The Osmotic Stress Response of Ale and Lager Yeast

Philip Andrew White

A thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy

Oxford Brookes University.

May 2004

In collaboration with, and co-supervised by Scottish Courage Brewing Ltd.

The following Figures have been omitted on request of the University –

Fig. 1.1 pg. 4

Fig. 1.2 pg. 12

Fig. 1.3 pg. 16

Fig. 1.4 pg. 19

Fig. 1.5 pg. 22

Fig. 1.7 pg. 27

For My Parents, Pauline and Trevor.

σοὶ δὲ θεοὶ τόσα δοῖεν, ὅσα φρεσὶ σῇσι μενοινᾷς,
ἄνδρα τε καὶ οἶκον, καὶ ὁμοφροσύνην ὀπάσειαν
ἐσθλήν· οὐ μὲν γὰρ τοῦ γε κρεῖσσον καὶ ἄρειον,
ἢ ὅθ' ὁμοφρονέοντε νοήμασιν οἶκον ἔχητον
ἀνὴρ ἠδὲ γυνή·

May the gods grant you all things which your heart desires, and may they give you a [wife] and a home and gracious concord, for there is nothing greater and better than this - when a man and wife keep a household in oneness of mind, a great woe to their enemies and joy to their friends, and win high renown.

Homer, The Odyssey (6.180-185)

Abstract

The Osmotic Stress Response of Ale and Lager Brewing Yeast Strains

Brewing ale (*Saccharomyces cerevisiae*) and lager (*Saccharomyces cerevisiae* (syn *S. pastorianus*)) yeast populations are subject to biological, chemical and physical stress during the brewing process. Osmotic stress is evident at discreet points throughout this industrial process, yet the impact of this stress on yeast tolerance, defence and repair has not been investigated. Haploid strains of *Saccharomyces cerevisiae* exhibit different tolerances to hyper-osmotic stress in which the solute concentration of the surrounding medium exceeds the cell's cytoplasm. The osmotolerance of ale and lager yeast to sorbitol and NaCl-induced osmotic stress has not been previously investigated but was observed to be strain, growth phase and solute dependent, reflecting inherent genetic differences in the management of this stress in all cases.

It has been postulated that the yeast vacuole plays a central role in the maintenance of physiological activity during osmotic stress. The gross morphological changes occurring in this organelle during osmotic stress were examined. The vacuole of brewing yeast strains was demonstrated to exhibit a highly fragmented morphology, independent of strain, growth phase and osmotic stress. The fragmentation of the yeast vacuole could not be correlated to one external parameter, and was therefore not observed to be a good biomarker of osmotic stress.

Saccharomyces cerevisiae exhibits a series of responses to osmotic stress. Cells may demonstrate an innate ability to withstand osmotic stress. In most instances however, osmotolerance is achieved when cells accumulate compatible solutes in order to increase intracellular osmolarity thus promoting retention of cellular water and as a result maintenance of turgor and viability. Yeast cells preferentially accumulate the polyhydric alcohol glycerol, although a detailed study on glycerol accumulation in brewing yeast strains during osmotic stress has not been previously reported.

Intracellular compatible solute accumulation was observed to be complex in brewing yeast strains. Analysis of the cytosolic pool of amino acids demonstrated that there were discreet compositional changes during osmotic stress, however no single amino acid was hyper-accumulated. Glycerol accumulation could not be correlated to the intensity of osmotic stress, however the response observed was strain, growth-phase and solute type dependent. PCR detection revealed that the elucidated genes involved in glycerol biosynthesis were present in all production brewing strains examined. Sequence analysis of the *GPD1* gene (encoding a cytoplasmic glycerol-3-phosphate dehydrogenase) revealed point mutations in the sequence of the SCB2 (lager) strain sufficient to affect the primary structure of Gpd1p but not its' functionality. In the ale strain SCB8 no such differences occurred. The unusual glycerol profile observed with both strains appeared to be a function of *GPD1* and Gpd1p production. However it was noted that glycerol export may have also contributed to this phenomenon.

Acknowledgements

This research was funded by the Morison-Inches trust and co-supervised by Scottish Courage Brewing Ltd. I would like to thank Dr Katherine Smart for her supervision and support, and Dr Alan Kennedy for his help. I would like to acknowledge the useful discussion of Professor Stefan Hohmann and Professor Lennart Adler at Göteborg University and for the provision of antibodies from the Adler lab. I would also like to give thanks to the staff and students of the Tonge building, especially to Kay Chambers for her sage advice and help, and to Jenny Brownsill for helping with microbiology matters in an emergency. Also thanks to Tony Clark for his great friendship and (sometimes funny) jokes. To my Family, without whom none of this would have been possible, especially to my parents Pauline and Trevor, for the infinite love, encouragement and support they have given. In memory of Peter Carter I give posthumous appreciation for a great mate, still remembered well and sorely missed. Last but not least, the greatest of all thanks to Alex, a support and help beyond measure, the one person above all for whom words cannot express my love and appreciation.

Abstract iv
Acknowledgements v
Abbreviations x
List of Figures xii
List of Tables xv
Publications and Presentations xvii

Chapter 1 Introduction 2
1.1 Yeast 2
1.2 *Saccharomyces* species and speciation 2
1.2.1 Ploidy and evolutionary history 4
1.3 Brewing yeast 5
1.3.1 Ale yeast 5
1.3.2 Lager strains 6
1.4 Yeast handling and brewing 6
1.4.1 Propagation 6
1.4.2 Pitching 7
1.4.2.1 Wort production 7
1.4.3 Fermentation 9
1.4.4 Cropping 10
1.4.5 Storage 10
1.4.6 Acid washing 11
1.4.7 Osmotic stress and yeast handling 11
1.5 Osmotic stress response 12
1.5.1 Osmotolerance 12
1.5.2 Osmoprotectants 13
1.5.3 Osmoadaption 13
1.5.4 Compatible solutes 14
1.5.5 Osmosensing and signal transduction 15
1.5.6 The high osmolarity glycerol (HOG) pathway 16
1.5.7 The calcineurin pathway 19
1.5.8 Stress-induced gene expression (the STRE response) 20
1.5.9 Glycerol biosynthesis 21
1.6 Impact of solute on the osmotic stress response 23
1.7 Cell size 23
1.8 Cell wall changes 23
1.9 Yeast cytology 24
1.9.1 Cell membrane 24
1.9.2 Aquaporins 24
1.9.3 Glyceroaquaporins 25
1.9.4 Vacuole 26
1.10 Objectives of this study 28

Chapter 2 Materials and Methods 30
2.1 Yeast strains 30
2.2 Bacterial strains 30
2.3 Vectors 30
2.4 Media and growth conditions 30
2.4.1 Bacteria 30
2.4.2 Yeast 31
2.5 Osmotic challenge 31
2.6 Viability 31
2.6.1 Citrate methylene violet (CMV) 32
2.6.2 Hemi-magnesium anilinonaphthalene sulphonic acid (MgANS) 32
2.6.3 Propidium iodide (PI) 32
2.7 Glycerol determination 32
2.8 Proline determination. 33
2.9 Thin layer chromatography of amino acids 33
2.10 Vacuole staining 34
2.10.1 Carboxy-DCFDA 34
2.10.2 MDY-64™ 34
2.10.3 Cell-tracker™ Blue CMAC 35
2.10.4 FUN®1 35
2.10.5 LysoTracker® Green 35
2.11 Confocal microscopy 36
2.12 Yeast genomic DNA extraction 36
2.13 Polymerase chain reaction (PCR) 37
2.13.1 Primer design 37
2.13.2 PCR reaction parameters 37
2.13.3 Thermal cycling 38
2.13.4 Quantitative (real-time) PCR 39
2.13.4.1 Primer and Taqman® probe design 39
2.14 PCR purification 39
2.15 Gel electrophoresis 40
2.16 Gene cloning 40
2.16.1 Amplification of DNA using proofreading enzymes 40
2.16.2 Gel extraction of DNA 41
2.16.3 Preparation of competent cells 41
2.16.4 Transformation of competent cells 42
2.16.5 Selection of transformants 42
2.16.6 Small scale plasmid preparation (mini-prep) 42
2.16.7 Large scale plasmid preparation (maxi-prep) 43
2.16.8 Enzymatic treatment of DNA 44
2.16.9 Ligations 44
2.17 DNA sequencing 45

2.18 *In vitro* transcription 45
2.19 Bioinformatics 45
2.20 RNA extraction and handling 45
2.20.1 Total RNA extraction 46
2.20.2 DNase treatment 46
2.20.3 RNA quantitation and storage 46
2.20.4 Reverse transcription 47
2.20.5 Formaldehyde gel electrophoresis 47
2.21 Protein extraction and analysis 47
2.21.1 Bradford assay 48
2.21.2 SDS-PAGE 48
2.21.3 Coomassie staining 49
2.21.4 Electroblot transfer of proteins 49
2.22.5 Immunodetection of immobilised protein 49

Chapter 3 The Impact of Osmotic Stress on Brewing Yeast Viability 51
3.1 Introduction 51
3.2 Results 53
3.2.1 Is osmotic stress tolerance strain dependent? 53
3.2.2 Is osmotic stress tolerance growth phase dependent? 61
3.2.3 Is osmotic stress tolerance solute dependent? 70
3.3 Discussion 76

Chapter4 Vacuolar Morphology and Changes in Brewing Yeast 83
4.1 Introduction 83
4.2 Results 86
4.2.1 Assessment of the utility and efficacy of yeast vacuolar dyes 86
4.2.1.1 MDY-64 86
4.2.1.2 LysoTracker™ yellow 88
4.2.1.3 FUN®1 89
4.2.1.4 C-DCFDA 90
4.2.1.5. Colocalisation using multiple stains 91
4.2.1.5.1 Cell Tracker™ blue CMAC and C-DCFDA 92
4.2.1.5.2 FUN®1 and MDY-64 92
4.2.1.5.3 MDY-64 and C-DCFDA 93
4.2.2 Vacuolar morphology of non-stressed cells 94
4.2.3 Vacuolar morphology of sorbitol-stressed cells 95
4.2.4 Growth phase and cell cycle dependent changes in vacuolar morphology. 96
4.3 Discussion 102

Chapter 5 Compatible Solute Accumulation in Osmotically Stressed *Saccharomyces* Species 110

5.1 Introduction 110

5.2 Results 113
5.2.1 Intracellular glycerol time course 113
5.2.2 Do brewing yeast strains accumulate glycerol during osmotic stress? 115
5.2.3 Intracellular glycerol accumulation and growth phase. 118
5.2.5 Intracellular glycerol accumulation and solute type. 121
5.2.6 Intracellular amino acid accumulation in brewing yeast 123

5.3 Discussion 129

Chapter 6 Osmotically-induced Gene Expression in Brewing Yeast Strains 135

6.1 Introduction 135

6.2 Results 139
6.2.1 Detection of glycerol biosynthesis genes in haploid and brewing strains 139
6.2.1.1 PCR primer design 139
6.2.1.2 PCR detection of genes 140
6.2.2 Cloning of the *GPD1* gene 142
6.2.3 Sequencing and sequence alignment of *GPD1* 146
6.2.4 Quantitative RT-PCR analysis of *GPD1* expression. 148
6.2.5 Western blot analysis of Gpd1p expression 151
6.2.6 Glycerol export 153

6.3 Discussion 155

Chapter 7 Conclusions 161

7.1 Conclusions 161

7.2 Future Work 165

References 167

8.1 References 167

Abbreviations

°C	Degrees Celsius
bp	Base-pairs
BSA	Bovine serum albumin
ca.	About/approximately (Lat. *circa*)
$CaCl_2$	Calcium chloride
C-DCFDA	5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate
cDNA	complementary DNA
CMV	Citrate-buffered methylene violet
kDa	Kilo Dalton
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid
et al.	And others (Lat. *et alli*,)
FRET	Fluorescence resonance energy transfer
g	Grams
GFP	Green fluorescent protein
HOG	High osmolarity glycerol (pathway)
kbp	Kilobase-pairs
kDa	Kilodalton
M	Molar
MAP(K)	Mitogen activated protein (kinase)
MgANS	Hemi-Magnesium1-anilinonaphthalene-8-sulfonic acid
mg	Milligram
ml	Milliliter
mm	Millimolar
mRNA	Messenger RNA
NaCl	Sodium Chloride
NAD+	Nicotinamide adenine dinucleotide (oxidised form)
Nm	Nanometre
ng	Nanogram
ORF	Open reading frame
PBS	Phosphate buffered saline buffer
PCR	Polymerase chain reaction
PI	Propidium iodide
pl	Plural
Pp	Pages
p.s.i	Pounds per square inch
RNA	Ribonucleic acid
RFP	Red fluorescent protein
r.p.m	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STRE	Stress responsive element
Sp.	Species
TAE	Tris/acetate/EDTA
TLC	Thin layer chromatography
μg	Microgram

μl	Microlitre
μm	Micrometre
w/v	Weight for volume
V	Volts

List of Figures

Figure 1.1. A cladogram indicating the phylogenetic arrangement of yeast species. 4
Figure 1.2. The sources of osmotic stress in the brewing process. 12
Figure 1.3. The five currently elucidated MAP kinase cascades in *S. cerevisiae*. 16
Figure 1.4. The HOG pathway in *S. cerevisiae*. 19
Figure 1.5. Glucose metabolism in *S. cerevisiae*. 22
Figure 1.7. Diagrammatic representation of vacuolar changes during replication 27
Figure 3.1. Tolerance of exponential phase lager, ale, and haploid yeast strains to sorbitol induced osmotic stress. 55
Figure 3.1.1 Examples of MgANS and propidium iodide viability staining in the ale strain SCB8. 56
Figure 3.2. Mortality profiles of exponential phase lager, ale and haploid yeast strains subjected to sorbitol induced osmotic stress.. 57
Figure 3.3. Tolerance of stationary phase lager, ale, and haploid yeast strains to sorbitol induced osmotic stress 64
Figure 3.4. Mortality profiles of stationary phase lager, ale and haploid yeast strains subjected to sorbitol induced osmotic stress. 65
Figure 3.5. Tolerance of stationary phase lager, ale, and haploid yeast strains to NaCl induced osmotic stress 73
Figure 3.6. Tolerance of exponential phase lager, ale, and haploid yeast strains to NaCl induced osmotic stress 75
Figure 4.1. Assessment of MDY-64 as a tonoplast specific marker. 87
Figure 4.2. Assessment of MDY-64 as a tonoplast specific marker. 87
Figure 4.3. Assessment of MDY-64 as a tonoplast specific marker. 88
Figure 4.4. Assessment of LysoTracker ™ yellow as a vacuole specific marker 88
Figure 4.5. Assessment of LysoTracker ™ yellow as a vacuole specific marker 89
Figure 4.6. Assessment of FUN®1 as a vacuole specific marker. 89
Figure 4.7. Assessment of C-DCFDA as a vacuole specific marker. 90
Figure 4.8. Assessment of C-DCFDA as a vacuole specific marker. 91
Figure 4.9. Assessment of C-DCFDA as a vacuole specific marker. 91
Figure 4.10. Assessment of the ability of C-DCFDA and CellTracker™ Blue CMAC to co-localise to the vacuole. 92
Figure 4.11. Assessment of the ability of FUN®1 and MDY-64 to co-localise to the vacuole. 93
Figure 4.12. Early stationary phase SCB8 (ale) cells stained with MDY-64 and C-DCFDA 94
Figure 4.13. SCB2 (lager) stationary phase YPD grown cell stained with C-DCFDA and MDY-64 95
Figure 4.14. SCB1 (lager) stationary phase 30% sorbitol stressed cell stained with C-DCFDA and MDY-64. 96
Figure 4.15. SCB5 (ale) stationary phase 30% sorbitol stressed cell stained with C-DCFDA and MDY-64. 96
Figure 4.16. Late exponential phase SCB8 (ale) cells stained with C-DCFDA and MY-64 97
Figure 4.17. Late exponential phase SCB8 (ale) cells stained with C-DCFDA and MY-64 97

