, pp. 191-6.

D'Amore, T., Panchal, C.J., Russell, I. and Stewart, G.G. 1990, 'A study of ethanol tolerance in yeast', *Crit Rev Biotechnol*, vol. 9, no. 4, pp. 287-304.

Dasari, G., Worth, M.A., Connor, M.A. and Pamment, N.B. 1990, 'Reasons for the apparent difference in the effects of produced and added ethanol on culture viability during rapid fermentation by *Saccharomyces cerevisiae*', *Biotechnol Bioeng*, vol. 35, no. 2, pp. 109-22.

Day, E., Bentham, P., Callaghan, R., Kuruvilla, T. and George, S. 2004, 'Thiamine for Wernicke-Korsakoff Syndrome in people at risk from alcohol abuse', *Cochrane Database Syst Rev*, no. 1, p. CD004033.

De Barros Lopes, M., Rehman, A.-U., Gockowiak, H., Heinrich, A.J., Langridge, P. and Henschke, P.A. 2000, 'Fermentation properties of a wine yeast overexpressing the *Saccharomyces cerevisiae* glycerol 3-phosphate dehydrogenase gene (GPD2).' *Aust J Grape Wine Res*, vol. 6, pp. 208-15.

de Carvalho Lima, K.G., Takahashi, C.M. and Alterthum, F. 2002, 'Ethanol production from corn cob hydrolysates by Escherichia coli KO11', *J Ind Microbiol Biotechnol*, vol. 29, no. 3, pp. 124-8.

De Risi, J.L., Iyer, V. and Brown, P. 1997, 'Exploring the metabolic and genetic control of gene expression on a genomic scale. ' *Science*, vol. 278, pp. 680-6.

De Virgilio, C., Hottiger, T., Dominguez, J., Boller, T. and Wiemken, A. 1994, 'The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant', *Eur J Biochem*, vol. 219, no. 1-2, pp. 179-86.

de Vries, S. and Grivell, L.A. 1988, 'Purification and characterization of a rotenone-insensitive NADH:Q6 oxidoreductase from mitochondria of *Saccharomyces cerevisiae*', *Eur J Biochem*, vol. 176, no. 2, pp. 377-84.

De Wever, V., Reiter, W., Ballarini, A., Ammerer, G. and Brocard, C. 2005, 'A dual role for PP1 in shaping the Msn2-dependent transcriptional response to glucose starvation', *Embo J*, vol. 24, no. 23, pp. 4115-23.

des Etages, S.A., Falvey, D.A., Reece, R.J. and Brandriss, M.C. 1996, 'Functional analysis of the PUT3 transcriptional activator of the proline utilization pathway in *Saccharomyces cerevisiae*', *Genetics*, vol. 142, no. 4, pp. 1069-82.

Doniger, S.W., Salomonis, N., Dahlquist, K.D., Vranizan, K., Lawor, S.C. and Conklin, B.R. 2003, 'MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data', *Genome Biol*, vol. 4, no. R7.

Dumond, H., Danielou, N., Pinto, M. and Bolotin-Fukuhara, M. 2000, 'A large-scale study of Yap1p-dependent genes in normal aerobic and H2O2-stress conditions: the role of Yap1p in cell proliferation control in yeast', *Mol Microbiol*, vol. 36, no. 4, pp. 830-45.

Durchschlag, E., Reiter, W., Ammerer, G. and Schuller, C. 2004, 'Nuclear localization destabilizes the stress-regulated transcription factor Msn2', *J Biol Chem*, vol. 279, no. 53, pp. 55425-32.

Eastmond, D.L. and Nelson, H.C. 2006, 'Genome-wide analysis reveals new roles for the activation domains of the *Saccharomyces cerevisiae* heat shock transcription factor (Hsf1) during the transient heat shock response', *J Biol Chem*, vol. 281, no. 43, pp. 32909-21.

Eleutherio, E.C., Araujo, P.S. and Panek, A.D. 1993, 'Protective role of trehalose during heat stress in *Saccharomyces cerevisiae*', *Cryobiology*, vol. 30, no. 6, pp. 591-6.

Eleutherio, E.C., Ribeiro, M.J., Pereira, M.D., Maia, F.M. and Panek, A.D. 1995, 'Effect of trehalose during stress in a heat-shock resistant mutant of *Saccharomyces cerevisiae*', *Biochem Mol Biol Int*, vol. 36, no. 6, pp. 1217-23.

Elliott, B., Haltiwanger, R.S. and Futcher, B. 1996, 'Synergy between trehalose and Hsp104 for thermotolerance in *Saccharomyces cerevisiae*', *Genetics*, vol. 144, no. 3, pp. 923-33.

Entian, K.D. and Barnett, J.A. 1992, 'Regulation of sugar utilization by *Saccharomyces cerevisiae*', *Trends Biochem Sci*, vol. 17, no. 12, pp. 506-10.

Estruch, F. 2000, 'Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast', *FEMS Microbiol Rev*, vol. 24, no. 4, pp. 469-86.

Estruch, F. and Carlson, M. 1993, 'Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*', *Mol Cell Biol*, vol. 13, no. 7, pp. 3872-81.

Faber-Barata, J. and Sola-Penna, M. 2005, 'Opposing effects of two osmolytes--trehalose and glycerol--on thermal inactivation of rabbit muscle 6-phosphofructo-1-kinase', *Mol Cell Biochem*, vol. 269, no. 1-2, pp. 203-7.

Feder, M.E., Parsell, D.A. and Lindquist, S. 1995, 'The stress response and stress proteins', *Cell Biology of Trauma*, vol. Boca Raton: CRC, pp. 177-91.

Fernandes, L., Rodrigues-Pousada, C. and Struhl, K. 1997, 'Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions', *Mol Cell Biol*, vol. 17, no. 12, pp. 6982-93.

Ferreira, C., van Voorst, F., Martins, A., Neves, L., Oliveira, R., Kielland-Brandt, M.C., Lucas, C. and Brandt, A. 2005, 'A member of the sugar transporter family, Stl1p is the glycerol/H+ symporter in *Saccharomyces cerevisiae*', *Mol Biol Cell*, vol. 16, no. 4, pp. 2068-76.

Fink, G.R. 1986, 'Translational control of transcription in eukaryotes', *Cell*, vol. 45, no. 2, pp. 155-6.

Folch-Mallol, J.L., Garay-Arroyo, A., Lledias, F. and Covarrubias Robles, A.A. 2004, '[The stress response in the yeast *Saccharomyces cerevisiae*]', *Rev Latinoam Microbiol*, vol. 46, no. 1-2, pp. 24-46.

Frankenburg, F.R. 2008, 'Alcohol use, thiamine deficiency, and cognitive impairment', *Jama*, vol. 299, no. 24, pp. 2854; author reply -5.

Franks, F., Hatley, R.H.M. and Mathias, S.F. 1991, 'Materials, science and the production of shelf stable biologicals', *Biopharm.-Technol.*, vol. 4, p. 38.

Fujita, K., Matsuyama, A., Kobayashi, Y. and Iwahashi, H. 2004, 'Comprehensive gene expression analysis of the response to straight-chain alcohols in *Saccharomyces cerevisiae* using cDNA microarray', *J Appl Microbiol*, vol. 97, no. 1, pp. 57-67.

Fujita, K., Matsuyama, A., Kobayashi, Y. and Iwahashi, H. 2006, 'The genome-wide screening of yeast deletion mutants to identify the genes required for tolerance to ethanol and other alcohols', *FEMS Yeast Res*, vol. 6, no. 5, pp. 744-50.

Gancedo, J.M. 1992, 'Carbon catabolite repression in yeast', *Eur J Biochem*, vol. 206, no. 2, pp. 297-313.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O. 2000, 'Genomic expression programs in the response of yeast cells to environmental changes', *Mol Biol Cell*, vol. 11, no. 12, pp. 4241-57.

Glover, J.R. and Lindquist, S. 1998, 'Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins', *Cell*, vol. 94, no. 1, pp. 73-82.

Gorner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H. and Schuller, C. 1998, 'Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity', *Genes Dev*, vol. 12, no. 4, pp. 586-97.

Green, J.L. and Angell, C.A. 1989, 'Phase relations and vitrification of saccharide-water solutions and the trehalose anomaly', *J. Phys. Chem.*, vol. 93, pp. 2880-2.

Gresham, D., Ruderfer, D.M., Pratt, S.C., Schacherer, J., Dunham, M.J., Botstein, D. and Kruglyak, L. 2006, 'Genome-wide detection of polymorphisms at nucleotide resolution with a single DNA microarray', *Science*, vol. 311, no. 5769, pp. 1932-6.

Gulshan, K., Rovinsky, S.A., Coleman, S.T. and Moye-Rowley, W.S. 2005, 'Oxidant-specific folding of Yap1p regulates both transcriptional activation and nuclear localization', *J Biol Chem*, vol. 280, no. 49, pp. 40524-33.