Figure 4.18. Exponential phase SCB8 (ale) cells stained with C-DCFDA and MDY-64 98
Figure 4.19. Exponential phase SCB8 (ale) cells stained with C-DCFDA and MDY-64 98
Figure 4.20. Exponential phase SCB8 (ale) cells stained with C-DCFDA and MDY-64 99
Figure 4.21. SCB8 (ale) late stationary phase cells stained with MDY-64. 100
Figure 4.22. SCB8 (ale) late stationary phase cells stained with MDY-64. 100
Figure 4.23. SCB8 late stationary phase cells stained with C-DCFDA and MDY-64 101
Figure 4.24 A composite diagram composed from images of actively replicating ale (SCB8) cells indicating the changes in vacuolar morphology 101
Figure 5.1. Zwitterionic quaternary ammonium compounds and their betaine derivatives utilised as compatible solutes in a range of organisms..... 111
Figure 5.2. SCB1 (lager) strain intracellular glycerol concentrations following exposure to 24% (w/v) sorbitol at 25°C for 48 hours 114
Figure 5.3. Intracellular glycerol levels in exponentially grown lager strain (SCB1-SCB4) populations subjected to sorbitol induced osmotic stress 116
Figure 5.4. Intracellular glycerol levels in exponentially grown ale (SCB5-SCB8) and laboratory haploid strain (S288C) populations subjected to sorbitol induced osmotic stress 117
Figure 5.5. Intracellular glycerol levels in stationary grown lager strain (SCB1-SCB4) populations subjected to sorbitol induced osmotic stress 120
Figure 5.6. Intracellular glycerol levels in stationary grown ale (SCB5-SCB8) and laboratory haploid strain (S288C) populations subjected to sorbitol induced osmotic stress. 120
Figure 5.7. Intracellular glycerol levels in stationary grown lager (SCB1 and SCB2) and ale (SCB5 and SCB8) strains populations.subjected to NaCl induced osmotic stress..... 122
Figure 5.8. Intracellular glycerol levels in exponentially grown lager (SCB1 and SCB2) and ale (SCB5 and SCB8) strains subjected to NaCl induced osmotic stress..... 123
Figure 5.9a. Silica TLC plate with standard amino acid solutions and extract of SCB1 12% sorbitol stressed cells. 125
Figure 5.9b. Silica TLC plate with standard amino acid solutions and extract of SCB8 12% sorbitol stressed cells. 125
Figure 5.10. Standard curve for proline absorbance at 520nm. 128
Figure 5.11. Intracellular proline accumulation in exponential phase SCB1 (lager) cells exposed to sorbitol stress, compared to a baseline of intracellular proline exhibited by non-stressed cells. 128
Figure 6.1. 0.7% agarose/TAE gel electrophoresis of PCR products formed using primers specific for *GPD1* (1176 bp) in strains S288C, SCB1, SCB2, SCB5 and SCB8. 141
Figure 6.2 0.7% agarose/TAE gel electrophoresis of PCR products formed using primers specific for *GPD1* (1176 bp) using *Pfu* Turbo ™ DNA polymerase in strains SCB2 (lager) and SCB8 (ale) 143
Figure 6.3 0.7% agarose/TAE gel electrophoresis of PCR products formed using primers specific for *GPD1* (1176 bp) using *Pfu* Turbo ™ following *BamHI*/*Not*I digests in strains SCB2 (lager) and SCB8 (ale) 144

Figure 6.4 Positive clone screening for SCB2 (lager) transformants..... 144
Figure 6.5 Positive clone screening for SCB2 (lager) transformants..... 145
Figure 6.6 Positive clone DNA screening for SCB2 (lager) and SCB8 (ale) transformants digested using *Bam*HI. 145
Figure 6.7. Positive clone DNA screening for SCB2 (lager) and SCB8 (ale) transformants digested using *Bam*HI and *Not*I. 146
Figure 6.6. Sequence of SCB2 (lager) *GPD1* translated into amino acid sequence 147
Figure 6.7. A phylogenetic tree demonstrating the relationships between the *GPD1* gene from 6 yeast species and SCB8 (ale) and SCB2 (lager)brewing yeast strains. 148
Figure 6.8. Denaturing (formaldehyde) gel electrophoresis of RNA extracted from brewing yeast cells. 149
Figure 6.9. *GPD1* expression in SCB2 (lager)cells as determined quantitative RT-PCR. 150
Figure 6.10 *GPD1* expression in SCB8 (ale) cells as determined quantitative RT-PCR 150
Figure 6.11. SDS-PAGE of yeast proteins extracted from stationary phase lager, ale and laboratory haploid cells. 152
Figure 6.12. Western-blot analysis of SCB2 (lager) yeast Gpd1p expression..... 153
Figure 6.13. Western-blot analysis of SCB8 (ale) yeast Gpd1p expression. 153
Figure 6.14. Intracellular and extracellular glycerol profiles for osmotically stressed exponentially grown SCB2 (lager) cell populations. 154
Figure 6.15. Intracellular and extracellular glycerol profiles for osmotically stressed exponentially grown SCB8 (ale) cell populations. 155

List of Tables

Table 1.1. Compositional analysis of a typical lager wort. 8
Table 2.1. Formula of PCR reaction mixture 38
Table 2.2. Generalised parameters for thermal cycling 39
Table 2.3. Generalised formula of restriction enzyme digests. 44
Table 3.0 Some commonly used fluorescent viability dyes and their proposed modes of action 52
Table 3.1. Relative percentages of sorbitol required to elicit a substantial (20%) reduction in viability (tolerance limit) for exponential phase lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) yeast strains 58
Table 3.2. Relative percentages of sorbitol required to elicit a reduction in viability by 50% (LD50) for exponential phase lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) yeast strains 58
Table 3.3 F test analysis of viability of ale and lager cells subjected to 12% sorbitol stress assessed using citrate buffered methylene violet. 59
Table 3.4. t-test analysis of viability of ale and lager cells subjected to 12% sorbitol stress 60
Table 3.5. F test analysis of viability of ale and lager cells subjected to 30% sorbitol stress assessed using citrate buffered methylene violet. 60
Table 3.6. t-test analysis of viability of ale and lager cells subjected to 30% sorbitol stress assessed using citrate buffered methylene violet. 60
Table 3.7. Relative percentages of sorbitol required to elicit a substantial (20%) reduction in viability (tolerance limit) for stationary phase lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) yeast strains 66
Table 3.8. Relative percentages of sorbitol required to elicit a reduction in viability by 50% (LD50) for exponential phase lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) yeast strains 66
Table 3.9. F test analysis of viability of lager cells subjected to 12% sorbitol stress in the exponential and stationary phases of growth 67
Table 3.10. F test analysis of viability of lager cells subjected to 30% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet 68
Table 3.11. t-test analysis of viability of lager cells subjected to 12% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet 68
Table 3.12. t-test analysis of viability of lager cells subjected to 30% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet 68
Table 3.13. F test analysis of viability of ale cells subjected to 12% sorbitol stress in exponential and stationary phases of growth assessed using citrate methylene violet 69
Table 3.14. F test analysis of viability of ale cells subjected to 30% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet 69

Table 3.15. t-test analysis of viability of ale cells subjected to 12% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.....69
Table 3.16. t-test analysis of viability of ale cells subjected to 30% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.....70
Table 4.1. Fluorescent dyes used in the detection of vacuolar structure.....85
Table 5.1. Intracellular glycerol accumulation for sorbitol stressed exponential phase lager (SCB1-4) ale (SCB5-8) and laboratory haploid (S288C) cell populations..117
Table 5.2. Intracellular glycerol levels in lager (SCB1-4) ale (SCB5-8) and laboratory haploid (S288C)strains stationary grown populations.....121
Table 5.3. Amino acids detected by TLC during a series of stress experiments on exponential lager (SCB1) and ale (SCB8) cells subject to a range of sorbitol concentrations for 48 hours.....127
Table 6.1 Genes encoding proteins involved in glycerol production and dissimilation.....138
Table 6.2 Oligonucleotide forward and reverse primers utilised in this study.....139
Table 6.3. Genes involved in glycerol biosynthesis screened for in this study using PCR.....141

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Yeast

Yeast belong to the taxonomic grouping the fungi. They are distinct from most other fungi in that they possess a unicellular type of thallus. Some yeast, especially animal (e.g. *Candida sp*) pathogens exhibit dimorphism between unicellular and mycelial (septate filamentous) forms. Yeasts may reproduce by budding or fission (Kreger van Rij and Veenhuis, 1975; Kurtzman, 1984; Kreger van Rij, 1987;), and can be further differentiated from related forms according to their biochemistry (Barnett, 1968; Fiol, 1976; Alegri *et al.*, 1978; Sobczak, 1985), physiology (Wickerham, 1951; Van der Walt, 1970) and genetic differences (Meyer and Phaff, 1969; Phaff, 1984; Lieckfeldt *et al.*, 1993; Barberio *et al.*, 1994; Kurtzman, 1994; Tornai-Lehoczki and Dlauchy, 1996; Josepa *et al.*, 2000). The *Saccharomycetaceae* is one of the most economically important families in yeast classification as it contains the genus *Saccharomyces.* It has been demonstrated that the genus *Saccharomyces* is phylogenetically diverse as demonstrated by partial 18S and 26S rRNA sequences (Ando *et al.*, 1996).

1.2 *Saccharomyces* species and speciation

The perennial and thorny question of *Saccharomyces* species taxonomic location has been addressed in many ways. Industrial strains of yeast are classified using methodology that differentiates between what are essentially discreetly dissimilar organisms (section 1.3). The industrial importance of members of the genus *Saccharomyces* and the utilisation of individual species as model organisms for the study of a plethora of applications has led to a high level of interest in comparative phylogenetic analysis of *Saccharomyces cerevisiae* and closely related yeast (Valente *et al*, 1996; Masneuf *et al.*, 1996; Jespersen *et al.*, 2000; Ramos *et al.*, 2001). *S. cerevisiae* has a number of fairly closely related species in the genii *Kluyveromyces*, *Zygosaccharomyces* and *Torulaspora* (Seoighe and Wolfe, 1999), and more obviously within the genus *Saccharomyces*.

Recent research has centred on the elucidation of the relationship between four physiologically and genetically comparable *Saccharomyces* species namely, *S. bayanus, S. pastorianus, S. paradoxus and S. cerevisiae*, known collectively as the *Saccharomyces sensu stricto* (*Lat.* in a narrow sense) species (Seoighe and Wolfe, 1999; Keogh *et al.*, 1998). It has been demonstrated that 18S ribosomal RNA phylogenetic analysis aligns these four species similarly, with very few genetic differences apparent (figure 1.1). Furthermore it has been demonstrated by Montrocher *et al.*, (1998) that the *Saccharomyces sensu stricto* species are readily differentiated into two subgroups according to the sequence of specific intergenic regions of their ribosomal RNA encoding DNA (rDNA). The so-called '*cerevisiae*' cluster contains *S. cerevisiae* and *S.paradoxus* whereas the '*bayanus*' cluster contains *S. bayanus* and *S. pastorianus* (Montrocher *et al.*, 1998).

It is possible that the *sensu stricto S. cerevisiae* species achieved speciation due to horizontal gene transfer during the formation of intermediate hybrid strains. Indeed it is now widely accepted that *S. pastorianus* is derived from an archaic hybridisation event between its sibling species *S. cerevisiae* and *S. bayanus* (Martini and Martini, 1987; Ryu *et al.*, 1996; Seoighe and Wolfe, 1999). Many of the biochemical and physiological differences that separate the *S. cerevisiae sensu stricto* species are thought to be the result of horizontal gene transfer and reciprocal translocations (Lockhart *et al.*, 2002). Furthermore, Colson *et al.*, (2004) have demonstrated that chromosomal translocations confer an evolutionary benefit adding further evidence for this mode of speciation. The resulting small genetic differences are sufficient to differentiate between species or yeast strains on the basis of their phenotypes.

Figure 1.1. A cladogram indicating the phylogenetic arrangement of yeast species based on conservation of 18S Ribosomal RNA sequences (Keogh *et al.*, 1998)

1.2.1 Ploidy and evolutionary history

Evolutionary changes in chromosome copy number (ploidy) can result from random deletions, transpositions, inversions or duplications (Keogh *et al.*, 1998). The selective pressures upon the specific organism are variable, and in *S. cerevisiae* it is suggested that an event occurred somewhere in evolutionary history which resulted in the formation of the current diploid (wild-type) state. Furthermore it is proposed that the *S. cerevisiae* ancestral lineage was tetraploid (*4n*) after an ancient genome duplication event 10^8 years ago (Fischer *et al.*, 2000), and the current wild type is essentially a degenerative tetraploid (Wolfe and Shields, 1997; James *et al.*, 1997; Keogh *et al.*, 1998).

Laboratory strains of *S. cerevisiae* such as the fully sequenced S288C (Goffeau *et al.*, 1996; Goffeau *et al.*, 1997; Mewes *et al.*, 1997) are stable haploids (n) being derived from diploid strains following sporulation and isolation. Brewing strains (section 1.3) are almost exclusively polyploid (variable ploidy more than *2n*) (Lewis *et al.*, 1976; Aigle *et al.*, 1984; Kielland-Brandt, 1994) and sometimes aneuploid (one or

more chromosome showing aberrant copy number) as a result of generations of specific selective pressure within the brewing process (Kielland-Brandt, 1994).

1.3 Brewing yeast

As with many taxonomic classifications, the naming of *Saccharomyces* species has been the subject of much discussion; originally 41 species of *Saccharomyces* were identified (Lodder, 1970). It is now generally accepted that there are seven true *Saccharomyces* species (Kreger van Rij, 1987). Brewing yeasts have been previously collectively classified as the species *S. cerevisiae* (Quain, 1986; Molzahn, 1993; Vaughan-Martini and Martini, 1995). The exact distinction between *S. cerevisiae* 'strains' has also been the subject of some conjecture and many of the differences in metabolism (Hammond, 1993), and flocculation characteristics (Stewart, 1975; Stewart and Russell, 1993) have led to recent reclassification (sections 1.3.1 and 1.3.2).

1.3.1 Ale yeast

The historical classification of ale yeast as *S. cerevisiae* 'top-fermenting' yeast (Boulton and Quain, 2001) has been reappraised many times, and this physiological parameter alone does not currently serve as a good mechanism for differentiation. Ale brewing has traditionally focussed upon the aggregation and collection of the yeast biomass at the top of the fermentation vessel after fermentation (hence the term 'top-fermenting' strains). The current use of cylindroconical fermentation vessels and 'bottom cropping' (collection of yeast biomass from the cone of the vessel) of ale strains specifically selected for the purpose has blurred the lines of categorisation (Hammond, 1993). A better consideration for isolation has been the inability of these strains to utilise the disaccharide melibiose (Kielland-Brandt, 1994), although the advances in molecular biology indicated in section 1.2 will undoubtedly prove invaluable in characterisation of these strains. Ale strains of brewing yeast have almost universally always been classified as *S. cerevisiae* (Hammond, 1993).

1.3.2 Lager strains

Lager or bottom-fermenting strains can be easily differentiated from ale strains by their respective capacities to grow at elevated temperatures. Ale strains will efficiently grow at 37°C, whereas lager yeast will not grow above 34°C (Vidal-Leiria *et al.*, 1979; Kreger Van Rij, 1987). Lager strains are able to utilise the disaccharide melibiose by cleavage with the enzyme α-galactosidase, and are therefore termed melibiose positive. These characteristics have led to the classification of *S. cerevisiae* lager strains as *Saccharomyces carlsbergensis* (Hough *et al.*, 1995), *Saccharomyces uvarum* (Lodder, 1970) and the more recently, and widely accepted *Saccharomyces cerevisiae* (syn. S. *pastorianus*) (Josepa *et al., 2000*; James *et al.*, 1997; Tamai *et al.*, 1998). Some debate still exists however, and lager strains in much of the related brewing literature are still referred to as melibiose positive *S. cerevisiae* or simply lager *S. cerevisiae* strains (Hammond, 1993; Naumov, 1996; Casey, 1996).