Gupta, S., Sharma, S.C. and Singh, B. 1994, 'Changes in the composition and peroxidation of yeast membrane lipids during ethanol stress', *Acta Microbiol Immunol Hung*, vol. 41, no. 2, pp. 197-204.

Hahn, J.S., Hu, Z., Thiele, D.J. and Iyer, V.R. 2004, 'Genome-wide analysis of the biology of stress responses through heat shock transcription factor', *Mol Cell Biol*, vol. 24, no. 12, pp. 5249-56.

Hallsworth, J.E., Nomura, Y. and Iwahara, M. 1998, 'Ethanol-induced water stress and fungal growth', *Journal of Fermentation and Bioengineering*, vol. 86, pp. 451-6.

Hanninen, A.L., Simola, M., Saris, N. and Makarow, M. 1999, 'The cytoplasmic chaperone hsp104 is required for conformational repair of heat-denatured proteins in the yeast endoplasmic reticulum', *Mol Biol Cell*, vol. 10, no. 11, pp. 3623-32.

Haslbeck, M. and Buchner, J. 2002, 'Chaperone function of sHsps', *Prog Mol Subcell Biol*, vol. 28, pp. 37-59.

Haslbeck, M., Braun, N., Stromer, T., Richter, B., Model, N., Weinkauf, S. and Buchner, J. 2004, 'Hsp42 is the general small heat shock protein in the cytosol of *Saccharomyces cerevisiae*', *Embo J*, vol. 23, no. 3, pp. 638-49.

Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H.E., Chen, S., Saibil, H.R. and Buchner, J. 1999, 'Hsp26: a temperature-regulated chaperone', *Embo J*, vol. 18, no. 23, pp. 6744-51.

Hazell, B.W., Nevalainen, H. and Attfield, P.V. 1995, 'Evidence that the *Saccharomyces cerevisiae* CIF1 (GGS1/TPS1) gene modulates heat shock response positively', *FEBS Lett*, vol. 377, no. 3, pp. 457-60.

He, X.J. and Fassler, J.S. 2005, 'Identification of novel Yap1p and Skn7p binding sites involved in the oxidative stress response of *Saccharomyces cerevisiae*', *Mol Microbiol*, vol. 58, no. 5, pp. 1454-67.

Henle, K., Nagle, W., Moss, A. and Herman, T.S. 1982, 'Polyhydroxy compounds and thermotolerance: a proposed concatenation. ' *Radiat. Res.*, vol. 92, pp. 445-51.

Herman, P.K. and Emr, S.D. 1990, 'Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae*', *Mol Cell Biol*, vol. 10, no. 12, pp. 6742-54.

Hirasawa, T., Yoshikawa, K., Nakakura, Y., Nagahisa, K., Furusawa, C., Katakura, Y., Shimizu, H. and Shioya, S. 2007, 'Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis', *J Biotechnol*, vol. 131, no. 1, pp. 34-44.

Holbrook, N.J. and Udelsman, R. 1994, 'Heat shock protein gene expression in response to physiologic stresses and aging', *The Biology of Heat Shock Proteins and Molecular Chaperones*, pp. 577-93.

Hottiger, T., Boller, T. and Wiemken, A. 1989, 'Correlation of trehalose content and heat resistance in yeast mutants altered in the RAS/adenylate cyclase pathway: is trehalose a thermoprotectant?' *FEBS Lett*, vol. 255, no. 2, pp. 431-4.

Hottiger, T., De Virgilio, C., Hall, M.N., Boller, T. and Wiemken, A. 1994, 'The role of trehalose synthesis for the acquisition of thermotolerance in yeast. II. Physiological concentrations of trehalose increase the thermal stability of proteins in vitro', *Eur J Biochem*, vol. 219, no. 1-2, pp. 187-93.

Hounsa, C.G., Brandt, E.V., Thevelein, J., Hohmann, S. and Prior, B.A. 1998, 'Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress', *Microbiology*, vol. 144 (Pt 3), pp. 671-80.

Hu, C.K., Bai, F.W. and An, L.J. 2003a, 'Enhancements in ethanol tolerance of a self-flocculating yeast by calcium ion through decrease in plasmalemma permeability', *Sheng Wu Gong Cheng Xue Bao*, vol. 19, no. 6, pp. 715-9.

Hu, C.K., Bai, F.W. and An, L.J. 2003b, 'Enhancing ethanol tolerance of a self-flocculating fusant of Schizosaccharomyces pombe and *Saccharomyces cerevisiae* by Mg2+ via reduction in plasma membrane permeability', *Biotechnol Lett*, vol. 25, no. 14, pp. 1191-4.

Ibeas, J.I. and Jimenez, J. 1997, 'Mitochondrial DNA loss caused by ethanol in Saccharomyces flor yeasts', *Appl Environ Microbiol*, vol. 63, no. 1, pp. 7-12.

Ikner, A. and Shiozaki, K. 2005, 'Yeast signaling pathways in the oxidative stress response', *Mutat Res*, vol. 569, no. 1-2, pp. 13-27.

Imazu, H. and Sakurai, H. 2005, '*Saccharomyces cerevisiae* heat shock transcription factor regulates cell wall remodeling in response to heat shock', *Eukaryot Cell*, vol. 4, no. 6, pp. 1050-6.

Ingram, L.O. 1986, 'Microbiol tolerance to alcohols: Role of the cell membrane', *Trends Biotechnol*, vol. 4, pp. 40-4.

Iwahashi, H., Nwaka, S., Obuchi, K. and Komatsu, Y. 1998, 'Evidence for the interplay between trehalose metabolism and Hsp104 in yeast', *Appl Environ Microbiol*, vol. 64, no. 11, pp. 4614-7.

Iwahashi, H., Kitagawa, E., Suzuki, Y., Ueda, Y., Ishizawa, Y.H., Nobumasa, H., Kuboki, Y., Hosoda, H. and Iwahashi, Y. 2007, 'Evaluation of toxicity of the mycotoxin citrinin using yeast ORF DNA microarray and Oligo DNA microarray', *BMC Genomics*, vol. 8, p. 95.

Jacquet, M., Renault, G., Lallet, S., De Mey, J. and Goldbeter, A. 2003, 'Oscillatory behavior of the nuclear localization of the transcription factors Msn2 and Msn4 in response to stress in yeast', *ScientificWorldJournal*, vol. 3, pp. 609-12.

Jakobsen, B.K. and Pelham, H.R. 1991, 'A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor', *Embo J*, vol. 10, no. 2, pp. 369-75.

Jimenez, J. and Benitez, T. 1988, *Selection of Ethanol-Tolerant Yeast Hybrids in pH-Regulated Continuous Culture*, 4, April 1, 1988, <http://aem.asm.org/cgi/content/abstract/54/4/917 >.

Johannson, A., Keightley, C.A., Smith, G.A., Richard, C.D., Hesketh, T.R. and Metcalf, J.C. 1981, 'The effect of bilayer thickness and n-alcanes on activity of the Ca^{+} and Mg^{++} dependant ATPase of the sarcoplasmic reticulum.' *Journal of Biological Chemistry*, vol. 256, pp. 1643-50.

Jones, D.L., Petty, J., Hoyle, D.C., Hayes, A., Ragni, E., Popolo, L., Oliver, S.G. and Stateva, L.I. 2003, 'Transcriptome profiling of a *Saccharomyces cerevisiae* mutant with a constitutively activated Ras/cAMP pathway', *Physiol Genomics*, vol. 16, no. 1, pp. 107-18.

Jones, R.P. and Greenfield, P.F. 1987, 'Ethanol and the fluidity of the yeast plasma membrane', *Yeast*, vol. 3, no. 4, pp. 223-32.

Kaida, D., Yashiroda, H., Toh-e, A. and Kikuchi, Y. 2002, 'Yeast Whi2 and Psr1-phosphatase form a complex and regulate STRE-mediated gene expression', *Genes Cells*, vol. 7, no. 6, pp. 543-52.

Kajiwara, S., Aritomi, T., Suga, K., Ohtaguchi, K. and Kobayashi, O. 2000, 'Overexpression of the OLE1 gene enhances ethanol fermentation by *Saccharomyces cerevisiae*', *Appl Microbiol Biotechnol*, vol. 53, no. 5, pp. 568-74.

Kajiwara, Y., Ogawa, K., Takashita, H. and Omori, T. 2000, 'Enhanced glycerol production in Shochu yeast by heat-shock treatment is due to prolonged transcription of GPD1', *J Biosci Bioeng*, vol. 90, no. 1, pp. 121-3.

Karreman, R.J. and Lindsey, G.G. 2005, 'A rapid method to determine the stress status of *Saccharomyces cerevisiae* by monitoring the expression of a Hsp12:green fluorescent protein (GFP) construct under the control of the Hsp12 promoter', *J Biomol Screen*, vol. 10, no. 3, pp. 253-9.

Kawai, R., Fujita, K., Iwahashi, H. and Komatsu, Y. 1999, 'Direct evidence for the intracellular localization of Hsp104 in *Saccharomyces cerevisiae* by immunoelectron microscopy', *Cell Stress Chaperones*, vol. 4, no. 1, pp. 46-53.

Khlebodarova, T.M. 2002, 'How cells protect themselfs against stress', *Genetika*, vol. 38, no. 4, pp. 437-52.

Kim, J., Alizadeh, P., Harding, T., Hefner-Gravink, A. and Klionsky, D.J. 1996, 'Disruption of the yeast ATH1 gene confers better survival after dehydration, freezing, and ethanol shock: potential commercial applications', *Appl Environ Microbiol*, vol. 62, no. 5, pp. 1563-9.

Kim, Y.J., Francisco, L., Chen, G.C., Marcotte, E. and Chan, C.S. 1994, 'Control of cellular morphogenesis by the Ip12/Bem2 GTPase-activating protein: possible role of protein phosphorylation', *J Cell Biol*, vol. 127, no. 5, pp. 1381-94.

Kobayashi, N. and McEntee, K. 1990, 'Evidence for a heat shock transcription factor-independent mechanism for heat shock induction of transcription in *Saccharomyces cerevisiae*', *Proc Natl Acad Sci U S A*, vol. 87, no. 17, pp. 6550-4.

Kong, Q.X., Cao, L.M., Zhang, A.L. and Chen, X. 2007, 'Overexpressing GLT1 in gpd1Delta mutant to improve the production of ethanol of *Saccharomyces cerevisiae*', *Appl Microbiol Biotechnol*, vol. 73, no. 6, pp. 1382-6.

Kong, Q.X., Gu, J.G., Cao, L.M., Zhang, A.L., Chen, X. and Zhao, X.M. 2006, 'Improved production of ethanol by deleting FPS1 and over-expressing GLT1 in *Saccharomyces cerevisiae*', *Biotechnol Lett*, vol. 28, no. 24, pp. 2033-8.

Krantz, M., Nordlander, B., Valadi, H., Johansson, M., Gustafsson, L. and Hohmann, S. 2004, 'Anaerobicity prepares *Saccharomyces cerevisiae* cells for faster adaptation to osmotic shock', *Eukaryot Cell*, vol. 3, no. 6, pp. 1381-90.

Kubota, S., Takeo, I., Kume, K., Kanai, M., Shitamukai, A., Mizunuma, M., Miyakawa, T., Shimoi, H., Iefuji, H. and Hirata, D. 2004, 'Effect of ethanol on cell growth of budding yeast: genes that are important for cell growth in the presence of ethanol', *Biosci Biotechnol Biochem*, vol. 68, no. 4, pp. 968-72.

Kuge, S., Jones, N. and Nomoto, A. 1997, 'Regulation of yAP-1 nuclear localization in response to oxidative stress', *Embo J*, vol. 16, no. 7, pp. 1710-20.

Kurita, O. and Nishida, Y. 1999, 'Involvement of mitochondrial aldehyde dehydrogenase ALD5 in maintenance of the mitochondrial electron transport chain in *Saccharomyces cerevisiae*', *FEMS Microbiol Lett*, vol. 181, no. 2, pp. 281-7.

Lane, P.G., Hutter, A., Oliver, S.G. and Butler, P.R. 1999, 'Selection of microbial mutants tolerant to extreme environmental stress using continuous culture-control design', *Biotechnol Prog*, vol. 15, no. 6, pp. 1115-24.

Larsson, C., Pahlman, I.L., Ansell, R., Rigoulet, M., Adler, L. and Gustafsson, L. 1998, 'The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*', *Yeast*, vol. 14, no. 4, pp. 347-57.

Larue, F., Lafon-Lafourcade, S. and Ribereau-Gayon, J. 1980, 'Relationship between the sterol content of yeast cells and their fermentation activity in grape must', *Appl Environ Microbiol*, vol. 39, pp. 808-11.

Leao, C. and Van Uden, N. 1984, 'Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*', *Biochim Biophys Acta*, vol. 774, no. 1, pp. 43-8.

Leão, C. and Van Uden, N. 1982, 'Effects of ethanol and other alkanols on the kinetics and the activation parameters of thermal death in *Saccharomyces cerevisiae*', *Biotechnol Bioeng*, vol. 24, no. 7, pp. 1581-90.

Leão, C. and Van Uden, N. 1983, 'Effects of ethanol and other alkanols on the ammonium transport system of *Saccharomyces cerevisiae*', *Biotechnol Bioeng*, vol. 25, no. 8, pp. 2085-9.

Leão, C. and van Uden, N. 1984, 'Effects of ethanol and other alkanols on the general amino acid permease of *Saccharomyces cerevisiae*', *Biotechnol Bioeng*, vol. 26, no. 4, pp. 403-5.

Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J. and Toledano, M.B. 1999, 'Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast', *J Biol Chem*, vol. 274, no. 23, pp. 16040-6.

Leslie, S.B., Teter, S.A., Crowe, L.M. and Crowe, J.H. 1994, 'Trehalose lowers membrane phase transitions in dry yeast cells', *Biochim Biophys Acta*, vol. 1192, no. 1, pp. 7-13.

Levine, H. and Slade, L. 1992, 'Another view of trehalose for drying and stabilizing biological materials', *Biopharm.-Technol.*, vol. May, pp. 36-40.

Lewis, J.G., Learmonth, R.P. and Watson, K. 1995, 'Induction of heat, freezing and salt tolerance by heat and salt shock in *Saccharomyces cerevisiae*.' *Microbiology*, vol. 141, pp. 687-94.

Li, N., Zhang, L.M., Zhang, K.Q., Deng, J.S., Prandl, R. and Schoffl, F. 2006, 'Effects of heat stress on yeast heat shock factor-promoter binding in vivo', *Acta Biochim Biophys Sin (Shanghai)*, vol. 38, no. 5, pp. 356-62.

Lillie, S.H. and Pringle, J.R. 1980, 'Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation', *J Bacteriol*, vol. 143, no. 3, pp. 1384-94.

Lindquist, S. 1993, 'Autoregulation of the heat-shock response', *Translational Regulation of Gene Expression*, pp. 279-320.

Lindquist, S. and Craig, E.A. 1988, 'The heat-shock proteins', *Annu Rev Genet*, vol. 22, pp. 631-77.

Lindquist, S., Patino, M.M., Chernoff, Y.O., Kowal, A.S., Singer, M.A., Liebman, S.W., Lee, K.H. and Blake, T. 1995, 'The role of Hsp104 in stress tolerance and [PSI+] propagation in *Saccharomyces cerevisiae*', *Cold Spring Harb Symp Quant Biol*, vol. 60, pp. 451-60.

Littlefield, O. and Nelson, H.C. 1999, 'A new use for the 'wing' of the 'winged' helix-turn-helix motif in the HSF-DNA cocrystal', *Nat Struct Biol*, vol. 6, no. 5, pp. 464-70.

Lopez-Diez, E.C. and Bone, S. 2004, 'The interaction of trypsin with trehalose: an investigation of protein preservation mechanisms', *Biochim Biophys Acta*, vol. 1673, no. 3, pp. 139-48.

Loureiro-Dias, M.C. and Peinado, J.M. 1982, 'Effect of ethanol and other alcanols on the maltose transport system of *Saccharomyces cerevisiae*.' *Biotechnol Lett*, vol. 4, pp. 721-4.

Loureiro-Dias, M.C. and Santos, H. 1990, 'Effects of ethanol on *Saccharomyces cerevisiae* as monitored by in vivo 31P and 13C nuclear magnetic resonance', *Arch Microbiol*, vol. 153, no. 4, pp. 384-91.

Lucero, P., Penalver, E., Moreno, E. and Lagunas, R. 1997, 'Moderate concentrations of ethanol inhibit endocytosis of the yeast maltose transporter', *Appl Environ Microbiol*, vol. 63, no. 10, pp. 3831-6.

Luo, Y., Li, W.-M. and Wang, W. 2008, 'Trehalose: protector of antioxidant enzymes or reactive oxigen species scavenger under heat stress?' *Environmental and Experimental Botany*, vol. 63, pp. 378-84.

Luttik, M.A., Overkamp, K.M., Kotter, P., de Vries, S., van Dijken, J.P. and Pronk, J.T. 1998, 'The *Saccharomyces cerevisiae* NDE1 and NDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH', *J Biol Chem*, vol. 273, no. 38, pp. 24529-34.

Mager, W.H. and Varela, J.C. 1993, 'Osmostress response of the yeast Saccharomyces', *Mol Microbiol*, vol. 10, no. 2, pp. 253-8.