1.4 Yeast handling and brewing

Yeast handling and management are critical to the brewing process, and alteration in the handling procedures will result in yeast of variable physiological condition. A deterioration of yeast quality adversely affects final product quality and influences process parameters such as attenuation and fermentation times (Boulton, 1991). Yeast handling represents the processes of propagation (section 1.4.1), pitching (1.4.2), fermentation (1.4.3), cropping (1.4.4), storage (1.4.5) and acid washing (1.4.6).

1.4.1 Propagation

Propagation is the process of replacement of recycled yeast biomass with fresh cultures. Propagation of yeast serves to introduce pure cultures back into the fermentation process that are in an optimal physiological state. This process also serves to avoid the accumulation of contaminant organisms, and succession of mutants or variants generated as a consequence of genetic drift due to continued use in consecutive fermentations (Kirsop, 1991, Hammond, 1993; Quain 1995).

Another major consideration during propagation is when bottom cropped fermentations with high hop contents are used; in this case a large accumulation of trub

(suspended solids) may occur (Kennedy, 2000). The reintroduction of newly propagated cultures restores rapid fermentation times, and desired beer profiles over a number of generations (Hulse *et al.*, 2000). New cultures are grown from laboratory stocks and increased in biomass by serial transfer into larger volumes of wort. A mirrored reduction in aeration and growth temperatures with increasing propagation volumes ensures that final propagations are acclimatised to fermentation vessel conditions (Kennedy, 2000).

1.4.2 Pitching

Pitching is the term used to describe the addition of the required yeast biomass to cooled brewers wort in the fermentation vessel (Stewart 1996). During pitching, yeast is aerated with pure oxygen or air, or added to oxygenated wort. The supply of oxygen to the yeast biomass at the start of fermentation is important in order that the yeast are able to synthesise sterols and unsaturated fatty acids (Wilson and McLeod; 1976; Parks, 1978; Nishino *et al.*, 1980; Lorenz and Parks; 1991) and therefore proliferate.

1.4.2.1 Wort production

Wort is the name given to the complex medium utilised in the brewing process, which contains all of the fermentable carbohydrates required in the production of alcohol, and all of the precursor molecules that eventually become important flavour compounds. The majority of the fermentable carbohydrates in wort are derived from barley (*Hordeum vulgare*); however other components such as sugar syrups (termed adjuncts) may be used in some cases (Boulton, 1991b). The first stage in wort production is termed malting, and is a process designed to allow barley grains to commence germination with the resultant production of a range of amylases and proteases (Boulton, 1991b).

The germination process is stopped during kilning, in which barley grains are exposed to temperatures ranging anywhere from 70-110°C dependent upon flavour requirements (Lewis and Young, 1995). The dried malt is milled to form a coarse residue termed grist, which is subsequently steeped in warm water in order to extract carbohydrates and other soluble elements (Boulton, 1991b) and the unwanted malt

debris is removed. It is at this stage that hops (the unfertilised and dried flowers of *Humulus lupulus*) are added to impart flavour, and the entire mixture is boiled to sterilise the medium and to deactivate barley derived degradative enzymes (Lewis and Young, 1995).

The wort is then cooled to a suitable temperature for fermentation, remaining unwanted solids (termed trub) are removed and the wort is aerated to provide oxygen for the impending fermentation. Wort composition is dependent entirely upon the raw materials utilised and as a result a high degree of variation is exhibited between batches (Kennedy *et al.*, 1997). To date, attempts to produce synthetic wort have not proven entirely successful; however Kennedy *et al.*, (1997) have produced a defined wort medium which was demonstrated to be satisfactory when used in conjunction with lager, but not ale yeast strains. The difficulty in reproducing wort artificially is that this medium is inherently complex as indicated in table 1.1

Table 1.1. Compositional analysis of a typical lager wort, (data kindly supplied by Scottish Courage Brewing Limited).

Component	*Units*	*Concentration*
Calcium	mg/l	55
Sodium	mg/l	70
Potassium	mg/l	318
Magnesium	mg/l	93
Fructose	g/l	1
Glucose	g/l	6.1
Sucrose	g/l	2.9
Maltose	g/l	38.8
Maltotriose	g/l	8
Chloride	mg/l	361
Nitrate	mg/l	11.2
Phosphate	mg/l	686
Sulphate	mg/l	396
Free amino nitrogen	mg/l	136
Iron	mg/l	0.14
Copper	mg/l	0.069
Zinc	mg/l	0.38

1.4.3 Fermentation

Pitching of yeast cells into brewers' wort is immediately followed by a lag phase, during which many cellular changes occur within the cells in preparation for growth and division. *S. cerevisiae* and *S. cerevisiae* (syn. *S. pastorianus*) will grow fermentatively in either anaerobic conditions, or where there is a surfeit of one or more exogenous carbon sources that can be utilised (a process known as the Crabtree effect) (Hommes, 1966; Postma *et al.*, 1989; Van Dijken *et al.*, 1993). The array of carbohydrates that are fermentable is wide and includes simple hexose sugars, disaccharides and some trisaccharides, although higher polymers of glucose are not assimilable (Stewart and Russell, 1993). Furthermore the range of carbohydrates that are utilised is dependent upon the species (and sometimes strain) of yeast employed (Boulton and Quain, 2001).

The regularity with which fermentation is performed in the brewing industry belies the complexity of the system. Apart from the intricate uptake and assimilation processes associated with carbohydrates in brewery worts, a range of other nutrients including nitrogenous compounds (Horak, 1986), lipids (Finnerty, 1989) and metal ions (Lie and Jacobsen, 1983; Lentini *et al.*, 1990) (section 1.4.2.1) are utilised by the yeast during fermentation. Indeed the biochemistry of fermentation is multifaceted and involves intricately linked degradation pathways, which result in the breakdown of wort components and the formation of yeast biomass and metabolic by-products (reviewed in Boulton and Quain, 2000).

At the end of the fermentation, assimilable sugars are consumed, the ethanol content of the green beer is elevated, and a range of esters and other flavour compounds are present. Flavour compounds are essentially yeast metabolites, and are extremely diverse and it has been demonstrated that over 200 compounds contribute to beer flavour (Meilgaard, 1975). Fermentation performance of a particular strain of brewing yeast is dependent upon a set of prerequisites, including efficient utilisation of fermentable carbohydrates (Stewart 1996) and adequate cell proliferation rates (Smart, 1997).

1.4.4 Cropping

Cropping is the term used to describe the collection of the yeast biomass from the fermentation vessel at the end of fermentation. As previously described, lager yeast is cropped from the bottom of the fermentation vessel, and ale strains have traditionally been recovered from the top of the fermentation vessel (Boulton and Quain, 2001). The use of cylindroconical fermentation vessels, and ale strains specifically selected for sedimentation into the cone of the vessel has resulted in many breweries adopting bottom cropping regimes for both ale and lager fermentations (O'Connor-Cox, 1997). Cropped yeast to be used in subsequent fermentations should have a high viability and be free from biological contaminants (Stewart, 1996). Furthermore, the amount of time that the yeast biomass is resident in the fermentation vessel at the end of fermentation is critical if the yeast is to be used in subsequent fermentations. The cone of the cylindroconical vessel is a highly stressful environment and it is recommended that the yeast is removed quickly to prevent a loss of viability (Loveridge *et al.*, 1999).

1.4.5 Storage

Yeast cropped at the end of the fermentation is usually stored for a period of time before re-use in a subsequent fermentation, the biomass is usually stored as a pressed cake or as a slurry (Knudsen 1985, Boulton 1991b). The conditions under which yeast are stored are highly influential in maintaining the physiological condition, viability and thus fermentative capacity of the biomass. The recommended conditions for storage in the brewery are a temperature below 4°C, with minimal agitation and as a slurry under beer or water (Boulton, 1991a; O'Connor-Cox, 1998; Smart 1999) in order to keep physiological activity low. It has been suggested that glycogen is the main storage carbohydrate in brewing yeast, and low levels in stored yeast may result in poor fermentation performance upon use in a following fermentation (Quain *et al.*, 1981). By the same token, higher glycogen levels in yeast cells cropped at the end of the fermentation will confer upon the cells the ability to better withstand storage conditions.

It is paramount to reduce metabolic activity in order that vital intracellular stores are not depleted, therefore yeast is routinely only stored for 3-5 days and this reduces the risk of a loss of both viability and vitality. When stored under beer, the yeast biomass is subjected to a definite osmotic stress coupled with ethanol stress (Odumeru

et al., 1992). The storage of yeast under beer however reduces the loss of vitality due to nutrient stress (Quain and Tubb, 1982), however prolonged storage will unquestionably lead to starvation (Knudsen, 1985).

1.4.6 Acid washing

Acid washing is a process used in the brewing industry to eliminate bacterial contaminants most notably *Pediococcus* spp and *Lactobacillus* spp which are less tolerant to low pH than brewing yeast. (Boulton and Quain, 2001). This process may be undertaken as a matter of course in some breweries, although there are important ramifications from the point of view of yeast physiology (Simpson and Hammond, 1989). Acid washing is conducted at low temperatures (ca. 4°C) as it has been demonstrated that high temperatures (and ethanol concentrations) have detrimental effects upon cell physiology (Fernandez *et al.*, 1993). Both ale and lager strains are resistant to acid washing conditions under the recommended parameters (Simpson and Hammond, 1989). It has also been noted that acid washing regimes affect the flocculation characteristics of some strains (Wilcocks and Smart, 1995).

1.4.7 Osmotic stress and yeast handling

During the brewing process, there are three major sources of osmotic stress (figure 1.2.). Acid washing regimes impart osmotic stress upon yeast, mainly due to the abundance of dissociated H^+ ions. Pitching in wort, a complex and highly concentrated medium provides another source of osmotic stress in the brewery, which is further exacerbated by the use of high gravity (or higher solute) worts (Panchal and Stewart, 1980). Storage of yeast also provides a source of osmotic stress especially where yeast is maintained underneath beer, which also represents a complex and concentrated medium.

Figure 1.2. The sources of osmotic stress in the brewing process, adapted according to Panchal and Stewart (1980) and Hammond *et al.*, (2001)

1.5 Osmotic stress response

Brewing yeast is subjected to biological, chemical and physical stress during fermentation and yeast handling (Smart, 2000). It has been recognised that the transfer of yeast from slurry storage to pitching environments represents a potential source of osmotic stress, exacerbated by the use of high gravity worts (Panchal and Stewart, 1980). Fundamentally two forms of osmotic stress are evident: hyperosmotic stress which occurs when the external medium is more concentrated than cellular fluids (leading to a net efflux of water), and hypo-osmotic stress which occurs when there is a drop in external osmolarity (resulting in influx of water) (Hohmann, 1997). Osmoregulation in yeast is dependent upon sensing external stimuli and the resultant changes in physiology, biochemistry and other cellular functions. There are two forms of "response" which any cell may apply following exposure to osmotic challenge: osmotolerance (section 1.5.1) and osmoadaption (section 1.5.3)

1.5.1 Osmotolerance

Eukaryotic and prokaryotic cells exposed to hyperosmotic stress utilise a range of strategies to withstand these stressful conditions. Cells may simply possess an innate ability to resist an increase in external solute conditions (be osmotolerant); in some cases this will involve the accumulation of osmoprotectant molecules (1.5.2)

1.5.2 Osmoprotectants

An osmoprotectant molecule is specifically defined as a macromolecule that can stabilise cellular membranes, enzymes and other proteins and possibly nucleic acids, with little affect on the intracellular water potential (Hernandez-Saavedra *et al.*, 1995). One example of a putative osmoprotectant molecule in yeast cells is trehalose (a dimeric form of D-glucose (α-D-glucopyranosyl α-D- glucopyranoside)) (Wiemken, 1990). Trehalose originally identified in 1832 from the fungal pathogen *Claviceps purpurea* as 'mycose' (Singer and Lindquist 1998) has been observed in many desiccation tolerant organisms and many so-called resurrection plants (Singer and Lindquist 1998). It also appears to play a role in thermotolerance and a range of other stresses (Wiemken, 1990). Interestingly, the major biological function of this non-reducing disaccharide appears to be in membrane stabilisation during desiccation (Crowe *et al.*, 1988; Leslie *et al.*, 1994; Leslie *et al.*, 1995) and not as an organic osmolyte (Wiemken, 1990).

In bacteria it has been observed that predominantly amino acids serve as osmoprotectants, *Bacillus subtilis* for example demonstrates increased osmotolerance when proline is supplied exogenously (Van Blohn *et al.*, 1997). Indeed Van Blohn *et al.*, (1997) have demonstrated that the proline uptake system in *B. subtilis* is positively upregulated upon exposure to osmotic stress. The accumulation of intracellular proline is also linked with an increase in intracellular osmolarity, and is thus considered to be a true compatible solute (section 1.5.4).

During osmotic stress, *B. subtilis* has also been associated with the uptake of the trimethylated glycine derivative, glycine betaine, when exogenously supplied (Kempf and Bremer 1995), which has been elucidated as a powerful osmoprotectant in a range of organisms (Csonka and Hanson, 1991; Lucht and Bremer 1994, Hanson *et al.*, 1994). This uptake mechanism is observed to work as an immediate response mechanism to an increase in external osmolarity, due to the relatively slow rate of *de novo* synthesis (Poolman and Glaasker 1998).

1.5.3 Osmoadaption

Osmoadaption represents the processes by which cells adjust their normal physiology in order to survive (if not to proliferate) in conditions of adverse water potential (Poolman and Glaasker 1998,). In many instances this involves the

accumulation of one or more types of molecule, termed *osmoticum* (pl = *osmotica*), within the cell in order to increase intracellular osmotic potential, and thus prevent cellular water loss (Yancey *et al.*, 1982, Wegmann, 1986; Blomberg and Adler, 1992, Hernández-saavedra *et al.*, 1995). Osmotica can be further categorised according to their specific effects upon cellular physiology. One subclass of osmotica, the compatible solutes (section 1.5.4) have very little effect on normal cellular functioning when accumulated at high levels (Poolman and Glaasker 1998).

1.5.4 Compatible solutes

The accumulation of compatible solutes by a range of eukaryotes and prokaryotes represents a fundamental response to hypertonic conditions. These solutes are sufficiently able to bind water molecules (Galinski *et al.*, 1997) and thus promote either retrieval of water from the environment, or prevent any further loss of intracellular water. Compatible solutes can be accumulated by the cell in large quantities, without any deleterious effect on cellular functioning, by protein denaturation, membrane degradation, or a reduction in enzyme activity (Gilbert *et al.*, 1998).

The range of compatible solutes utilised by an array of organisms is extensive and includes simple ions (K^+, Na^+), sugars (trehalose, raffinose) (Gilbert *et al.*. 1998), amino acids and their derivatives (proline, glutamic acid) (Poolman and Glaasker, 1998), quaternary amines (glycine betaine, carnitine) (Welsh and Herbert, 1999) and ectoines (tetrahydropyrimidines) (Galinski and Trüper, 1994). In most cases the compatible solute accrued is either non-charged or zwitterionic, as these molecules are more favourable to protein stabilisation (Poolman and Glaasker, 1998).

It has been demonstrated that yeasts appear to preferentially accumulate sugar alcohols and in *S. cerevisiae*, the role of glycerol has been examined intensively (Panchal and Stewart, 1980; Hohmann, 1997; Hounsa *et al.*, 1998). It has been demonstrated that haploid laboratory *S. cerevisiae* cells exposed to osmotic stress accumulated glycerol intracellularly (Brown, 1978; Reed *et al.*, 1987; Bellinger and Larher, 1987; Meikle *et al.*, 1991; Blomberg and Adler, 1992). Glycerol accumulation in osmotically stressed *S. cerevisiae* cells may be due to *de novo* synthesis, reduced dissimilation, increased uptake of an exogenous source, and reduced permeability of the plasmalemma (Nevoigt and Stahl, 1997, Hohmann, 1997).