Mager, W.H. and Ferreira, P.M. 1993, 'Stress response of yeast', *Biochem J*, vol. 290 (Pt 1), pp. 1-13.

Mager, W.H. and Siderius, M. 2002, 'Novel insights into the osmotic stress response of yeast', *FEMS Yeast Res*, vol. 2, no. 3, pp. 251-7.

Mansure, J.J., Panek, A.D., Crowe, L.M. and Crowe, J.H. 1994, 'Trehalose inhibits ethanol effects on intact yeast cells and liposomes', *Biochim Biophys Acta*, vol. 1191, no. 2, pp. 309-16.

Marchler, G., Schuller, C., Adam, G. and Ruis, H. 1993, 'A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions', *Embo J*, vol. 12, no. 5, pp. 1997-2003.

Marczak, J.E. and Brandriss, M.C. 1991, 'Analysis of constitutive and noninducible mutations of the PUT3 transcriptional activator', *Mol Cell Biol*, vol. 11, no. 5, pp. 2609-19.

Marres, C.A., de Vries, S. and Grivell, L.A. 1991, 'Isolation and inactivation of the nuclear gene encoding the rotenone-insensitive internal NADH: ubiquinone oxidoreductase of mitochondria from *Saccharomyces cerevisiae*', *Eur J Biochem*, vol. 195, no. 3, pp. 857-62.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. and Estruch, F. 1996, 'The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE)', *Embo J*, vol. 15, no. 9, pp. 2227-35.

Matsutani, K., Fukuda, Y., Mutrata, K., Kimura, A. and Yajima, N. 1992, 'Adaptation mechanism of yeast to extreme environments: construction of salt-tolerance mutants of the yeast *Saccharomyces cerevisiae*', *J Ferm. Bioeng.*, vol. 73, no. 228-229.

Matsuura, K. and Takagi, H. 2005, 'Vacuolar functions are involved in stress-protective effect of intracellular proline in *Saccharomyces cerevisiae*', *J Biosci Bioeng*, vol. 100, no. 5, pp. 538-44.

Meaden, P.G., Arneborg, N., Guldfeldt, L.U., Siegumfeldt, H. and Jakobsen, M. 1999, 'Endocytosis and vacuolar morphology in *Saccharomyces cerevisiae* are altered in response to ethanol stress or heat shock', *Yeast*, vol. 15, no. 12, pp. 1211-22.

Menees, T.M. and Sandmeyer, S.B. 1996, 'Cellular stress inhibits transposition of the yeast retrovirus-like element Ty3 by a ubiquitin-dependent block of virus-like particle formation', *Proc Natl Acad Sci U S A*, vol. 93, no. 11, pp. 5629-34.

Michnick, S., Roustan, J.L., Remize, F., Barre, P. and Dequin, S. 1997, 'Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for GPD1 encoding glycerol 3-phosphate dehydrogenase', *Yeast*, vol. 13, no. 9, pp. 783-93.

Miroliaei, M., Ranjbar, B., Naderi-Manesh, H. and Nemat-Gorgani, M. 2007, 'Thermal denaturation of yeast alcohol dehydrogenase and protection of seconary and tertiary structural changes by sugars: CD and fluorescence studies', *Enzyme and Microbial Technology*, vol. 40, pp. 896-901.

Mishra, P. and Prasad, R. 1989, 'Relationship between fluidity and L-alanine transport in a fatty acid auxotroph of *Saccharomyces cerevisiae*', *Biochem Int*, vol. 19, no. 5, pp. 1019-30.

Miyahara, K., Hirata, D. and Miyakawa, T. 1996, 'yAP-1- and yAP-2-mediated, heat shock-induced transcriptional activation of the multidrug resistance ABC transporter genes in *Saccharomyces cerevisiae*', *Curr Genet*, vol. 29, no. 2, pp. 103-5.

Modig, T., Granath, K., Adler, L. and Liden, G. 2007, 'Anaerobic glycerol production by *Saccharomyces cerevisiae* strains under hyperosmotic stress', *Appl Microbiol Biotechnol*, vol. 75, no. 2, pp. 289-96.

Mohammad, I. 2007, *Osmotic stress in Saccharomyces cerevisiae.*, Victoria University, School of Molecular Sciences, Melbourne.

Monteiro, G.A. and Sa-Correia, I. 1998, 'In vivo activation of yeast plasma membrane H+-ATPase by ethanol: effect on the kinetic parameters and involvement of the carboxyl-terminus regulatory domain', *Biochim Biophys Acta*, vol. 1370, no. 2, pp. 310-6.

Moraitis, C. and Curran, B.P. 2007, 'Can the different heat shock response thresholds found in fermenting and respiring yeast cells be attributed to their differential redox states?' *Yeast*, vol. 24, no. 8, pp. 653-66.

Morano, K.A., Santoro, N., Koch, K.A. and Thiele, D.J. 1999, 'A trans-activation domain in yeast heat shock transcription factor is essential for cell cycle progression during stress', *Mol Cell Biol*, vol. 19, no. 1, pp. 402-11.

Morillon, A., Springer, M. and Lesage, P. 2000, 'Activation of the Kss1 invasive-filamentous growth pathway induces Ty1 transcription and retrotransposition in *Saccharomyces cerevisiae*', *Mol Cell Biol*, vol. 20, no. 15, pp. 5766-76.

Mosser, D.D., Ho, S. and Glover, J.R. 2004, '*Saccharomyces cerevisiae* Hsp104 enhances the chaperone capacity of human cells and inhibits heat stress-induced proapoptotic signaling.' *Biochemistry*, vol. 43, pp. 8107-15.

Muller, E.H., Richards, E.J., Norbeck, J., Byrne, K.L., Karlsson, K.A., Pretorius, G.H., Meacock, P.A., Blomberg, A. and Hohmann, S. 1999, 'Thiamine repression and pyruvate decarboxylase autoregulation independently control the expression of the *Saccharomyces cerevisiae* PDC5 gene', *FEBS Lett*, vol. 449, no. 2-3, pp. 245-50.

Natarajan, K., Meyer, M.R., Jackson, B.M., Slade, D., Roberts, C., Hinnebusch, A.G. and Marton, M.J. 2001, 'Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast', *Mol Cell Biol*, vol. 21, no. 13, pp. 4347-68.

Nevitt, T., Pereira, J. and Rodrigues-Pousada, C. 2004, 'YAP4 gene expression is induced in response to several forms of stress in *Saccharomyces cerevisiae*', *Yeast*, vol. 21, no. 16, pp. 1365-74.

Nevoigt, E. and Stahl, U. 1996, 'Reduced pyruvate decarboxylase and increased glycerol-3-phosphate dehydrogenase [NAD+] levels enhance glycerol production in *Saccharomyces cerevisiae*', *Yeast*, vol. 12, no. 13, pp. 1331-7.

Nevoigt, E. and Stahl, U. 1997, 'Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*', *FEMS Microbiol Rev*, vol. 21, no. 3, pp. 231-41.

Nguyen, D.T., Alarco, A.M. and Raymond, M. 2001, 'Multiple Yap1p-binding sites mediate induction of the yeast major facilitator FLR1 gene in response to drugs, oxidants, and alkylating agents', *J Biol Chem*, vol. 276, no. 2, pp. 1138-45.

Nieto-Sotelo, J., Wiederrecht, G., Okuda, A. and Parker, C.S. 1990, 'The yeast heat shock transcription factor contains a transcriptional activation domain whose activity is repressed under nonshock conditions', *Cell*, vol. 62, no. 4, pp. 807-17.

Novotny, C., Flieger, M., Panos, J. and Karst, F. 1992, 'Effect of 5,7-unsaturated sterols on ethanol tolerance in *Saccharomyces cerevisiae*', *Biotechnol Appl Biochem*, vol. 15, no. 3, pp. 314-20.

Novotny, C., Dolezalova, L., Flieger, M., Panos, J. and Karst, F. 1992, 'Ethanol-induced death and lipid composition of *Saccharomyces cerevisiae*: a comparative study of the role of sterols', *Folia Microbiol (Praha)*, vol. 37, no. 4, pp. 286-8.

Nwaka, S., Kopp, M., Burgert, M., Deuchler, I., Kienle, I. and Holzer, H. 1994, 'Is thermotolerance of yeast dependent on trehalose accumulation?' *FEBS Lett*, vol. 344, no. 2-3, pp. 225-8.

Ogawa, Y., Nitta, A., Uchiyama, H., Imamura, T., Shimoi, H. and Ito, K. 2000, 'Tolerance mechanism of the ethanol-tolerant mutant of sake yeast', *J Biosci Bioeng*, vol. 90, no. 3, pp. 313-20.

Oliveira, R., Lages, F., Silva-Graca, M. and Lucas, C. 2003, 'Fps1p channel is the mediator of the major part of glycerol passive diffusion in *Saccharomyces cerevisiae*: artefacts and re-definitions', *Biochim Biophys Acta*, vol. 1613, no. 1-2, pp. 57-71.