1.5.5 Osmosensing and signal transduction

The ability of *S. cerevisiae* cells to sense external (environmental) osmotic stimuli is central to the survival of this organism. The means by which this sensing and signalling occurs, has been shown to involve the ubiquitous mitogen activated protein (MAP) kinase cascade mechanism of signal amplification and transduction (Toone and Jones 1998).

MAP kinase cascades usually involve three proteins which act sequentially, activated by a previous signal, and essentially passing it on to another by the phosphorylation switching mechanism. In short, the first protein in the cascade (MAP kinase kinase kinase (MAPKKK)) is activated and phosphorylates the second protein (MAP kinase kinase (MAPKK)) which in turn phosphorylates the final protein (MAP Kinase (MAPK)) (Madhani *et al.*, 1998; Posas *et al.*, 1998; Gustin *et al.*, 1998; Alberts *et al.*, 2002).

The full activation of a MAPK requires that phosphorylation occurs on both a threonine and tyrosine residue which are topologically adjacent, this ensures that the proteins remain inactive (and subsequently the downstream effects are turned off) unless activated specifically by its corresponding kinase. The eventual target of the MAPK varies according to the nature of the induction of the cascade system, for example MAPK can phosphorylate various proteins, and in many cases these include gene regulatory proteins, or protein complexes (Madhani *et al.*, 1998; Alberts *et al.*, 2002; Uesono and Toh, 2002). The downstream protein phosphorylation results in many cases in the eventual activation of transcription of one or more target genes (Uesono and Toh, 2002).

The ubiquitous and varied nature of MAPK cascades means that there are multiple target sites for the action of the MAPK, and so there is a potential for unrelated downstream effects. The non-specific activation of a MAP kinase pathway is termed 'cross-talk' and has been demonstrated to occur (Buehrer and Errede, 1997). An ingenious strategy utilised by many organisms to circumvent this problem is the grouping together of MAP kinases into functional 'cassettes' held together by either scaffold proteins, or scaffold sections of one of the functional MAP kinases (Whitmarsh, and Davis, 1998; Alberts *et al.*, 2002). The MAPK cascade is highly conserved throughout eukaryotic organisms, and the reason for possessing this

mechanism seems to be in order that graduated external stimuli can be converted into a more switch-like activation signal (Cohen 1992).

Figure 1.3. The five currently elucidated MAP kinase cascades in *S. cerevisiae*, adapted from Gustin *et al.*, (1998).

In *S. cerevisiae* there are four cellular and one sporulation associated MAPK cascades (Posas *et al.*, 1998; Gustin *et al.*, 1998) (Figure 1.3). Of these five MAPK pathways, the most well understood are the mating-pheromone response involved in the aggregation and eventual fusion of the opposite mating types MATa and MATα, mediated by the mating factors a and α respectively (Buehrer and Errede, 1997; Schrick *et al.*, 1997; Elion, 2000; van Drogen *et al.*, 2001), and the response to high external osmolarity (Brewster *et al.*, 1993, Varela and Mager, 1996; Hohmann 1997,).

1.5.6 The high osmolarity glycerol (HOG) pathway

Cells subjected to high external osmolarity are able to effectively sense this external stimulus and respond by activation of the HOG (MAP kinase) pathway. This stress signalling pathway has been shown to be induced by two surface sensor proteins

Sln1p and Sho1p which control the pathway induction (Maeda *et al.*, 1994; Maeda *et al.*, 1995). It has been shown that these two inputs for the HOG pathway can respond independently to hyperosmolarity (Maeda *et al.*, 1995). The independent function of Sln1p and Sho1p demonstrate that these proteins are redundant (= having a functionally interchangeable paralogue) as shown by the ability of the single Δ*sln1* or Δ*sho1* mutants to activate the HOG pathway (Maeda *et al.*, 1995).

The detailed function of the Sln1p and Sho1p proteins has remained a subject of some conjecture, and until recently a comprehensive mechanism for the activation of these 'osmosensor' proteins was unknown. It has been shown that the HOG pathway is activated by a change in turgor rather than an osmotic effect *per se* (Tamas *et al.*, 2000) indicating a turgor-related induction of the membrane osmosensors. Reiser *et al.*, (2003) have subsequently successfully demonstrated that Sln1p is activated by turgor changes mediated by the separation of the plasmalemma and the cell wall during hyperosmotic stress. Indeed it has been suggested that Sln1p mediates a physical link between the cell membrane and the cell wall via its periplasmic ectodomain (Reiser *et al.*, 2003). The reduction in cytoplasmic volume during osmotic stress would therefore be sufficient to produce a conformational change in the essentially tethered Sln1p protein.

Although the mode of activation of Sho1p remains unclear, it is known that osmotic stress affects an autophosphorylation (or cessation of autophosphorylation) of both Sln1p and Sho1p which results in an interaction with a specific downstream MAPK cascade (Maeda *et al.*, 1994; Maeda *et al.*, 1995; Posas and Saito, 1997; Posas *et al.*, 1998). The realisation that there are two upstream inputs into the HOG pathway has led to the elucidation of two distinct upstream branches of this highly specific signalling pathway. The activation of the surface receptor protein Sln1p results in the movement of a phosphate group from a histidine residue to an aspartate group in the protein. This phosphate group is then sequentially transferred to an intermediate protein Ypd1p and eventually to a third protein Ssk1p (Posas *et al.*, 1996). This system is known as a three component phosphor-relay system and is analogous to those found in many other organisms.

The phosphorylating effects of Ssk1p upon two partially redundant MAPKK kinases Ssk2p/Ssk22p, serves to initiate the conserved section of the HOG signalling pathway via the MAPKK Pbs2p and the MAPK Hog1p (Posas *et al.*,1996 ; Posas and

Saito, 1997; Posas *et al.*, 1998). The first two proteins of the three component Sln1p phosphorelay serve to negatively regulate the regulatory Ssk1p. When the osmosensing Sln1p detects an increase in external osmolarity the phosphorylation chain is broken, allowing Ssk1p, in its non-phosphorylated form, to activate the MAPKK kinases (Posas *et al.*, 1996).

The second upstream input in the HOG pathway is via the surface osmosensing protein Sho1p, which acts via a positive two component phosphorelay mechanism to initiate the HOG cascade (Maeda *et al.*, 1994; Maeda *et al.*, 1995) (figure 1.4). Sho1p is activated by an increase in external osmolarity, and again undergoes an intramolecular relay of a phosphate group. The resultant activation gives rise to a more linear activation of the HOG pathway via the MAPKKK Ste11p, which is also involved in the pheromone mating response (figure 1.4) (Gustin *et al.*, 1998; O'Rourke and Herskowitz, 1998). It is believed that there is some cross-talk between the osmosensing and mating MAPK (O'Rourke and Herskowitz, 1998; Van Drogen *et al.*, 2001) cascades but this is reduced with the utilisation of a scaffold mechanism between Pbs2p, Hog1p and Ste11p, suggesting two cytosolic pools of the protein Ste11p which are distinct from each other.

The effects of the initiation of the HOG pathway are all designed to promote survival of the organism during a period of physiological stress. Hog1p can mediate the expression of a global stress response via stress responsive elements (STRE) (section 1.5.8) which initiate the transcription of genes *HSP12* and *CTT1* (responsible for the heat shock 12 and catalase proteins respectively), as well as other putative upregulations, such as the genes coding for trehalose producing enzymes (Gustin *et al.*, 1998; Stark, 1999). It is also believed that Hog1p can initiate a cell cycle arrest as seen in many cases where the cell is exposed to conditions of physiological stress.

The major consequence of the activation of the HOG pathway is the increase in transcription of genes involved in glycerol production, specifically *GPD1* (Albertyn *et al.*, 1994) (encoding glycerol-3-phosphate dehydrogenase) and its isoform encoding homologue *GPD2* (Remize *et al.*, 2001) (section 1.5.9). The HOG pathway also appears to mediate the activity of the glyceroaquaporin Fps1p (Luyten *et al.*, 1994; Tamás *et al.*, 1999) (section 1.9.3) which reduces the net efflux of glycerol and thus aids the accumulation of intracellular glycerol.

Figure 1.4, The HOG pathway in *S. cerevisiae* adapted from Hohmann (1997). Key; Purple: Transmembrane receptor protein, Blue: Phosphorelay protein, Orange: MAPKKK, Green: MAPKK, Red: MAPK, White: Transcription factor. White arrows indicate positive activation; white diamond indicates negative protein activation.

1.5.7 The calcineurin pathway

The adaptation to salt stress, and the subsequent halotolerance of yeast has been the subject of extensive research (Gaxiola *et al.*, 1992; Murgia *et al.*, 1995, Murgia *et al.*, 1996; Ganster *et al.*, 1998) As with many higher organisms, a specific Ca^{2+} mediated response exists in *S. cerevisiae* upon exposure to saline conditions (Mendoza *et al.*, 1994; Mendoza *et al.*, 1995; Matsumoto *et al.*, 2002). In the case of *S. cerevisiae* this response is mediated by the protein phosphatase IIB, otherwise known as calcineurin (Gaxiola *et al.*, 1992).

The major defence mechanism utilised by *S. cerevisiae* during Na^+ stress, is the increase in activity and expression of a Na^+ and Li^+ ion efflux pumps specifically Pmrp2p (Ganster *et al.*, 1998; Matsumoto *et al.*, 2002) . Upon exposure to hypersaline conditions, *S. cerevisiae* cells exhibit an increase in cytosolic Ca^{2+} (possibly mediated by the HOG osmosensing proteins Sln1p and Sho1p), which in turn activates the

ubiquitous calcium binding protein calmodulin (Gaxiola *et al.*, 1992, Mendoza *et al.*, 1996).

The binding of the calcium/calmodulin complex to calcineurin affects a conformational change in this protein phosphatase thereby acting as a transducer of the initial Ca^{2+} 'message'. The subsequent calcineurin signalling gives rise to the transcription of the genes, *ENA1, PMR2A, PMC1* and *PMR*1 (Mendoza *et al.*, 1994, Mendoza *et al.*, 1996; Matsumoto *et al.*, 2002), responsible for the production of transmembrane ion pump proteins. Activated calcineurin is also believed to activate the Trk1-Trk2 K^+ transport system post-transcriptionally, resulting in reduced influx of Na^+ ions (Matsumoto *et al.*, 2002).

1.5.8 Stress-induced gene expression (the STRE response)

S. cerevisiae demonstrates two major stress response pathways. One is the heat shock response (HSR) which is activated in a complex manner by sublethal heat stress (Chatterjee *et al.*, 2000) mediated by the so-called heat shock transcription factor (HSF) (Bienz and Pelham, 1986; Morimoto *et al.*, 1996). The other is, the general (or global) stress response (GSR) which is activated by a number of environmental stresses including oxidative, pH, heat and osmotic stresses and nitrogen starvation (Ruis and Schuller, 1995; Schmitt, and McEntee, 1996; Martinez-Pastor *et al.*, 1996). The general stress response is believed to be an evolutionary adaptation that allows yeast to adapt to adverse environmental conditions in a non-specific manner, in order that cellular fecundity is retained whilst specific responses are activated (Ruis and Schuller, 1995; Martinez-Pastor *et al.*, 1996). The general stress response is typified by the upregulation of a wide array of genes and their corresponding proteins which are involved in a diverse array of cellular functions (Ruis and Schuller, 1995). The expression of these genes has been demonstrated to occur in a process dependent upon the pentameric cis-acting sequence CCCCT within the promoter region of the induced genes. This so-called stress responsive element (STRE) was first identified in reference to the stress induced expression of the *CTT1* gene encoding cytosolic catalase T (Marchler *et al.*, 1993) and subsequently in control of expression of the *DDR2* gene which encodes a putative chaperone protein (Kobayashi and McEntee, 1993). It has subsequently been demonstrated that the activation of the STRE element of inducible

genes is dependent upon two zinc finger transcriptional activators (Msn2p and Msn4p) (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996; Treger *et al.*, 1998) which are active during a wide array of stresses (Ruis and Schuller, 1995) and, interestingly during the diauxic shift (Boy-Marcotte *et al.*, 1998). The identification of this mode of activation has given a mechanism to earlier findings that exposure to one type of stress confers resistance to another, unrelated form of stress (Lindquist, 1986). It has been demonstrated that the transcription factors Msn2p, Msn4p and the more recently identified Hot1p (Rep *et al.*, 1999) are under the control of the Hog1p protein, and therefore become activated in a specific manner during osmotic stress, which mediates a cell cycle arrest, and altered transcriptional profile (Rep *et al.*, 2000; Posas *et al.*, 2000; Nevitt *et al.*, 2003) (figure 1.4).

1.5.9 Glycerol biosynthesis

The induction of the HOG pathway (section 1.5.6) has the ultimate result of stimulating the hyperproduction and hyperaccumulation of glycerol as a compatible solute in order that the external and internal osmolarities are balanced. This occurs via over expression of key genes in the glycerol biosynthetic pathways, most importantly the genes *GPD1/GPD2* (coding for NAD^+ dependent glycerol-3-phosphate dehydrogenases) (Albertyn *et al.*, 1994) and *GPP1/GPP2* (coding for glycerol-3-phosphatases) (Remize *et al.*, 2001; Pahlman *et al.*, 2001).

The glycolytic pathway in *S. cerevisiae* is seen to branch at fructose 1,6 bisphosphate, giving rise to pyruvate and eventually ethanol via the phoshpoenolpyruvate (PEP) pathway, or glycerol via dihydroxyacetone-phosphate (DHAP) intermediate (figure 1.5) (Dickinson, 1999). Glycerol biosynthesis and dissimilation can occur freely and a number of important enzymes are involved in this process. Glycerol can be dissimilated via the action of glycerol kinase (encoded by *GUT1*) to glycerol-3-phoshphate (Pavlik *et al.*, 1993) and back to dihydroxyacetone phosphate upon the action of a mitochondrial glycerol-3-phosphate dehydrogenase encoded by the gene *GUT2* (Ronnow and Kielland-Brandt, 1993).

Figure 1.5. Glucose metabolism in *S. cerevisiae*, showing the two biosynthetic arms which lead to glycerol and pyruvate (and eventually ethanol) respectively adapted from Dickinson (1999). Open reading frames which encode enzymes in red

An alternative route of dissimilation is via dihydroxyacetone, the reaction being putatively catalysed by the action of one of two poorly characterised glycerol dehydrogenases encoded by *GCY1* and *YPR1* (Norbeck and Blomberg, 1997.). The dihydroxyacetone intermediate is returned to its phosphate containing form under the action of a dihydroxyacetone kinase, the product of the gene *DAK1* (Norbeck and Blomberg, 1997). It is possible that glycerol synthesis represents a precursor for other compatible solutes or as an endogenous energy source to enable cells to alter their physiology during osmotic stress, although this remains hitherto untested.

1.6 Impact of solute on the osmotic stress response

In marine yeast it has been observed that the type of osmoticum accumulated by osmotically stressed cells depends greatly upon the type of solute used to elicit the osmotic stress (Hernandez-Saavedra *et al.*, 1995). This process has not been previously demonstrated in brewing yeast strains, however, the response of 0.7M NaCl on haploid laboratory *S. cerevisiae* strains has been reported to involve modified protein expression and amino acid uptake (Norbeck and Blomberg, 1996; Norbeck and Blomberg, 1998). Metabolic and regulatory changes during 1.4M NaCl stress have also been studied and it has been observed that glycerol dissimilation occurs via dihydroxyacetone under these conditions (Norbeck and Blomberg, 1997). This work provides evidence to support the hypothesis that osmotic responses are solute dependent.