Overkamp, K.M., Bakker, B.M., Kotter, P., Luttik, M.A., Van Dijken, J.P. and Pronk, J.T. 2002, 'Metabolic engineering of glycerol production in *Saccharomyces cerevisiae*', *Appl Environ Microbiol*, vol. 68, no. 6, pp. 2814-21.

Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kameyama, T., Nonaka, H., Hirano, H., Matsuura, Y. and Takai, Y. 1996, 'Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in *Saccharomyces cerevisiae*', *Embo J*, vol. 15, no. 9, pp. 2196-207.

Pahlman, A.K., Granath, K., Ansell, R., Hohmann, S. and Adler, L. 2001, 'The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress', *J Biol Chem*, vol. 276, no. 5, pp. 3555-63.

Pahlman, I.L., Gustafsson, L., Rigoulet, M. and Larsson, C. 2001, 'Cytosolic redox metabolism in aerobic chemostat cultures of *Saccharomyces cerevisiae*', *Yeast*, vol. 18, no. 7, pp. 611-20.

Panozzo, C., Nawara, M., Suski, C., Kucharczyka, R., Skoneczny, M., Becam, A.M., Rytka, J. and Herbert, C.J. 2002, 'Aerobic and anaerobic NAD+ metabolism in *Saccharomyces cerevisiae*', *FEBS Lett*, vol. 517, no. 1-3, pp. 97-102.

Parsell, D.A., Kowal, A.S., Singer, M.A. and Lindquist, S. 1994, 'Protein disaggregation mediated by heat-shock protein Hsp104', *Nature*, vol. 372, no. 6505, pp. 475-8.

Pascual, C., Alonson, A., Garcia, I. and Romay, C. 1988, 'Effect of ethanol on glucose transport, key glycolytic enzymes, and proton extrusion in *Saccharomyces cerevisiae*', *Biotechnology and bioengineerings*, vol. 32, pp. 374-8.

Perisic, O., Xiao, H. and Lis, J.T. 1989, 'Stable binding of Drosophila heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit', *Cell*, vol. 59, no. 5, pp. 797-806.

Perkel, J. 2008, 'SNP genptyping: six technologies that keyed a revolution.' *Nat Methods*, vol. 5, no. 5, pp. 447-54.

Peteranderl, R. and Nelson, H.C. 1992, 'Trimerization of the heat shock transcription factor by a triple-stranded alpha-helical coiled-coil', *Biochemistry*, vol. 31, no. 48, pp. 12272-6.

Petrov, V.V. and Okorokov, L.A. 1990, 'Increase of the anion and proton permeability of Saccharomyces carlsbergensis plasmalemma by n-alcohols as a possible cause of its de-energization', *Yeast*, vol. 6, no. 4, pp. 311-8.

Piper, P.W. 1993, 'Molecular events associated with acquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*', *FEMS Microbiol Rev*, vol. 11, no. 4, pp. 339-55.

Piper, P.W., Ortiz-Calderon, C., Holyoak, C., Coote, P. and Cole, M. 1997, 'Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H(+)-ATPase', *Cell Stress Chaperones*, vol. 2, no. 1, pp. 12-24.

Plesset, J., Palm, C. and McLaughlin, C.S. 1982, 'Induction of heat shock proteins and thermotolerance by ethanol in *Saccharomyces cerevisiae*', *Biochem Biophys Res Commun*, vol. 108, no. 3, pp. 1340-5.

Rachenko, E.I., Rikhvanov, E.G., Varakina, N.N., Rusaleva, T.M., Borovskii, G.B. and Voinikov, V.K. 2004, 'The effect of sodium azide on basic and induced thermotolerance in *Saccharomyces cerevisiae*', *Fiziol. Rast. (Moscow)*, vol. 51, pp. 222-7

Raitt, D.C., Johnson, A.L., Erkine, A.M., Makino, K., Morgan, B., Gross, D.S. and Johnston, L.H. 2000, 'The Skn7 response regulator of *Saccharomyces cerevisiae* interacts with Hsf1 in vivo and is required for the induction of heat shock genes by oxidative stress', *Mol Biol Cell*, vol. 11, no. 7, pp. 2335-47.

Randez-Gil, F., Bojunga, N., Proft, M. and Entian, K.D. 1997, 'Glucose derepression of gluconeogenic enzymes in *Saccharomyces cerevisiae* correlates with phosphorylation of the gene activator Cat8p', *Mol Cell Biol*, vol. 17, no. 5, pp. 2502-10.

Remize, F., Roustan, J.L., Sablayrolles, J.M., Barre, P. and Dequin, S. 1999, 'Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in By-product formation and to a stimulation of fermentation rate in stationary phase', *Appl Environ Microbiol*, vol. 65, no. 1, pp. 143-9.

Replogle, K., Hovland, L. and Rivier, D.H. 1999, 'Designer deletion and prototrophic strains derived from *Saccharomyces cerevisiae* strain W303-1a', *Yeast*, vol. 15, no. 11, pp. 1141-9.

Rikhvanov, E.G., Varakina, N.N., Rusaleva, T.M., Rachenko, E.I., Knorre, D.A. and Voinikov, V.K. 2005, 'Do mitochondria regulate the heat-shock response in *Saccharomyces cerevisiae*?' *Curr Genet*, vol. 48, no. 1, pp. 44-59.

Robinson, M.D., Grigull, J., Mohammad, N. and Hughes, T.R. 2002, 'FunSpec: a web-based cluster interpreter for yeast', *BMC Bioinformatics*, vol. 3, no. 35.

Rodrigues-Pousada, C.A., Nevitt, T., Menezes, R., Azevedo, D., Pereira, J. and Amaral, C. 2004, 'Yeast activator proteins and stress response: an overview', *FEBS Lett*, vol. 567, no. 1, pp. 80-5.

Rolfe, M. 1985, 'UV-inducible transcripts in *Saccharomyces cerevisiae*', *Curr Genet*, vol. 9, no. 7, pp. 533-8.

Rosa, M.F. and Sa-Correia, I. 1991, 'In vivo activation by ethanol of plasma membrane ATPase of *Saccharomyces cerevisiae*', *Appl Environ Microbiol*, vol. 57, no. 3, pp. 830-5.

Rose, A.H. 1993, 'Composition of the envelope layers of *Saccharomyces cerevisiae* in relation to flocculation and ethanol tolerance', *J Appl Bacteriol*, vol. 74 Suppl, pp. 110S-8S.

Ruis, H. and Schuller, C. 1995, 'Stress signaling in yeast', *Bioessays*, vol. 17, no. 11, pp. 959-65.

Sacerdot, C., Mercier, G., Todeschini, A.L., Dutreix, M., Springer, M. and Lesage, P. 2005, 'Impact of ionizing radiation on the life cycle of *Saccharomyces cerevisiae* Ty1 retrotransposon', *Yeast*, vol. 22, no. 6, pp. 441-55.

Saint-Prix, F., Bonquist, L. and Dequin, S. 2004, 'Functional analysis of the ALD gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the NADP+-dependent Ald6p and Ald5p isoforms play a major role in acetate formation', *Microbiology*, vol. 150, no. Pt 7, pp. 2209-20.

Sajbidor, J. and Grego, J. 1992, 'Fatty acid alterations in *Saccharomyces cerevisiae* exposed to ethanol stress', *FEMS Microbiol Lett*, vol. 72, no. 1, pp. 13-6.

Sakurai, H. and Fukasawa, T. 2001, 'A novel domain of the yeast heat shock factor that regulates its activation function', *Biochem Biophys Res Commun*, vol. 285, no. 3, pp. 696-701.

Sales, K., Brandt, W., Rumbak, E. and Lindsey, G. 2000, 'The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress', *Biochim Biophys Acta*, vol. 1463, no. 2, pp. 267-78.

Sampedro, J.G., Cortes, P., Munoz-Clares, R.A., Fernandez, A. and Uribe, S. 2001, 'Thermal inactivation of the plasma membrane H+-ATPase from Kluyveromyces lactis. Protection by trehalose', *Biochim Biophys Acta*, vol. 1544, no. 1-2, pp. 64-73.

Sanchez, Y. and Lindquist, S.L. 1990, 'HSP104 required for induced thermotolerance', *Science*, vol. 248, no. 4959, pp. 1112-5.

Sanchez, Y., Taulien, A., Borkovich, A. and Lindquist, S. 1992, 'HSP104 is required for tolerance to many forms of stress.' *Embo J*, vol. 11, pp. 2357-64.

Sanchez, Y., Parsell, D.A., Taulien, J., Vogel, J.L., Craig, E.A. and Lindquist, S. 1993, 'Genetic evidence for a functional relationship between Hsp104 and Hsp70', *J Bacteriol*, vol. 175, no. 20, pp. 6484-91.