1.7 Cell size

It has been previously shown that despite the rigid cell wall, yeast cells show a change in cell size concurrent with external osmolarity changes (Hohmann, 1997). An increase in external osmolarity results in a rapid loss of intracellular water and thus cell shrinkage (Morris *et al.*, 1986; Hohmann, 1997; Blomberg and Adler, 1992). Restoration of favourable (isotonic) solute conditions to hyperosmotically stressed cells does not however result in cells regaining their former volume (Hohmann, 1997). Conversely, when cells are exposed to low external solute concentrations, the net influx causes an increase in cell size.

1.8 Cell wall changes

Cell wall architecture and ultrastructure has not been significantly examined in *S. cerevisiae* production strains during osmotic stress, however some literature exists concerning cell wall changes during starvation (Rhymes and Smart, 1996), and differing growth phases (Smart *et al.*, 1994). It has been demonstrated that cell wall changes, sufficient to alter flocculation characteristics occurred as a result of starvation (Rhymes and Smart, 1996).

1.9 Yeast cytology

1.9.1 Cell membrane

The yeast cell membrane (plasma membrane) is reported as being *ca.* 8 nm in thickness (Hough *et al.*, 1995) and freeze etching studies have revealed that it possesses a number of elongated pits or invaginations (Webster, 1993). The generalised morphology of the yeast plasmalemma is concurrent with cell membrane systems of other organisms, comprising lipid, phospholipid and a range of proteins and sterols. SDS polyacrylamide gel electrophoresis analysis has identified a number of protein and glycoprotein elements including one glycoprotein with a molecular mass of 27.5 kDa which is the main structural component (Santos *et al.*, 1982).

Radioiodination has elucidated at least five polypeptides in the outer layer of the membrane and two glycopeptides which span the phospholipid bilayer (Santos *et al.*, 1982). As with other cellular membranes, the yeast plasma membrane is a highly dynamic organelle, and contains many bound enzymes involved in transport and uptake. Transmembrane proteins also facilitate uptake and excretion of a myriad of nutrient and waste products respectively. Interestingly, recent research has isolated a number of open reading frames (ORFs) in the genome of the haploid S288C strain that encode water (aquaporins (section 1.9.2)) and glycerol (aquaglyceroporins (section 1.9.3)) transport proteins (Carbrey *et al.*, 2001).

The presence or absence of these aquaporin channels in the *S. cerevisiae* plasmalemma has been demonstrated by Carbrey *et al.*, (2001) to influence cell surface properties including flocculence and hydrophobicity. The structural and physiological characteristics of the yeast plasmalemma are homologous to that of higher eukaryotes (Bertl *et al.*, 1998; Bihler *et al.*, 1998) and it is suggested that many of these characteristics are evolutionarily conserved in mammalian plasma membranes, and other membrane systems (Bertl, *et al.*, 1998; Bihler *et al.*, 1999).

1.9.2 Aquaporins

Aquaporins are transmembrane proteins which facilitate the movement of water across the plasma membrane (section 1.9.1) (Agre *et al.*, 1998). The first aquaporin to be elucidated was Aquaporin-CHIP (channel forming intrinsic protein) (*synonym*

Aquaporin 1) from human erythrocytes (Moon *et al.*, 1993) and renal tubules, and subsequently from the same tissues in *Mus musculus* (Moon *et al.*, 1995), and eventually in a wide range of other organisms.

The 3D crystal structure of this protein has subsequently been shown (Li *et al.*, 1997), demonstrating its strucutural complexity. Aquaporins (AQP's) represent the largest group in the major intrinsic protein (MIP) family of integral transmembrane proteins (Laizé *et al.*, 2000) which also include the plasma membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs) in yeast. AQP's mediate rapid fluxes in water (and solute) balance within cells and vacuoles (Borginia *et al.*, 1999, Laizé *et al.*, 2000) and consequently have important roles in water, ionic, solute and hence osmotic balance. In *S. cerevisiae*, four putative aquaporins were initially reported (Goffeau *et al.*, 1996), two of these, ORF YFL054C and *FPS1* have subsequently been attributed with glycerol transport capabilities (section 1.9.3) (Sutherland *et al.*, 1997, Bonhivers *et al.*, 1998)

The remaining two ORFs, YPR192w and YLL052c-053c (overlapping) (now renamed *AQY1* and *AQY2* respectively) exhibit highly conserved structural similarity with plant aquaporins (André, 1995) and they contribute similar function in *S. cerevisiae* (Bonhivers *et al.*, 1998; Laizé *et al.*, 2000). Moreover, *AQY1* and *AQY2* are expressed differentially according to growth conditions, and *AQY2* expression is suppressed in hyper-osmotic culture conditions (Meyrial *et al.*, 2001). Meyrial *et al.*, (2001) also demonstrated an exclusive water channel function for *AQY2* in the laboratory *S. cerevisiae* strain Σ1278b, confirming previous reports concerning the function of *AQY1* by Bonhivers *et al.*, (1998) in the same strain.

1.9.3 Glyceroaquaporins

The two MIP encoding ORFs *YFL054C* and *FPS1* exhibit solute transport capabilities, although the latter has been more extensively studied, and as a consequence its glycerol transport capability is better understood (Luyten *et al.*, 1994; Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamàs *et al.*, 1999). The accumulation of the compatible solute glycerol (section1.5.4) occurs in an Fps1p dependent manner, the regulation of this membrane channel being dependent on the osmolarity of the medium in which the yeast is grown (Luyten *et al.*, 1994; Tamàs *et al.*, 1999). Glycerol efflux from *S. cerevisiae* cells can be facilitated or partly regulated by the *FPS1* and *YFL054C* but

glycerol can passively diffuse across biological membranes (Sutherland *et al.*, 1997). However it has been demonstrated that the rate of transmembrane efflux from *S. cerevisiae* cells is lower than one would expect, and that the yeast plasma membrane/cell wall barrier appears to be relatively impermeable to glycerol (Tamas *et al.*, 1999).

1.9.4 Vacuole

The yeast vacuole is a predominant and highly dynamic organelle (Klionsky, 1998; Schwencke, 1977). In many instances homology has been drawn between the yeast and plant vacuole in its generalised appearance, and more recently biochemical and physiological characteristics (Klionsky, 1998). The yeast vacuole is, however more analogous with the mammalian lysosome (Spormann *et al.*, 1992), due to its low pH and high content of hydrolases. The vacuole is a dynamic organelle, and is involved in a number of diverse functions, from sequestration of cationic products and compartmentalisation of hydrolytic enzymes (Schwencke, 1977; Nishikawa *et al.*, 1990, Sporman *et al.*, 1992), to protein sorting (Banta *et al.*, 1988; Chiang, 1995) and cell division (Schwencke, 1991). The vacuole also plays roles in pH homeostasis, osmoregulation and other regulatory processes that require degradation by intravacuolar enzymes (Klionsky 1998; Chiang, 1995).

The exact morphological appearance of the yeast vacuole is variable as it is prone to flux between fragmented and non fragmented forms during the cell cycle (figure. 1.7) and under stress conditions (Schwencke, 1977; Schwencke, 1991; Sporman *et al.*, 1992; Guthrie and Wickner, 1988; Wonsich *et al.*, 2001) (see also chapter 4). The dynamicity of the vacuole is, in part due to its association with other cellular organelles. Indeed the vacuole, endoplasmic reticulum, Golgi complex, secretory and endocytic vesicles are all interrelated as they are essentially components of the same system (Schwencke, 1991). The high turnover of the organelles of this secretory/transport system led de Duve (1973) to coin the term the "vacuome" to describe the entire endomembrane system. The vacuolar membrane or tonoplast is as dynamic as the plasmalemma, although there are distinct differences between these two organelles. Analysis of the tonoplast has shown a lack of the invaginations observed with the plasmalemma (Kramer *et al.*, 1978). Matile and Wiemken (1967) also

demonstrated the high degree of elasticity of the tonoplast in comparison with the relatively inelastic plasmalemma.

The differences in elasticity are undoubtedly a consequence of biochemical differences, and reflect the different roles of these two membranes. Ultrastructural differences are few, however, the tonoplast has a lower proportion of carbohydrate as demonstrated by its inability to bind the plant derived agglutinin concanavalin A (Boller *et al.*, 1976).

Figure 1.7. Diagrammatic representation of vacuolar changes during the yeast cell division cycle (adapted from Schwencke, 1977).

1.10 Objectives of this study

The objectives of this study were to establish the relative tolerances and responses of production ale and lager brewing yeasts to hyperosmotic stress.

The innate capacity of a given strain to retain viability following acute and chronic exposure to hyperosmotic stress, induced by sorbitol and NaCl solutions was examined in an attempt to establish the relative osmotolerances of ale and lager brewing yeast strains. The aim of this assessment was to establish methods, by which osmotolerant production strains could be identified for use in high gravity brewing environments.

The aim of the investigation into the cellular responses elicited following exposure to osmotic stress, was to identify biomarkers that could be utilised to monitor osmotic stress phenotypes in an industrial context. To achieve this latter objective morphological responses including changes in vacuolar integrity, physiological responses involving the accumulation of compatible solutes, and molecular responses involving the expression of key osmotic defence genes were examined.

Chapter 2

Materials and methods

Chapter 2 Materials and Methods

2.1 Yeast strains

Four production strains of lager brewing yeast (designated SCB1 – 4) and four production strains of ale brewing yeast (designated SCB 5 – 8) were obtained from Scottish Courage Brewing Ltd, Technical Centre, Edinburgh, UK. One haploid laboratory *Saccharomyces cerevisiae* strain designated S288C was obtained from the laboratory of Professor Stephen Oliver, University of Manchester, Manchester. UK.

2.2 Bacterial strains

XL10-Gold® ultracompetent *E. coli* cells of genotype Tet^RΔ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 lac Hte [F′ proAB $lacI^qZ$ M15 Tn10 (Tet^R) Amy Cam^R]^a (Bullock *et al*, 1987; Jerspeth *et al.*, 1997) and Jm109 competent *E. coli* cells of genotype e14–(McrA–) *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 (rK– mK+) supE44 relA1 Δ(lac-proAB) [F′ traD36 proAB lacIqZΔM15] were obtained from Stratagene Inc. (USA).

2.3 Vectors

The purified phagemid vector pBlueScript® II SK+, derived from a pUC plasmid, was obtained form Stratagene Inc. (USA) and stored at -20°C indefinitely until required. Amplification and isolation/purification of this vector was carried out as detailed in section 2.16.

2.4 Media and growth conditions

All media components were supplied by Oxoid (Hampshire, UK) unless otherwise stated.

2.4.1 Bacteria

Bacterial strains were stored on beads at -80°C in nutrient broth (Oxoid) containing 15% [v/v] glycerol. Stock cultures were maintained and grown on L-Broth medium (1% [w/v] Tryptone, 0.05 [w/v] sodium 3hloride, 0.5 [w/v] yeast extract) solidified with 1.2% [w/v] agar. Where ampicillin resistance was utilised as a selectable marker, L-Broth medium was autoclaved as previously described, allowed to cool to

approximately 50°C and ampicillin (Sigma-Aldrich, UK) was added to a final concentration of 100μg ml^{-1}. Bacteria were grown to the required phase of growth aerobically in 250ml Erlenmeyer flasks at 37°C on an orbital shaker at 150 r.p.m. in L-Broth medium. Cell growth was monitored using a Shimadzu spectrophotometer at 600nm. All media was sterilised immediately.

2.4.2 Yeast

Yeast strains were stored on beads at -80°C in YPD (1% [w/v] yeast extract, 2% [w/v] bacteriological peptone and 2% [w/v] D-Glucose) containing 20% [v/v] glycerol. Stock cultures were maintained and grown on YPD medium solidified with 1.2% [w/v] agar as 10ml Slopes or as 20ml agar plates. All media were sterilised immediately following preparation by autoclaving at 120°C and 15 p.s.i for 15 minutes unless otherwise stated. Agar cultures were grown at 25°C until defined colonies were visible, and then stored at 4°C for up to 2 weeks as agar plates, and up to 3 months as slope cultures. Yeasts were grown to the required phase of growth aerobically at 25°C in Erlenmeyer flasks with constant agitation at 120 r.p.m. in YPD medium. Cell growth was monitored using a Shimadzu spectrophotometer at 600nm.

2.5 Osmotic challenge

Cells were osmotically challenged with either D-Sorbitol (Sigma –Aldrich, UK) or NaCl (Sigma-Aldrich) at the indicated concentrations. Yeast cells were harvested at the required phase of growth and washed twice in phosphate buffer (pH 5.9) (0.1M NaH_2PO_4: 0.1M Na_2HPO_4). Cells were resuspended in 100 ml of either sterile de-ionised water, sorbitol (6, 12, 18, 24 and 30% [w/v]) or NaCl (6, 12, 18, 24 and 30% [w/v] to 1×10^6 cells ml^{-1}, and incubated on an orbital shaker at 25°C and 120 r.p.m. for 48 hours.

2.6 Viability

One millilitre aliquots of yeast cell suspension were washed in phosphate buffer (pH 5.9) and resuspended to 1×10^6 cells ml^{-1} in single strength phosphate buffered saline (PBS) (Sigma-Aldrich). The bright field dye citrate methylene violet (2.6.1), and the fluorescent dyes hemi-MgANS (2.6.2) and propidium iodide (2.6.3) were used as described below. In all cases, enumeration of 100 cells was performed in triplicate.

2.6.1 Citrate methylene violet (CMV)

Methylene Violet 3RAX (Sigma-Aldrich UK) was dissolved in 2% [w/v] sodium citrate solution to a final concentration of 0.01% [w/v]. A yeast suspension (0.5ml) was mixed with 0.5ml of citrate methylene violet using a vortex agitator and examined microscopically after 5 minutes. Samples were examined using light microscopy, unstained cells were assumed to be viable. Total and viable cell counts were enumerated as previously described (Smart *et al.*, 1999).

2.6.2 Hemi-magnesium anilinonaphthalene sulphonic acid (MgANS)

Hemi-Magnesium salt of 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) (Molecular Probes, the Netherlands) (0.3g) was dissolved in 2ml of absolute ethanol and diluted to 100ml with sterile redistilled water to a final concentration of 0.3% [w/v]. The stock solution was stored for up to six months at 4°C, and protected from light. 10μl of MgANS solution was added to 200μl of yeast suspension and examined using epifluorescence microscopy after 5 minutes. Cells showing obvious total cellular fluorescence were assumed to be non-viable. Non-fluorescent cells or cells exhibiting a 'halo' of fluorescence were assumed to be viable (McCaig, 1990).

2.6.3 Propidium iodide (PI)

Solid propidium iodide of purity approx 98% (Sigma-Aldrich, UK) was diluted in sterile redistilled water to a 1mg/ml solution [1.5mM]. Aliquots of this stock solution were frozen and stored at -20°C for up to 1 year. Working aliquots were thawed and stored, protected from light at 4°C for up to six months. 3μl of propidium iodide solution was added to 200μl of yeast suspension, and incubated for 15 minutes at 4°C protected from light. Samples were examined using epifluorescence microscopy, cells showing fluorescence were assumed to be non-viable, non-fluorescent cells were assumed to be viable. (Deere *et al.*, 1998).

2.7 Glycerol determination

Triplicate samples of 1×10^9 cells were washed twice in phosphate buffer (pH 5.9) and resuspended in 1ml boiling Tris-HCl (pH 7.0) (0.5M Tris(hydroxymethyl) aminomethane:0.5M HCl diluted to a total of 200ml) (Sigma-Aldrich, UK) , and boiled for 10 minutes. The lysed cells were centrifuged at 4000 r.p.m for 10 minutes to

remove cellular debris, and the supernatant assayed for glycerol enzymatically, according to the method of Hounsa *et al* (1998) using Boehringer-Mannheim kit no. E0148 270, the principle of which is as follows. Glycerol in suspension is phosphorylated to form L-glycerol-3-phosphate, catalysed by glycerokinase in the presence of adenosine-5'-triphosphate (ATP) (which is reduced to adenosine-5'-diphosphate (ADP)). The ADP from the first catalytic step is reconverted into ATP by phosphoenolpyruvate (PEP) and pyruvate kinase (PK) with the formation of pyruvate. Pyruvate is reduced to L-lactate in the presence of L-lactate dehydrogenase (L-LDH) by reduced nicotinamide-adenine dinucleotide (NADH) with a resultant oxidation of NADH to NAD^+. The amount of NADH oxidised is stoichiometric to the amount of glycerol, and this is recorded on the basis of its light absorption at 340nm.

2.8 Proline determination.

Triplicate samples of 1×10^9 total cells were washed twice in phosphate buffer (pH 5.9), and resuspended in 1ml of absolute ethanol, and boiled for 10 minutes. The lysed cells were centrifuged at 4000 r.p.m for 10 minutes to remove cellular debris, and the supernatant was carefully removed. 0.5ml of the retained supernatant was mixed with 2ml of a 1% [w/v] ninhydrin : 60% [v/v] glacial acetic acid solution and placed in a boiling water bath for 1 hour. The samples were subsequently removed to a room temperature water bath to cool. 1.5ml of analytical (spectrophotometric) grade Toluene (methylbenzene) (Sigma-Aldrich, UK) was added, and the sample was agitated for 1 minute. The sample was allowed to separate and the upper phase removed, this was subsequently assayed for optical density at 520nm using optical glass 1ml cuvettes. The concentration of proline was determined by reference to a standard curve prepared using known concentrations of D-Proline (Sigma-Aldrich, UK) suspended in absolute ethanol (modified from the methods of Troll and Lindsley (1955) and Magné and Larher (1992)).

2.9 Thin layer chromatography of amino acids

A Whatman® K6 Silica gel 20cm×20cm thin layer chromatography (TLC) plate of pore size 60Å and layer thickness of 250μm was marked using graphite pencil with a horizontal line 2cm from one edge of the plate. The horizontal demarcation line was subdivided into 21 equidistant divisions. To this horizontal line, 2μl of standard amino

acids solutions (dissolved in double distilled H_2O) were added. Individual cellular extracts were obtained as per the glycerol extraction procedure (section 2.7) with the exception that distilled H_2O and not Tris-HCl was used. Cellular extracts were added to the final sample point. The entire plate was transferred to a chromatography chamber containing 200ml of a motile phase (n-butanol: glacial acetic acid: distilled water 80:20:20 [v/v]) and allowed to run until the solvent had migrated to within 2cm of the top of the plate. The plate was allowed to dry and was sprayed with a ninhydrin solution (ninhydrin 0.2% [w/v] in absolute ethanol). The plate was allowed to dry further and subsequently developed at 110°C for 15minutes. The retention factor (R_f) values for each of the standard samples that corresponded to each blot in the sample were calculated (Mondal *et al.*, 1998).

2.10 Vacuole staining

Following exposure to osmotic stress, cells were washed twice in phosphate buffer (pH 5.9), and stained, using specific fluorescent probes for vacuole lumen or tonoplast.

2.10.1 Carboxy-DCFDA

500μg of 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (C-DCFDA) (Molecular Probes, the Netherlands) was dissolved in 95μl of anhydrous Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, UK) to give a stock concentration of 10mM. Stock aliquots were frozen and stored at -20°C for at least six months. Working aliquots were thawed to room temperature, and 4μl of C-DCFDA/DMSO solution added to yeast cells resuspended to 1×10^6 cells ml^{-1} in 196μl 50mM Sodium Citrate (pH 5) (Sigma-Aldrich, UK), 2% [w/v] glucose, to give a final concentration of 20μM. Samples were incubated for 10 minutes at room temperature, washed twice in single strength PBS and examined using confocal laser scanning microscopy (section 2.11).

2.10.2 MDY-64™

1mg of MDY-64™ (Molecular Probes, the Netherlands) was dissolved in 2.6ml of anhydrous (DMSO) to give a stock concentration of 10mM. Stock aliquots were frozen and stored at -20°C for at least six months. Working aliquots were thawed to room temperature, and 4μl of MDY-64™/DMSO solution added to yeast cells resuspended to 1×10^6 cells ml^{-1} in 196μl 10mM HEPES (2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) Buffer (pH 7.4) (Sigma-Aldrich, UK), 5% [w/v] glucose to give a

final concentration of 20μM. Samples were incubated for 10 minutes at room temperature, washed twice in single strength PBS and examined using confocal laser scanning microscopy (section 2.11).

2.10.3 Cell-tracker™ Blue CMAC

500μg of 7-amino-4-chloromethyl-coumarin (Molecular Probes, the Netherlands) was dissolved in 237μl of anhydrous DMSO to give a stock concentration of 10mM. Stock aliquots were frozen and stored at -20°C for at least six months. Working aliquots were thawed to room temperature, and 40μl of CMAC/DMSO solution added to yeast cells resuspended to 1×10^6 cells ml^{-1} in 160μl 10mM HEPES Buffer (pH 7.4) (2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 5% [w/v] glucose to give a final concentration of 200μM. Samples were incubated for 20 minutes at room temperature, washed twice in single strength PBS and examined using confocal laser scanning microscopy (section 2.11).

2.10.4 FUN®1

A 10mM solution of FUN®1 dissolved in 100μl anhydrous DMSO was obtained from Molecular probes, the Netherlands and stored as stock aliquots at -20°C for at least 1 year. Working aliquots were thawed to room temperature and the FUN®1 Solution added to yeast cells resuspended to 1×10^6 cells ml^{-1} in 10mM HEPES Buffer (pH 7.4), 2% [w/v] glucose to a final concentration of 15μM. Samples were incubated for 20 minutes at room temperature protected from light, washed twice in single strength PBS (and examined using confocal laser scanning microscopy (section 2.11).

2.10.5 LysoTracker® Green

A 1mM solution of LysoTracker® Green dissolved in 50μl anhydrous DMSO was obtained from Molecular probes, the Netherlands and stored as stock aliquots at -20°C for at least 6 months. Working aliquots were thawed to room temperature and the LysoTracker® Green Solution added to yeast cells resuspended to 1×10^6 cells ml^{-1} in single strength PBS to a final concentration of 100μM. Samples were incubated for 10 minutes at room temperature, washed twice in single strength PBS and examined using confocal laser scanning microscopy (section 2.11).

2.11 Confocal microscopy

Cells were immobilised on microscope slides in 2% [w/v] low gelling temperature (LGT) agarose and examined using a Zeiss® 510 confocal laser scanning microscope. Fluorochromes were excited using three laser lines, Argon at 488nm and two Helium/Neon lines at 543nm and 633nm respectively. Detection of fluorescence was achieved using a ×63 plan neofluor objective and the appropriate filter sets. Images were stored as digital tagged image file format (TIFF) files and processed for colour balance using Adobe® Photoshop® version 6.0 software. Processed images were saved using joint photographic experts group (JPEG) compression technology, and printed using high quality colour laser printing. Three dimensional reconstructions were processed using Adobe® Premiere® version 6.0 Software, and saved using motion pictures expert group (MPEG) compression technology as audio video interleaved (AVI) files.

2.12 Yeast genomic DNA extraction

Overnight 400ml YPD cultures of each of the strains (SCB1-8 and S288C) were harvested by centrifugation at 4000 rpm/4°C and washed in phosphate buffer (pH 5.9) then resuspended to give 2.5×10^9 total cells. This cell suspension was further centrifuged at 4000 rpm/4°C and the pellet resuspended in 4ml of TE buffer (10 mM Tris Cl, pH 8.0; 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) in order to remove any remaining media components. DNA extraction was performed on the prepared samples using a Qiagen® genomic buffer kit, and utilising Qiagen® genomic tips; the procedure outline is as follows. Cell suspensions were incubated in yeast lysis buffer "Y1" (1 M sorbitol; 100 mM EDTA; 14 mM β –mercaptoethanol) at 30°C in the presence of lyticase (1000 U/ml) for 1 hour in order to generate spheroplasts. The spheroplasts were harvested by centrifugation at 4700 rpm at 4°C for 10 minutes and resuspended in digestion buffer "G2" (800 mM guanidine HCl; 30 mM Tris Cl, pH 8.0; 30 mM EDTA, pH 8.0; 5% [v/v] Tween®-20; 0.5% [v/v] Triton X®-100) in the presence of 10μl (100mg/ml) RNase (Sigma-Aldrich, UK) and 100 μl (1000U/ml) of Proteinase K (Sigma-Aldrich, UK) in order to lyse cells and denature endogenous RNA and proteins. The cellular debris was removed by centrifugation at 4700 rpm/4°C for 10 minutes and the supernatant retained. A Qiagen® 100/G genomic tip (pre-packed anion-exchange resin column with 100μg DNA binding capacity) was equilibrated with

buffer "QBT" (1.0 M NaCl; 50 mM 3-Morpholinopropanesulfonic acid (MOPS), pH 7.0; 15% [v/v] isopropanol; 0.15% [v/v] Triton® X-100) to which was added the supernatant from the lysis steps. The genomic tip was washed with 15 ml of low salt wash buffer "QC" (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% [v/v] isopropanol) to remove RNA and protein fragments, leaving genomic DNA bound to the positively charged resin. 5ml of a high salt buffer ("QF") (1.25 M NaCl; 50 mM Tris Cl, pH 8.5; 15% [v/v] isopropanol) was used to elute the bound DNA by ion exchange. The eluate from the genomic tip procedure was subsequently collected and the DNA precipitated using 0.7 volumes (3.5ml) of isopropanol. The precipitated DNA was collected by centrifugation at 4700 rpm/4°C for 15 minutes and then washed in cold 70% [v/v] ethanol. The ethanol supernatant was aspirated from the DNA pellet, which was air dried briefly to remove traces of ethanol. The purified genomic DNA was resuspended in 150μl of distilled water (pH 8.0). The purity and concentration was determined as described in section 2.15 and the DNA was placed in aliquots at -80°C until required. Working aliquots were thawed for use and stored at 4°C.

2.13 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was carried out using a Stratagene Robocycler® thermal cycler, and *Taq* DNA polymerase obtained from Promega Corporation UK unless otherwise stated.

2.13.1 Primer design

The coding sequences for the genes examined in this study were retrieved from the Stanford *Saccharomyces* genome database (http://www.yeastgenome.org) and analysed for restriction enzyme sites using webcutter version 2.0 software (http://www.firstmarket.com/cutter/cut2.html). Complimentary sequences between 20 and 30 bases were designed for the ends of each strand. Unique restriction sites were added to facilitate later cloning (section 2.16). The oligonucleotide sequences were synthesised and supplied freeze-dried by Invitrogen Life Technologies Ltd UK.

2.13.2 PCR reaction parameters

Magnesium chloride (25mM), deoxynucleotide triphosphates (dNTP's) (100mM) comprising deoxyadenosine triphosphate (dATP) deoxycytosine triphosphate (dCTP) deoxyguanosine triphosphate (dGTP) deoxythymidine triphosphate (dTTP), *Taq*

(*Thermus aquaticus*) DNA polymerase (5U/μl) and 10× reaction buffer (500mM KCl, 100mM Tris-HCl, pH 9.0 (at 25°C), and 1.0% [v/v] Triton® X-100) were obtained from Promega Corporation UK and added according to the formula shown in Table 2.1, Genomic DNA and oligonucleotide primers were added at the indicated concentrations. For optimisation reasons these parameters were sometimes altered: wherever this was undertaken this is indicated in the relevant results section.

Table 2.1. Formula of PCR reaction mixture

Reagent	*Initial concentration*	*Final concentration*	*Volume*
Template DNA	Depended on extraction	Up to100ng/μl	5μl
Forward Primer	1μg/μl	200ng per reaction	2μl
Reverse Primer	1μg/μl	200ng per reaction	2μl
dNTP's	2μM	200μM	5μl
$MgCl_2$	25mM	2.5mM	5μl
10× Reaction Buffer	10×	1×	5μl
Taq Polymerase	5U/μl	5 units	1μl
dH_2O	-	-	25μl
Total	-	-	50μl

2.13.3 Thermal cycling

Thermal cycling was carried out using a gradient programme for annealing temperatures, according to the conditions shown in Table 2.2. For optimisation reasons, thermal cycling was sometimes altered; wherever this was undertaken this is indicated in the relevant results and discussion section.

Table 2.2. Generalised parameters for thermal cycling

	Temperature	*Duration*	*No. of cycles*
Step 1	95°C	5 minutes	1
Step 2	95°C	1minute	30 – 35
	44 - 65°C	2 minutes	
	72°C	2 minutes	
Step 3	72°C	10 minutes	1
Step 4	6°C	Hold	

2.13.4 Quantitative (real-time) PCR

The Applied Biosystems® 7000 sequence detection system (ABI SDS7000) was used in accordance with the manufacturers' instructions.

2.13.4.1 Primer and Taqman® probe design

The Applied Biosystems® Primer Express™ software was employed to design Primers and Fluorescence Resonance Energy Transfer (FRET) Taqman® probes for the genes of interest. Primers were used at a final concentration of 300μM (Invitrogen, UK). Taqman® probes were obtained freeze dried (5' 6-FAM - 3'TAMRA), rehydrated to 5mM, stored at -20°C in aliquots and used at a final concentration of 130μM. PCR master mix (Applied Biosystems, UK) was used in all cases and contained the passive reference dye ROX.

2.14 PCR purification

A Qiagen® QIAquick® PCR purification kit was used to remove excess PCR components (DNA polymerase, dNTP's, $MgCl_2$, primers) using the following protocols. 5 volumes of buffer "PB"* were added to 1 volume of PCR reaction sample and mixed and added to a QIAprep® spin column (pre-packed anion-exchange resin column in 1.5ml Eppendorf tube format) and centrifuged at 13,000 rpm for 1 minute to bind DNA. The eluate was discarded, and the column washed with 0.75 ml of buffer "PE" * and the column again centrifuged at 13,000 rpm for 1 minute. The eluate was discarded and the

* The composition of this buffer are not publicised by Qiagen® USA

column centrifuged at 13,000 rpm for a further 1 minute to remove any traces of ethanol. The DNA was eluted by applying 50μl of distilled water (adjusted to pH 8.0 with 0.5 M NaOH) to the spin column and performing a final centrifugation step at 13,000 rpm for 1minute.

2.15 Gel electrophoresis

DNA samples (From PCR (section 2.13), genomic extraction (section 2.12), plasmid extraction and restriction enzyme digests (section 2.16.8) were analysed by gel electrophoresis. A 0.8-2% [w/v] agarose gel was prepared in TAE buffer (242g/l Tris; 57.1ml/l Glacial Acetic Acid; 18.6 g/l EDTA for 50×, diluted to 1×) in a standard 20cm×20cm electrophoresis tank (Anachem, UK) using gel casting combs in order to prepare wells. When set, the gel was placed into the electrophoresis tank and covered with TAE buffer and the casting combs removed. 10 μl of DNA sample was combined with 5μl of loading dye (15% [w/v] Ficoll-400, 0.25% [w/v] xylene cyanol FF, 0.25% [w/v] bromophenol blue) and loaded into the well. A 5μl aliquot of DNA 10kb or 1kb Smartladder® (Eurogentec, the Netherlands) was added into a reference well, and a voltage of 100mV applied to the gel for 30-60 minutes. The gel was removed and incubated in ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) (20μl of 100mg/ml solution in 200ml TAE buffer) for 20 minutes in order to stain nucleic acids. The nucleic acids were visualised using a standard UV transilluminator.

2.16 Gene cloning

Cloning of S. *cerevisiae* genes was undertaken using molecular biochemicals and reagents from Promega Corporation (UK and New England Biolabs (UK) unless otherwise stated using the procedures described in sections 2.16.1- 2.16.5.

2.16.1 Amplification of DNA using proofreading enzymes

The PCR reaction was carried out as described in section 2.13 with the addition of DNA polymerase that exhibit 3'-5' exonuclease (proofreading) activity. *Pfu* Turbo® (*Pyrococcus furiosus*) (Stratagene Inc. USA) or $Vent_R$® (*Thermococcus litoralis*) (New England Biolabs USA) were added to a final concentration of 5 Units per reaction.