Sandmeyer, S.B. 1992, 'Yeast retrotransposons', *Curr Opin Genet Dev*, vol. 2, no. 5, pp. 705-11.

Santoro, N., Johansson, N. and Thiele, D.J. 1998, 'Heat shock element architecture is an important determinant in the temperature and transactivation domain requirements for heat shock transcription factor', *Mol Cell Biol*, vol. 18, no. 11, pp. 6340-52.

Schacherer, J., Ruderfer, D.M., Gresham, D., Dolinski, K., Botstein, D. and Kruglyak, L. 2007, 'Genome-wide analysis of nucleotide-level variation in commonly used Saccharomyces cerevisiae strains', *PLoS ONE*, vol. 2, no. 3, p. e322.

Schellenberger, A. 1998, 'Sixty years of thiamin diphosphate biochemistry', *Biochim Biophys Acta*, vol. 1385, no. 2, pp. 177-86.

Schmitt, A.P. and McEntee, K. 1996, 'Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*', *Proc Natl Acad Sci U S A*, vol. 93, no. 12, pp. 5777-82.

Schnell, N., Krems, B. and Entian, K.D. 1992, 'The PAR1 (YAP1/SNQ3) gene of *Saccharomyces cerevisiae*, a c-jun homologue, is involved in oxygen metabolism', *Curr Genet*, vol. 21, no. 4-5, pp. 269-73.

Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D. and Emr, S.D. 1993, 'Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting', *Science*, vol. 260, no. 5104, pp. 88-91.

Schuler, E., Lin, F. and Worman, H.J. 1994, 'Characterization of the human gene encoding LBR, an integral protein of the nuclear envelope inner membrane', *J Biol Chem*, vol. 269, no. 15, pp. 11312-7.

Schut, G.J., Zhou, J. and Adams, M.W. 2001, 'DNA microarray analysis of the hyperthermophilic archaeon Pyrococcus furiosus: evidence for the new type of sulfur-reducing enzyme complex.' *J Bacteriol*, vol. 183, p. 7027.

Sekine, T., Kawaguchi, A., Hamano, Y. and Takagi, H. 2007, 'Desensitization of feedback inhibition of the *Saccharomyces cerevisiae* gamma-glutamyl kinase enhances proline accumulation and freezing tolerance', *Appl Environ Microbiol*, vol. 73, no. 12, pp. 4011-9.

Shinohara, T., Singh, D.P. and Fatma, N. 2002, 'LEDGF, a survival factor, activates stress-related genes', *Prog Retin Eye Res*, vol. 21, no. 3, pp. 341-58.

Singer, M.A. and Lindquist, S. 1998, 'Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose', *Trends Biotechnol*, vol. 16, no. 11, pp. 460-8.

Smith, A., Ward, M.P. and Garrett, S. 1998, 'Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation', *Embo J*, vol. 17, no. 13, pp. 3556-64.

Smith, B.J. and Yaffe, M.P. 1991, 'A mutation in the yeast heat-shock factor gene causes temperature-sensitive defects in both mitochondrial protein import and the cell cycle', *Mol Cell Biol*, vol. 11, no. 5, pp. 2647-55.

Smith, S.J., Crowley, J.H. and Parks, L.W. 1996, 'Transcriptional regulation by ergosterol in the yeast *Saccharomyces cerevisiae*', *Mol Cell Biol*, vol. 16, no. 10, pp. 5427-32.

Sola-Penna, M. and Meyer-Fernades, J.R. 1994, 'Protective role of trehalose in thermal denaturation of yeast pyrophosphatase', *Z Naturforsch [C]*, vol. 49, no. 5-6, pp. 327-30.

Sola-Penna, M. and Meyer-Fernandes, J.R. 1998, 'Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: why is trehalose more effective than other sugars?' *Arch Biochem Biophys*, vol. 360, no. 1, pp. 10-4.

Sorger, P.K. and Pelham, H.R. 1988, 'Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation', *Cell*, vol. 54, no. 6, pp. 855-64.

Sorger, P.K. and Nelson, H.C. 1989, 'Trimerization of a yeast transcriptional activator via a coiled-coil motif', *Cell*, vol. 59, no. 5, pp. 807-13.

Soto, T., Fernandez, J., Vicente-Soler, J., Cansado, J. and Gacto, M. 1999, 'Accumulation of trehalose by overexpression of tps1, coding for trehalose-6-phosphate synthase, causes increased resistance to multiple stresses in the fission yeast schizosaccharomyces pombe', *Appl Environ Microbiol*, vol. 65, no. 5, pp. 2020-4.

Stanley, G.A. and Pamment, N.B. 1993, 'Transport and intracellular accumulation of acetaldehyde in *Saccharomyces cerevisiae*.' *Biotechnol Bioeng*, vol. 42, pp. 24-9.

Stanley, G.A., Hobley, T.J. and Pamment, N.B. 1997, 'Effect of acetaldehyde on *Saccharomyces cerevisiae* and Zymomona mobilis subjected to environmental shocks.' *Biotechnol Bioeng*, vol. 53, pp. 71-8.

Stanley, G.A., Douglas, N.G., Everu, E.J., Tzanatos, T. and Pamment, N.B. 1993, 'Inhibition and stimulation of yeast growth by acetaldehydee.' *Biotechnol Lett*, vol. 15, pp. 1199-204.

Sterner, D.E., Wang, X., Bloom, M.H., Simon, G.M. and Berger, S.L. 2002, 'The SANT domain of Ada2 is required for normal acetylation of histones by the yeast SAGA complex', *J Biol Chem*, vol. 277, no. 10, pp. 8178-86.

Strahl, T. and Thorner, J. 2007, 'Synthesis and function of membrane phosphoinositides in budding yeast, *Saccharomyces cerevisiae*', *Biochim Biophys Acta*, vol. 1771, no. 3, pp. 353-404.

Strumilo, S.A., Senkevich, S.B. and Vinogradov, V.V. 1984, 'Effect of oxythiamine on adrenal thiamine pyrophosphate-dependent enzyme activities', *Biomed Biochim Acta*, vol. 43, no. 2, pp. 159-63.

Suihko, M.L., Blomqvist, K., Penttila, M., Gisler, R. and Knowles, J. 1990, 'Recombinant brewer's yeast strains suitable for accelerated brewing', *J Biotechnol*, vol. 14, no. 3-4, pp. 285-300.

Susek, R.E. and Lindquist, S.L. 1989, 'hsp26 of *Saccharomyces cerevisiae* is related to the superfamily of small heat shock proteins but is without a demonstrable function', *Mol Cell Biol*, vol. 9, no. 11, pp. 5265-71.

Sussich, F., Skopec, C., Brady, J. and Cesaro, A. 2001, 'Reversible dehydration of trehalose and anhydrobiosis: from solution state to an exotic crystal?' *Carbohydr Res*, vol. 334, no. 3, pp. 165-76.

Tachibana, T., Astumi, S., Shioda, R., Ueno, M., Uritani, M. and Ushimaru, T. 2002, 'A novel non-conventional heat shock element regulates expression of MDJ1 encoding a DnaJ homolog in *Saccharomyces cerevisiae*', *J Biol Chem*, vol. 277, no. 25, pp. 22140-6.

Takagi, H., Iwamoto, F. and Nakamori, S. 1997, 'Isolation of freeze-tolerant laboratory strains of *Saccharomyces cerevisiae* from proline-analogue-resistant mutants', *Appl Microbiol Biotechnol*, vol. 47, no. 4, pp. 405-11.

Takagi, H., Takaoka, M., Kawaguchi, A. and Kubo, Y. 2005, 'Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*', *Appl Environ Microbiol*, vol. 71, no. 12, pp. 8656-62.

Takahashi, T., Shimoi, H. and Ito, K. 2001, 'Identification of genes required for growth under ethanol stress using transposon mutagenesis in *Saccharomyces cerevisiae*', *Mol Genet Genomics*, vol. 265, no. 6, pp. 1112-9.

Thevelein, J.M. and de Winde, J.H. 1999, 'Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*', *Mol Microbiol*, vol. 33, no. 5, pp. 904-18.

Thomas, B.J. and Rothstein, R. 1989, 'Elevated recombination rates in transcriptionally active DNA', *Cell*, vol. 56, no. 4, pp. 619-30.

Thomas, D.S. and Rose, A.H. 1979, 'Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition', *Arch Microbiol*, vol. 122, no. 1, pp. 49-55.

Thomas, D.S., Hossack, J.A. and Rose, A.H. 1978, 'Plasma-membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*', *Arch Microbiol*, vol. 117, no. 3, pp. 239-45.

Todeschini, A.L., Morillon, A., Springer, M. and Lesage, P. 2005, 'Severe adenine starvation activates Ty1 transcription and retrotransposition in *Saccharomyces cerevisiae*', *Mol Cell Biol*, vol. 25, no. 17, pp. 7459-72.