2.16.2 Gel extraction of DNA

A Qiagen® QIAquick® gel extraction kit was used to isolate required DNA fragments following gel electrophoresis (section 2.15). The DNA fragment of interest was excised using a scalpel from the agarose gel in as small a quantity of agarose as possible and transferred to a 1.5ml Eppendorf tube. 3 volumes of guanidine thiocyanate containing solubilisation buffer "QG" * was added and the mixture was then incubated at 50°C for 10 minutes until the agarose was completely dissolved. Upon dissolution of the agarose, 1 gel volume of isopropanol was added to the solubilisation mixture. The resulting mixture was loaded onto a QIAquick column (pre-packed anion-exchange resin column in 1.5ml Eppendorf tube format) and centrifuged at 13,000 rpm for 1 minute to bind DNA. The eluate was discarded, and the column washed with 0.5 ml of buffer QG and centrifuged at 13,000 rpm for 1 minute. The eluate was again discarded and the column washed with 0.75ml of the ethanol based wash buffer "PE" *and the column again centrifuged at 13,000 rpm for 1 minute. The eluate was discarded and the column centrifuged at 13,000 rpm for a further 1 minute to remove any traces of ethanol. The DNA was eluted by applying 50μl of distilled water (adjusted to pH 8.0 with 0.5 M NaOH) to the spin column and performing a final centrifugation step at 13,000 rpm for 1 minute.

2.16.3 Preparation of competent cells

XL10-Gold® ultracompetent or JM109 competent *Escherichia coli* cells were grown in L-Broth medium overnight and expanded into a 1 litre culture by performing a 1:100 dilution. The diluted cultures were grown until the optical density at 600nm reached between 0.4 – 0.5. The cells were harvested by centrifugation at 3000 rpm for 15 minutes at 4°C and washed in 100ml of cold calcium chloride solution (50mM $CaCl_2$; 15% [v/v] glycerol). The cells were pelleted by centrifugation and resuspended in 50ml of cold calcium chloride solution. This process was repeated, using reducing volumes of calcium chloride solution until the final volume reached 2ml. The final pellet was resuspended in 2ml of cold calcium chloride solution and 50 μl aliquots were prepared into pre-chilled 1.5ml Eppendorf tubes and stored at -80°C until required. The competent cells were tested for transformation efficiency with 1ng of known plasmid

* The composition of this buffer are not publicised by Qiagen® USA

according to the procedure detailed in section 2.15 and the results recorded as colony forming units per μg of DNA.

2.16.4 Transformation of competent cells

A 50μl aliquot of competent XL10-Gold® or JM109 *Escherichia coli* cells was removed from -80°C storage and defrosted on ice. The required amount of plasmid DNA was defrosted and added to the competent cells, and mixed gently by tapping the Eppendorf tube. The tube was left on ice for 15 minutes and the cells briefly heat shocked at 42°C for 2 minutes and then placed on ice for 2 minutes. 900μl of sterile L-broth was added to the cells which were subsequently allowed to recover for 1 hour at 37°C with shaking at 150 rpm. A 50μl and a 100μl sample of the transformation mixture was inoculated onto an L-Broth agar plate containing the appropriate antibiotic using the spread plate method. The transformants were allowed to grow at 37°C overnight in a standard laboratory incubator.

2.16.5 Selection of transformants

Overnight plates containing transformation mixtures were examined for the presence of transformed bacteria. Those cells transformed with a specific plasmid containing an antibiotic resistance sequence were able to grow on the corresponding antibiotic containing plates. Well isolated colonies were removed using a sterile plastic pipette tip and transferred to 1-10ml (mini prep section 2.16.6) or 100-500ml (maxi-prep section 2.16.7) of antibiotic containing L-broth and grown overnight at 37°C with shaking at 150 rpm.

2.16.6 Small scale plasmid preparation (mini-prep)

Plasmid DNA was extracted using a Qiagen® QIAprep® plasmid extraction kit. 1.5ml of overnight cultures were selected as described in section 2.16.5 and placed into a 1.5ml Eppendorf tube. The bacteria were pelleted by centrifugation at 4000 rpm for 5 minutes in a conventional bench-top microcentrifuge. The supernatant was discarded and the pellet was resuspended in 250μl of resuspension buffer "P1" (50 mM Tris Cl, pH 8.0; 10 mM EDTA; 100 μg/ml RNase A) and 250μl of lysis buffer "P2" (200 mM NaOH, 1% SDS [w/v]) and mixed by inversion 4-6 times. The lysis reaction was allowed to proceed for 4 minutes and was stopped with the addition of 350μl of neutralisation buffer "P3" (3.0 M potassium acetate, pH 5.5) and mixed by inversion 3-6

times. The cellular debris was pelleted by centrifugation at 13,000 rpm for 10 minutes in a bench top microcentrifuge. The supernatant was carefully removed and added to a QIAprep® spin column (pre-packed anion-exchange resin column in 1.5ml Eppendorf tube format) and centrifuged at 13,000 rpm for 1 minute to bind plasmid DNA. The eluate was discarded, and the column washed with 0.5 ml of low salt buffer "PB"* and centrifuged at 13,000 rpm for 1 minute. The eluate was again discarded and the column washed with 0.75ml of the ethanol based wash buffer "PE"* and further centrifuged at 13,000 rpm for 1 minute. The eluate was discarded and the column centrifuged at 13,000 rpm for 1 minute to remove any traces of ethanol. The plasmid DNA was eluted by applying 50μl of distilled water (adjusted to pH 8.0 with 0.5 M NaOH) to the spin column and performing a final centrifugation step. Plasmid DNA purity was verified by restriction enzyme digest treatment (section 2.16.8) and gel electrophoresis (section 2.15).

2.16.7 Large scale plasmid preparation (maxi-prep)

Large scale plasmid extraction was performed using a Qiagen® plasmid Maxi kit. Selected bacterial transformants were prepared according to the protocol outlined in section 2.16.5 and harvested from culture by centrifugation at 3500 rpm for 10 minutes. The supernatant fraction was discarded and the pellet resuspended in 10ml of resuspension buffer "P1" (50 mM Tris Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A) and 10ml of lysis buffer "P2" (200 mM NaOH, 1% SDS [w/v]) and mixed by inversion 4-6 times. The lysis reaction was allowed to proceed for 4 minutes and was stopped with the addition of 10ml of cold neutralisation buffer "P3" (3.0 M potassium acetate, pH 5.5), mixed by inversion 3-6 times and placed on ice for 15 minutes. The lysis reaction mixture was centrifuged at 4700rpm for 15 minutes and the supernatant removed and retained. The supernatant was further centrifuged at 4700 rpm for 15 minutes to remove any particulate matter. A Qiagen® 500 plasmid tip (pre-packed anion-exchange resin column with 500μg DNA binding capacity) was equilibrated with 10ml of buffer "QBT" (1.0 M NaCl; 50 mM 3-Morpholinopropanesulfonic acid (MOPS), pH 7.0 ;15% [v/v] isopropanol; 0.15% [v/v] Triton® X-100) to which was added the supernatant from the lysis steps. The tip was washed with 60 ml of low salt wash buffer "QC" (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% [v/v] isopropanol) to

* The composition of this buffer are not publicised by Qiagen® USA

remove RNA and protein fragments, leaving plasmid DNA bound to the positively charged resin. 15ml of a high salt buffer ("QF") (1.25 M NaCl; 50 mM Tris Cl, pH 8.5; 15% [v/v] isopropanol) was used to elute the bound DNA by ion exchange chromatography. The eluate from the genomic tip procedure was subsequently collected and the DNA precipitated using 0.7 volumes (10.5ml) of isopropanol. The precipitated plasmid DNA was collected by centrifugation at 4700 r.p.m /4°C for 15 minutes and then washed in cold 70% [v/v] ethanol. The ethanol supernatant was aspirated from the DNA pellet, which was air dried briefly to remove traces of ethanol. The plasmid DNA was resuspended in 500μl of distilled water (adjusted to pH 8.0 with 0.5M NaOH) and stored at -20°C until required. Plasmid DNA purity was verified by restriction enzyme digest treatment (section 2.16.8) and gel electrophoresis (section 2.15)

2.16.8 Enzymatic treatment of DNA

DNA was treated with restriction endonucleases as previously described (Sambrook *et al.*, 1989). DNA of the correct molar concentration was treated with restriction enzymes at 37°C overnight according to the manufacturers' optimum conditions in the presence of the correct buffer. The generalised restriction enzyme digest formula is shown intable 2.3.

Table 2.3. Generalised formula of restriction enzyme digests.

DNA	*500ng – 1μg*
Buffer	1×
Enzyme	2.5 – 5 units
Distilled water	To volume
Total	50 – 100μl

2.16.9 Ligations

The catalysis of the joining of two strands of DNA between the 5′-phosphate and the 3′-hydroxyl groups of adjacent nucleotides was performed using T4 DNA ligase (Promega Corporation UK). A 3:1 ratio of vector DNA to insert DNA was used in the

presence of buffer (300mM Tris-HCl (pH 7.8), 100mM $MgCl_2$, 100mM DTT (1,4-dithiothreitol) and 10mM ATP) and 0.1- 1 units of T4 DNA ligase. The ligation reaction was allowed to occur overnight at 4°C, or at room temperature for 3-4 hours.

2.17 DNA sequencing

Sequencing of constructs was performed at the Biochemistry Department, the University of Oxford using the ABI® version 3 Big Dye Terminators® protocols in an automated sequencer.

2.18 *In vitro* transcription

Probes for Northern analysis were produced from genes cloned into the pBlueScript® II SK+ vector using T7 or T3 RNA polymerase (Promega, UK). 1μg of plasmid was linearised by performing a single restriction enzyme cut downstream of the cloned gene, which was subsequently PCR purified to remove restriction buffers and enzyme. The purified template DNA was added to an *in vitro* transcription mix containing the appropriate RNA polymerase, optimal buffer and RNA bases, including digoxigenin (DIG) labelled Uracil. The reaction was allowed to proceed for 1 hour at 37°C, and then stopped by the addition of 0.2M EDTA.

2.19 Bioinformatics

DNA sequences were viewed using Chromas 1.4x sequence viewer, and analysed using ClustalX sequence alignment software.

2.20 RNA extraction and handling

All Glassware and plasticware utilised for RNA extraction and handling was double autoclaved to remove contaminating RNases. Where this was not possible, treatment with RNase ZAP® (Sigma-Aldrich, UK) was employed, and the excess washed off with RNase-free water. All solutions were made in RNase-free water (treated with 0.1% [v/v] diethylpyrocarbonate (DEPC) overnight at 37°C and then double autoclaved). All handling was performed according to Sambrook *et al.*, (1989) in order to minimise RNase contamination.

2.20.1 Total RNA extraction

Total yeast RNA was extracted using the Qiagen® RNeasy™ extraction kit. 1×10^7 cells were harvested from osmotic challenge (section 2.5) and resuspended in 2ml buffer Y1 (section 2.12) supplemented with 50 units of lyticase (Sigma-Aldrich UK). The suspension was incubated at 30°C for 15 minutes with gentle agitation to generate spheroplasts. Spheroplasts were harvested by centrifugation and resuspended in 350μl of buffer "RLT"* to lyse and subsequently 250μl RNase-free ethanol (Sigma-Aldrich, UK) was added. The sample was added to a RNeasy mini column®, and centrifuged at 10,000 rpm for 15 seconds. The eluate was discarded and 700μl of buffer "RW1"* added to the column which was subsequently centrifuged at 10,000 rpm to wash. The eluate was discarded and 500μl of buffer "RPE"* was added to the column which was subsequently centrifuged at 10,000 RPM to wash; this step was then repeated. RNA was eluted by the addition of 50μl of RNase-free water and centrifugation for 1 minute at 10,000 rpm.

2.20.2 DNase treatment

The final eluate from 2.20.1 was incubated for 1hour in the presence of 5 units of RQ1 RNase-free DNase (Promega, UK) in order to remove any possible contaminating genomic DNA template. The reaction was stopped with the addition of 20mM (pH 8.0) ethylene glycol-bis-(b-amino-ethyl ether) N,N,N′,N′-tetra-acetic acid (EGTA). The enzyme was subsequently deactivated by incubation at 95°C for 10 minutes.

2.20.3 RNA quantitation and storage

The amount and quantity of RNA eluted from the final step in section 2.20.1 was determined spectrophotometerically. A 10μl aliquot of RNA was diluted 1:100 and the absorbance at 260nm was determined using RNase-free quartz cuvettes. The amount of RNA present was determined using the equation Concentration of RNA sample = 40 x A_{260} x dilution factor. Purity of RNA was determined using the ratio of absorbance at 260nm and 280nm (A_{260}/A_{280}), a ratio of between 1.9 and 2.1 indicated pure RNA. RNA was aliquoted and stored at -70°C until required.

* The composition of this buffer are not publicised by Qiagen® USA

2.20.4 Reverse Transcription

Complimentary DNA (cDNA) was generated by reverse transcription of mRNA extracted from yeast cells. Omniscript™ reverse transcriptase (Qiagen, UK) was added to a 20μl reaction mixture containing 2μg of RNA, 1μM final concentration of oligo(pdT) primer (New England Biolabs, UK) and appropriate buffer. The reaction was allowed to proceed for 1 hour at 37°C and stopped by incubation at 95°C for 5 minutes. Concentration of the generated single-stranded cDNA was determined spectrophotometerically.

2.20.5 Formaldehyde gel electrophoresis

RNA for analysis purposes was separated electrophoretically under denaturing conditions. A 1.2% formaldehyde gel was prepared by the addition of 10ml of 10× FA buffer (200mM MOPS, free acid, 50mM sodium acetate, 10mM EDTA, adjusted to pH 7.0 with RNase-free NaOH) to 1.2g of agarose and the volume adjusted to 100ml with DEPC-treated water. The mixture was heated in a microwave to dissolve the agarose and subsequently cooled to 65°C in a water bath, after which the flask was transferred to a fume hood and 1.8ml of 37% (12.3M) formaldehyde and 1μl of a 10mg/ml solution of ethidium bromide solution were added (for the purpose of northern transfer ethidium bromide was not added). The gel solution was added to a standard electrophoresis tank with appropriate combs and allowed to set. RNA samples were diluted 5 times in 5× RNA loading buffer (0.25% [w/v] bromophenol blue, 4mM EDTA, 0.9% [v/v] formaldehyde, 20% [w/v] glycerol, 30.1% [v/v]formamide, 4× FA gel buffer), denatured for 4 minutes at 65°C and subsequently chilled on ice for 5 minutes. The denatured samples were added to the formaldehyde gel together with a reference size marker (6,583 – 281bp RNA size marker, Promega, UK). The samples were electrophoresed at 7 V/cm until the dye had migrated half-way through the gel. For quantitation, the gel was removed to a standard UV transilluminator, or otherwise used as described in section 2.20.4.

2.21 Protein extraction and analysis

2×10^7 cells were combined with 0.1g of glass beads (425-600μM) (Sigma-Aldrich, UK) and sonicated for 3 minutes in a Camlab transonic T420 bench-top sonicator. All

samples were maintained on ice and the resultant samples were assayed as described in section 2.21.1.

2.21.1 Bradford assay

Protein concentration was determined using the Bio-Rad protein assay solution. The concentrated dye reagent (modified Bradford's acidic Coomassie brilliant blue G-250) was diluted 1:4 in water and filtered through a Whatman No.1 paper. The diluted reagent was mixed with 100μl of protein extract and the absorbance of the solution was measured at 595nm and compared to a standard curve for bovine serum albumin (BSA). Samples were diluted to the same concentration and analysed as described in 2.21.2.