Tran, T. 2007, *Characterisation of ethanol-toerant mutants SM1 and CM1*, Victoria university, Melbourne.

Treger, J.M., Magee, T.R. and McEntee, K. 1998, 'Functional analysis of the stress response element and its role in the multistress response of *Saccharomyces cerevisiae*', *Biochem Biophys Res Commun*, vol. 243, no. 1, pp. 13-9.

Trotter, E.W., Berenfeld, L., Krause, S.A., Petsko, G.A. and Gray, J.V. 2001, 'Protein misfolding and temperature up-shift cause G1 arrest via a common mechanism dependent on heat shock factor in Saccharomycescerevisiae', *Proc Natl Acad Sci U S A*, vol. 98, no. 13, pp. 7313-8.

Trotter, E.W., Kao, C.M., Berenfeld, L., Botstein, D., Petsko, G.A. and Gray, J.V. 2002, 'Misfolded proteins are competent to mediate a subset of the responses to heat shock in *Saccharomyces cerevisiae*', *J Biol Chem*, vol. 277, no. 47, pp. 44817-25.

Ueom, J., Kwon, S., Kim, S., Chae, Y. and Lee, K. 2003, 'Acquisition of heat shock tolerance by regulation of intracellular redox states', *Biochim Biophys Acta*, vol. 1642, no. 1-2, pp. 9-16.

Valadi, A., Granath, K., Gustafsson, L. and Adler, L. 2004, 'Distinct intracellular localization of Gpd1p and Gpd2p, the two yeast isoforms of NAD+-dependent glycerol-3-phosphate dehydrogenase, explains their different contributions to redox-driven glycerol production', *J Biol Chem*, vol. 279, no. 38, pp. 39677-85.

Valadi, H., Larsson, C. and Gustafsson, L. 1998, 'Improved ethanol production by glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*', *Appl Microbiol Biotechnol*, vol. 50, no. 4, pp. 434-9.

Valadi, H., Valadi, A., Ansell, R., Gustafsson, L., Adler, L., Norbeck, J. and Blomberg, A. 2004, 'NADH-reductive stress in *Saccharomyces cerevisiae* induces the expression of the minor isoform of glyceraldehyde-3-phosphate dehydrogenase (TDH1)', *Curr Genet*, vol. 45, no. 2, pp. 90-5.

van Dijken, J.P., van den Bosch, E., Hermans, J.J., de Miranda, L.R. and Scheffers, W.A. 1986, 'Alcoholic fermentation by 'non-fermentative' yeasts', *Yeast*, vol. 2, no. 2, pp. 123-7.

van Voorst, F., Houghton-Larsen, J., Jonson, L., Kielland-Brandt, M.C. and Brandt, A. 2006, 'Genome-wide identification of genes required for growth of *Saccharomyces cerevisiae* under ethanol stress', *Yeast*, vol. 23, no. 5, pp. 351-9.

Varela, J.C., van Beekvelt, C., Planta, R.J. and Mager, W.H. 1992, 'Osmostress-induced changes in yeast gene expression', *Mol Microbiol*, vol. 6, no. 15, pp. 2183-90.

Veal, E.A., Ross, S.J., Malakasi, P., Peacock, E., & Morgan, B.A. (2003). 'Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor.' *J Biol Chem*,vol. 278, no. 33, pp 30896-904.

Vemuri, G.N., Eiteman, M.A., McEwen, J.E., Olsson, L. and Nielsen, J. 2007, 'Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*', *Proc Natl Acad Sci U S A*, vol. 104, no. 7, pp. 2402-7.

Vogel, J.L., Parsell, D.A. and Lindquist, S. 1995, 'Heat-shock proteins Hsp104 and Hsp70 reactivate mRNA splicing after heat inactivation', *Curr Biol*, vol. 5, no. 3, pp. 306-17.

von Jagow, G. and Klingenberg, M. 1970, 'Pathways of hydrogen in mitochondria of Saccharomyces carlsbergensis', *Eur J Biochem*, vol. 12, no. 3, pp. 583-92.

Voos, W. and Rottgers, K. 2002, 'Molecular chaperones as essential mediators of mitochondrial biogenesis', *Biochim Biophys Acta*, vol. 1592, no. 1, pp. 51-62.

Vriesekoop, F. and Pamment, N.B. 2005, 'Acetaldehyde addition and pre-adaptation to the stressor together virtually eliminate the ethanol-induced lag phase in *Saccharomyces cerevisiae*', *Lett Appl Microbiol*, vol. 41, no. 5, pp. 424-7.

Vriesekoop, F., Barber, A.R. and Pamment, N.B. 2007, 'Acetaldehyde mediates growth stimulation of ethanol-stressed *Saccharomyces cerevisiae*: evidence of a redox-driven mechanism', *Biotechnol Lett*, vol. 29, no. 7, pp. 1099-103.

Walker-Caprioglio, H.M. and Parks, L.W. 1987, 'Autoconditioning factor relieves ethanol-induced growth inhibition of *Saccharomyces cerevisiae*', *Appl Environ Microbiol*, vol. 53, no. 1, pp. 33-5.

Walker-Caprioglio, H.M., Casey, W.M. and Parks, L.W. 1990, '*Saccharomyces cerevisiae* membrane sterol modifications in response to growth in the presence of ethanol', *Appl Environ Microbiol*, vol. 56, no. 9, pp. 2853-7.

Walker, G.M. 1994, 'The roles of magnesium in biotechnology', *Crit Rev Biotechnol*, vol. 14, no. 4, pp. 311-54.

Walker, G.M. 1998, *Yeast Physiology and Biotechnology*, John Wiley & Sons Ltd., England.

Wang, H.T., Rahaim, P., Robbins, P. and Yocum, R.R. 1994, 'Cloning, sequence, and disruption of the Saccharomyces diastaticus DAR1 gene encoding a glycerol-3-phosphate dehydrogenase', *J Bacteriol*, vol. 176, no. 22, pp. 7091-5.

Wang, J. and White, A.L. 1999, '6-Aminohexanoic acid as a chemical chaperone for apolipoprotein(a)', *J Biol Chem*, vol. 274, no. 18, pp. 12883-9.

Wang, T. and Bretscher, A. 1995, 'The rho-GAP encoded by BEM2 regulates cytoskeletal structure in budding yeast', *Mol Biol Cell*, vol. 6, no. 8, pp. 1011-24.

Wang, X., Watt, P.M., Louis, E.J., Borts, R.H. and Hickson, I.D. 1996, 'Pat1: a topoisomerase II-associated protein required for faithful chromosome transmission in *Saccharomyces cerevisiae*', *Nucleic Acids Res*, vol. 24, no. 23, pp. 4791-7.

Wang, X., Watt, P.M., Borts, R.H., Louis, E.J. and Hickson, I.D. 1999, 'The topoisomerase II-associated protein, Pat1p, is required for maintenance of rDNA locus stability in *Saccharomyces cerevisiae*', *Mol Gen Genet*, vol. 261, no. 4-5, pp. 831-40.

Warner, J.R. 1999, 'The economics of ribosome biosynthesis in yeast', *Trends Biochem Sci*, vol. 24, no. 11, pp. 437-40.

Wati, L., Dhamija, S.S., Singh, D., Nigam, P. and Marchant, R. 1996, 'Characterization of of genetic control of thermotolerance in mutants of *Saccharomyces cerevisiae*', *Gen. Eng. Biotechnol.*, vol. 16, no. 19-28.

Watson, K. and Cavicchioli, R. 1983, 'Acquisition of ethanol tolerance in yeast cells by heat shock.' *Biotechnol Lett*, vol. 5, no. 10, pp. 683-8.

Weitzel, G. and Li, G.C. 1993, 'Thermal response of yeast cells overexpressing hsp70 genes', *Int J Hyperthermia*, vol. 9, no. 6, pp. 783-97.

Wemmie, J.A., Szczypka, M.S., Thiele, D.J. and Moye-Rowley, W.S. 1994, 'Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1', *J Biol Chem*, vol. 269, no. 51, pp. 32592-7.

Werner-Washburne, M. and Craig, E.A. 1989, 'Expression of members of the *Saccharomyces cerevisiae* hsp70 multigene family', *Genome*, vol. 31, no. 2, pp. 684-9.

Werner-Washburne, M., Stone, D.E. and Craig, E.A. 1987, 'Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*', *Mol Cell Biol*, vol. 7, no. 7, pp. 2568-77.

Wieser, R., Adam, G., Wagner, A., Schuller, C., Marchler, G., Ruis, H., Krawiec, Z. and Bilinski, T. 1991, 'Heat shock factor-independent heat control of transcription of the CTT1 gene encoding the cytosolic catalase T of *Saccharomyces cerevisiae*', *J Biol Chem*, vol. 266, no. 19, pp. 12406-11.

Winkler, K., Kienle, I., Burgert, M., Wagner, J.C. and Holzer, H. 1991, 'Metabolic regulation of the trehalose content of vegetative yeast', *FEBS Lett*, vol. 291, no. 2, pp. 269-72.

Wojda, I., Alonso-Monge, R., Bebelman, J.P., Mager, W.H. and Siderius, M. 2003, 'Response to high osmotic conditions and elevated temperature in *Saccharomyces cerevisiae* is controlled by intracellular glycerol and involves coordinate activity of MAP kinase pathways', *Microbiology*, vol. 149, no. Pt 5, pp. 1193-204.

Wotton, D., Freeman, K. and Shore, D. 1996, 'Multimerization of Hsp42p, a novel heat shock protein of *Saccharomyces cerevisiae*, is dependent on a conserved carboxyl-terminal sequence', *J Biol Chem*, vol. 271, no. 5, pp. 2717-23.

Wu, A., Wemmie, J.A., Edgington, N.P., Goebl, M., Guevara, J.L. and Moye-Rowley, W.S. 1993, 'Yeast bZip proteins mediate pleiotropic drug and metal resistance', *J Biol Chem*, vol. 268, no. 25, pp. 18850-8.

Yamamoto, A., Mizukami, Y. and Sakurai, H. 2005, 'Identification of a novel class of target genes and a novel type of binding sequence of heat shock transcription factor in *Saccharomyces cerevisiae*', *J Biol Chem*, vol. 280, no. 12, pp. 11911-9.

Yan, C., Lee, L.H. and Davis, L.I. 1998, 'Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor', *Embo J*, vol. 17, no. 24, pp. 7416-29.

Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. and Somero, G.N. 1982, 'Living with water stress: evolution of osmolyte systems', *Science*, vol. 217, no. 4566, pp. 1214-22.

Yang, Y.H., Dudoit, S., Luu, P. and Speed, T.P. 2001, 'Normalisation for cDNA microarray', *Microarrays: Optical Technologies and Informatics SPIE, Society for Optical Engineering, San Jose, C.A.*, vol. 4266, pp. 141-52.

Yomano, L.P., York, S.W. and Ingram, L.O. 1998, 'Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production', *J Ind Microbiol Biotechnol*, vol. 20, no. 2, pp. 132-8.

You, K.M., Rosenfield, C.L. and Knipple, D.C. 2003, 'Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content', *Appl Environ Microbiol*, vol. 69, no. 3, pp. 1499-503.

Zancan, P. and Sola-Penna, M. 2005, 'Trehalose and glycerol stabilize and renature yeast inorganic pyrophosphatase inactivated by very high temperatures', *Arch Biochem Biophys*, vol. 444, no. 1, pp. 52-60.

Zarzov, P., Boucherie, H. and Mann, C. 1997, 'A yeast heat shock transcription factor (Hsf1) mutant is defective in both Hsc82/Hsp82 synthesis and spindle pole body duplication', *J Cell Sci*, vol. 110 (Pt 16), pp. 1879-91.

Zimatkin, S.M. and Zimatkina, T.I. 1996, 'Thiamine deficiency as predisposition to, and consequence of, increased alcohol consumption', *Alcohol Alcohol*, vol. 31, no. 4, pp. 421-7.

GUIDE THROUGH THE APPENDIX CD

Due to the large amount of data generated during this project the appendices have been provided in electronic format on the attached CD. This guide provides details of the various appendices and aims to help the reader explore the data and locate the required files with ease. The guide should be used concurrently with the CD. All file names have been shortened to adhere to Microsoft Windows® requirements regarding maximum path for each file.

APPENDIX 1

Appendix 1.1 Buffers and solutions

This folder describes buffers and solutions used during the experimental work.

Appendix 1.2 Growth curve statistics

This folder contains a detailed statistical analysis of the *S. cerevisiae* growth curves performed during this project. The analysis was done using KY-Plot version 2 beta 15, statistical software.

APPENDIX 2

This appendix includes the original microarray results (reference and direct design experiments) and includes details of the various statistical analyses performed with this data. The raw microarray data is included in this appendix CD (Appendix 2.5) for further analysis at the reader's discretion. In some cases the folder, sample and condition names have been abbreviated (as indicated).

The order of samples is the same for all reference design heatmaps and other graphs, as follows: triplicates of W303-1A at 0% (v/v) ethanol, W303-1A at 6.5% (v/v) ethanol, SM1 at 0% (v/v) ethanol, SM1 at 6.5% (v/v) ethanol, CM1 at 0% (v/v) ethanol and CM1 at 6.5% (v/v) ethanol.

Direct design comparisons include each strain directly hybridised at 0% (v/v) ethanol vs. 6.5% (v/v) ethanol. The order of samples in all direct design heatmaps and graphical presentations is: W303-1A, SM1 and CM1 (each with 0% vs. 6.5% (v/v) ethanol).

Appendix 2.1 Reference design 2-way ANOVA

Folder contains results of 2-way ANOVA analysis with p-value<0.001 (Genowiz).

2.1.1 Gene lists and heatmaps

This folder contains Excel (.xls) spreadsheets of ethanol significant genes (ESG), strain significant genes (SSG) and interaction significant genes (ISG) with corresponding p-values. It also contains folders with heatmaps of ESG, SSG and ISG. The Venn diagram (.jpg) presents the intersection of ESG, SSG and ISG as defined by 2-way ANOVA ($p < 0.001$).

2.1.2 Gene ontology

This folder contains Genowiz GO bioanalysis (Genowiz^{TM} 4.0.2.1 software) and Funspec cluster interpreter analysis (Robinson *et al.*, 2002) outputs, in form of Excel (.xls) spreadsheets for ESG, ISG and SSG. Please note that the ESG analysis includes all stress induced and repressed genes. These two groups of ESG were subsequently separated using K-Means and are presented as separated lists in Appendix 2.1.5.

2.1.3 Previous publications

Folder contains provides lists of genes reported as ethanol stress-induced in previous microarray studies (Alexandre *et al.*, 2001; Chandler *et al.*, 2004) or as growth impaired mutants in yeast deletion library studies (Fujita *et al.*, 2006; Kubota *et al.*, 2004; van Voorst *et al.*, 2006). The lists of reported genes were imported into Genowiz software and their expression profiles in reference design (Appendix 2.1.3.1) and in direct design experiments (Appendix 2.1.3.2) were inspected.

The folder "2.1.3.1 Reference design" presents reported genes that also passed 2-way ANOVA in the current study ("2-way ANOVA genes" folder) and full ethanol stress related gene lists as reported by the respective authors ("all genes" folder).

2.1.4 Retrotransposons in 2way ANOVA

Presents graphical views of GO category "Retrotransposition RNA mediated" imported into Genowiz from *Saccharomyces* Genome Database (SGD).

2.1.5 K-Means

Presents K-Means clustering results for ESG, SSG and ISG. Each folder contains genelists with p-values and heatmaps of all clusters and GO analysis of clusters of interest. It also contains line graphs, pie-charts, mean graphs and PCA views of all 10 clusters. K-Means of SSG also contains Blast analysis of YNL134C encoded protein.

Appendix 2.2 Direct design

This folder presents lists of up-regulated and down-regulated genes during ethanol stress from the direct design experiment. Data is presented in the form of heatmaps and mean graphs. Mean graphs present on y-axes mean intensities of all up/down regulated genes on $\log_2$ transformed scale (intensity of 1 is 2 fold induced, intensity of 2 is 4 fold and 0 represents unchanged intensity).

Appendix 2.3 Pathway inspection

2.3.1 Pathway images

Contains Windows picture files (.bmp) showing selected metabolic and functional pathways. Individual experimental conditions are described by the first two letters of the strain and first number of ethanol concentration. For example, "w6sm6" represents a comparison of triplicates of W303-1A at 6.5% (v/v) ethanol to triplicates of SM1 at 6.5% (v/v) ethanol. Genes more highly expressed in probe (SM1 at 6.5% v/v) are coloured in shades of red as per legend and genes more highly expressed in control

(W303-1A at 6.5% v/v) are coloured in shades of green. Actual fold change differences are given on the right side of each gene.

2.3.2 GenMAPP expression datasets

Contain .gex files or expression datasets experiments for GenMAPP (Doniger *et al.*, 2003). These can be opened using GenMAPP (available freely at http://www.genmapp.org/) and hundreds of GO categories can be further inspected in the same form as presented in pathway images.

Appendix 2.4 Folds

Contains lists of genes in Excel format (.xls) differentially expressed between any two defined experimental conditions (in triplicate). Genes with fold difference of greater than 2 that also passed 2-way ANOVA (p-value ≤ 0.05) and used in pathway inspection, are presented.

Appendix 2.5 Microarray data gpr files

This folder contains the original microarray data in the form of .gpr files for the reference and direct design experiments.

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