2.21.2 SDS-PAGE

Proteins were resolved by electrophoresis in a 12% acrylamide gel using the Bio-Rad Mini-Protean-3® electrophoresis system. 6.0ml of acrylamide:bis-acryalmide solution (Protogel, UK) was added together with 3.8ml of 1.5M Tris (pH 8.8), 150μl of 10% [w/v] SDS, 150μl of 10% [w/v] ammonium persulphate (APS) and 4.9ml of water as described by Sambrook *et al.*, (1989). The solution was degassed for 10 minutes using a standard laboratory pump and 6μl of N,N,N',N'-Tetramethylethylenediamine (TEMED) was added. The solution was added to the gel casting plates according to the instructions of the manufacturer, and 3ml of H_2O-saturated n-butanol was added to promote the formation of a level upper surface of the gel. The gel was allowed to set for 10 minutes and the butanol overlay was removed. A 5% stacking gel was prepared containing 2.7ml of water, 670μl of acrylamide:bis-acryalmide solution, 500μl of 1M Tris (pH 6.8), 40μl of 10% [w/v] SDS, 40μl of 10% [w/v] APS and 4μl of TEMED. Gel casting combs were inserted into the stacking gel and removed after 10 minutes. The cast gel was placed into the gel-running apparatus and submerged in Tris-glycine electrophoresis buffer (25mM Tris, 250mM glycine (pH 8.3), 0.1%[w/v] SDS). Samples were prepared for electrophoresis by boiling at 100°C for 3 minutes in SDS gel loading buffer (50mM Tris·Cl (pH 6.8), 100mM dithiothreeitol (DTT), 2% [w/v] SDS, 0.1% [w/v] bromophenol blue, 10% [v/v] glycerol) and subsequently added to the polyacrylamide gel. A voltage of 200V was applied to the gel until the dye reached the end of the glass holding plates.

2.21.3 Coomassie staining

Resolved SDS-polyacrylamide gels were placed in 50ml of Coomassie® staining solution (0.05% [w/v] Coomassie Brilliant Blue R-250, 40% [v/v] ethanol, 10% [v/v] glacial acetic acid, 50% [v/v] water) for 1hour. The gels were removed and washed for 3 × 30minutes in destaining solution (40% [v/v] ethanol, 10% [v/v] glacial acetic acid, 50% [v/v} water) and fixed onto cellophane using a gel-drying frame (Sigma-Aldrich, UK). Alternatively proteins were transferred to a nitrocellulose membrane as described in section 2.21.4.

2.21.4 Electroblot transfer of proteins

Resolved SDS-polyacrylamide gels were placed upon a nitrocellulose membrane (Optitran® Schleicher and Schuell, Germany) and sandwiched between two layers of Whatman 3MM paper and then one layer of sponge padding. The entire sample was subsequently placed into a Trans-Blot® electrophoresis chamber (Bio-Rad, UK), submerged in transfer buffer (39 mM glycine, 48mM Tris, 0.037% [w/v] SDS. 20% [v/v] methanol) and a current of 20V was applied overnight.

2.22.5 Immunodetection of immobilised protein

The protein containing nitrocellulose membrane was placed in blocking solution (0.1% Tween® 20, 5% [w/v] non-fat dried milk in PBS) for 1 hour with gentle agitation. The membrane was subsequently transferred to a solution containing the primary antibody (monoclonal antibody to Gpd1p obtained from Prof. Lennart Adler Goteborg University) (primary antibody diluted 1:3000 in blocking solution) and incubated for 1 hour with gentle agitation. Excess primary antibody was removed using wash solution (0.1% Tween® 20 in PBS) by incubating the membrane for 10 minutes per wash, with removal of the previously applied buffer between washes. Secondary antibody conjugated to alkaline phosphatase was applied to the blot diluted 1:10,000 in blocking solution for 1 hour and subsequently washed three times. The localisation of the target protein was achieved by incubation of the membrane for 10 minutes in alkaline phosphatase buffer (100mM NaCl, 5mM $MgCl_2$, 100mM Tris·Cl (pH 9.5)) containing 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium (BCIP/NBT) as described by Sambrook *et al.*, (1989).

Chapter 3

The Impact of Osmotic Stress on Brewing Yeast Viability

Chapter 3 The Impact of Osmotic Stress on Brewing Yeast Viability

3.1 Introduction

The assessment of microbial cell physiological condition is a subject that has important applications in many areas of biotechnology and medicine (Ulber *et al.*, 2003). However, the exact definition of viability remains somewhat ambiguous, and subsequently the field of viability determination has been dedicated to the prediction of cell physiological condition, using a number of techniques (Lloyd and Hayes, 1995). Traditional methods of analysis, such as serial dilutions of microorganisms and subsequent plating on suitable medium are time-consuming, cumbersome and prone to error (Heidelberg *et al.*, 1997). Direct dye treatment of a sample of cells and subsequent microscopic or flow-cyometric analysis, therefore, represents the fastest, most accurate means of determining viability.

The determination of yeast viability is no less important to the brewing industry, than in other biotechnological applications. Accurate assessment of cell viability is critical before inoculation into fermentation vessel as overpitching of dead cells may lead to the generation of autolytic haze and flavour defects in the final product. The industry standard to measure viability involves the use of the brightfield stain methylene blue (Pierce, 1970), which is reduced intracellularly to a colourless form by live cells (Sami *et al.*, 1996). However, it has been demonstrated that this dye may be inaccurate at viabilities lower than 90% (O'Connor-Cox *et al.*, 1997; Smart *et al.*, 1999) and is subjective due to the presence of derivatives of the dye molecule (Smart *et al.*, 1999). Alternatives to methylene blue have been assessed including the brightfield stain methylene violet (3RAX) (Smart *et al.*, 1999). Methylene violet is oxidatively stable and therefore less subjective to use, furthermore it has been demonstrated to correspond well to fluorescent stains, which may also be used to determine cell viability (Van Zandycke *et al.*, 2003; Powell *et al.*, 2003).

As early as 1966, Rotman and Papermaster described the potential of dyes that were taken up by the cell and converted to a fluorescent form. In 1969, Graham and Caiger demonstrated the advantage of using fluorophores as an alternative to methylene blue. More recently, fluorescence has become an increasingly common way of assessing viability (VanZandycke *et al.*, 2003). A number of fluorescent dyes have

been proposed as good indicators of physiological condition, including the magnesium salt of 1-anilinio-8-napthalene sulphonic acid (MgANS) (McCaig, 1990), fluorescein diacetate (Raynal *et al.*, 1994; Tsuji *et al.*,1995; ; Yang *et al.*, 1995; Prudencio *et al.*, 1998), erythrosin B (Sano *et al.*,1993), propidium iodide (Deere *et al.*, 1998), ethidium bromide (Jayapal *et al.*, 1991) and many others (reviewed by Van Zandycke *et al.*, 2003).

The mechanisms by which fluorescent stains determine cell viability varies, however, there are two principle modes of action: membrane exclusion and intracellular conversion (Table 3.0). Many fluorescent stains are excluded by live cells, such as oxonol (Humphreys *et al.*, 1994), and therefore fluorescence equates to cell death. Some stains bind specific components within a cell that lacks membrane potential and is therefore considered to be non-viable. Examples of this latter group include propidium iodide (Deere *et al.*, 1998) and MgANS (McCaig, 1990), which bind to nucleic acids and cytoplasmic proteins, respectively.

Table 3.0 Some commonly used fluorescent viability dyes and their proposed modes of action (adapted from Lloyd and Hayes, 1995).

Dye	*Reference*	*Mode of Action*
Dihexyloxacarbocyanine	Diaper *et al.*, 1991	Transmembrane electrochemical potential
Rhodamine 123	Darzynkiewicz *et al.*,1982	
Bis(1,3-dibutylbarbituric acid) trimethine (DiBaC,(3))	Humphreys *et al.*, 1994	
Fluorescein diacetate (FDA)	Prudencio *et al.*, 1998	Esterase activity and membrane integrity
Carboxyfluorescein diacetate (CFDA)	Breeuwer *et al.*, 1994	
4'6Diamidino-2-phenylindole (DAPI)	Jenkins *et al.*,1997	Dye exclusion
Propidium iodide	Deere *et al.*, 1998	
Ethidium homodimer	Kaneshiro *et al.*, 1991	
1-anilinio-8-napthalene sulphonic acid (MgANS)	McCaig, 1990	
Chemichrome Y	Lloyd and Hayes, 1995	Fungal enzymatic activity and membrane integrity
Fungolite	Lloyd and Hayes, 1995	

The ability of a cell to withstand deleterious external environmental conditions can be ascertained with reference to the changes in viability observed during exposure to these stresses. The link between yeast viability and tolerance to a specific environmental stress therefore is clear, as the (osmo) tolerance is indicated by a drop in cellular viability. The point, at which viability of a population of cells falls below a predetermined value, can consequently be used as a valuable predictor of relative resistance.

The impact of osmotic challenge on the tolerance of ale and lager brewing yeast has not been previously reported, and only limited analyses have been conducted with laboratory haploid strains of *Saccharomyces cerevisiae,* most notably the strain CBS1171 (Marechal and Gervais, 1994; Beney *et al.,* 2000; Beney *et al.,* 2001). In these studies, methylene blue and standard plating techniques were utilised to assess physiological condition.

In this study, the effect of solute on the osmotolerance of production brewing strains was determined, using the brightfield stain methylene violet, and the fluorescent stains MgANS and propidium iodide.

3.2 Results

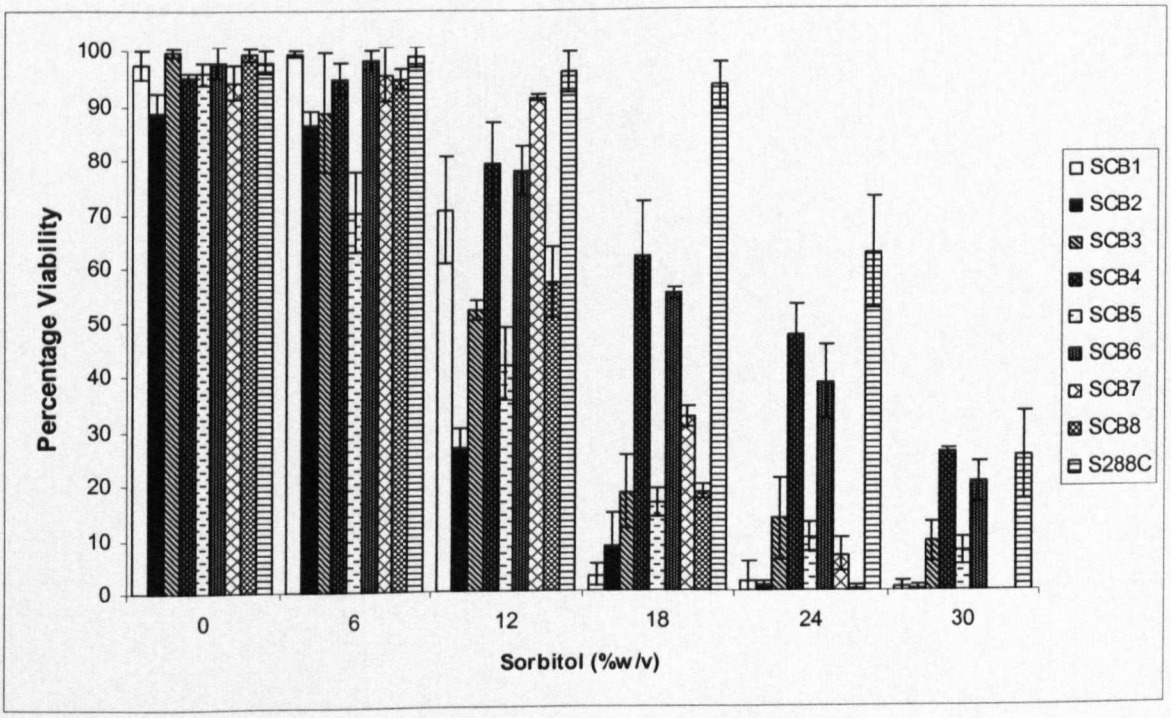
All results presented in this chapter represent the mean of 3 replicates of each culture, unless otherwise stated. The standard deviation was calculated and represented graphically as error bars, where appropriate. The two-tailed T-test was used to determine the occurrence of significant differences between specific data sets as appropriate.

3.2.1 Is osmotic stress tolerance strain dependent?

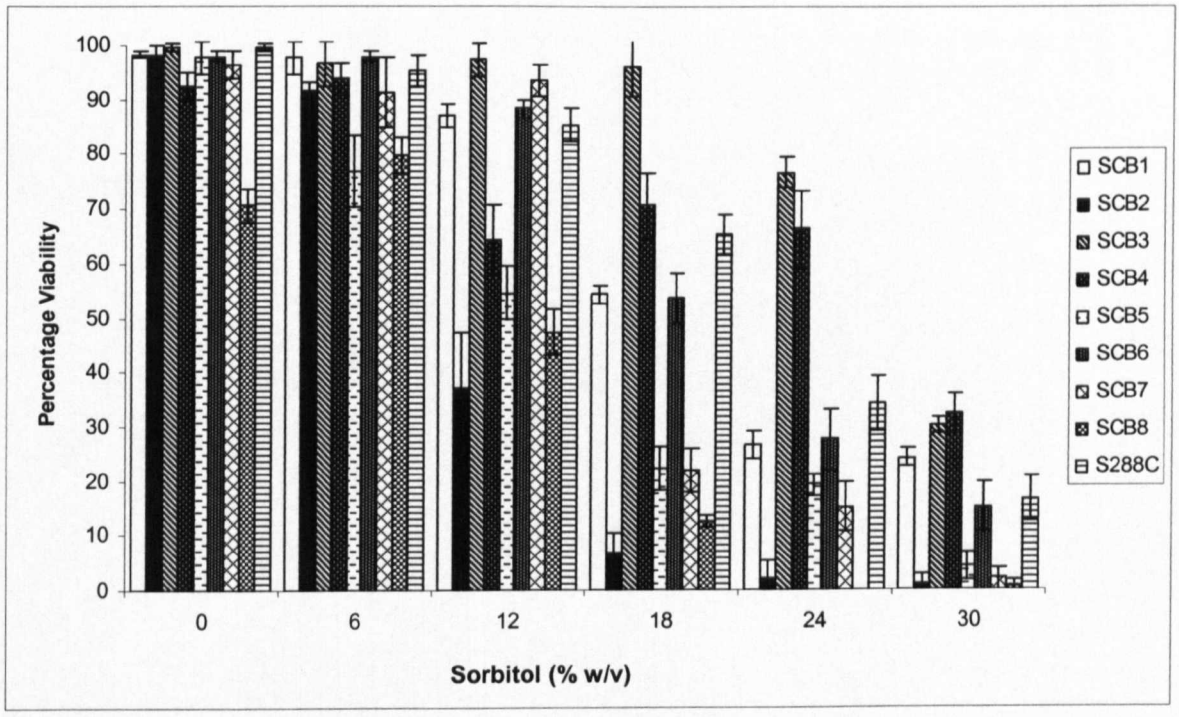
Resistance to osmotic stress has been observed to be strain dependent in haploid laboratory strains of *Saccharomyces cerevisiae* (Blomberg, 1997), but the relative tolerance of ale and lager brewing yeast production strains has not been previously assessed. In order to investigate brewing yeast tolerance to osmotic stress, the sensitivity of four ale (SCB5-8) and four lager (SCB1-4) strains to high solute concentrations was examined and compared to that of the haploid laboratory strain

S288C. Mid-exponential phase cells, grown in YPD (Section 2.4.2), were exposed to different concentrations of sorbitol, which cannot be metabolised by *Saccharomyces sp.* and represents the standard means of eliciting osmotic stress in yeast (Brown, 1978; Panchal and Stewart, 1980; Blomberg and Adler, 1992, Hohmann, 1997). Viability was determined using methylene violet (section 2.6.1) (figure 3.1a), MgANS (section 2.6.2) (figure 3.1b), and propidium iodide (section 2.6.3) (figure 3.1c). Figure 3.1.1 shows examples of cells stained with MgANS (figure 3.1.1a) and propidium iodide (figure 3.1.1b).

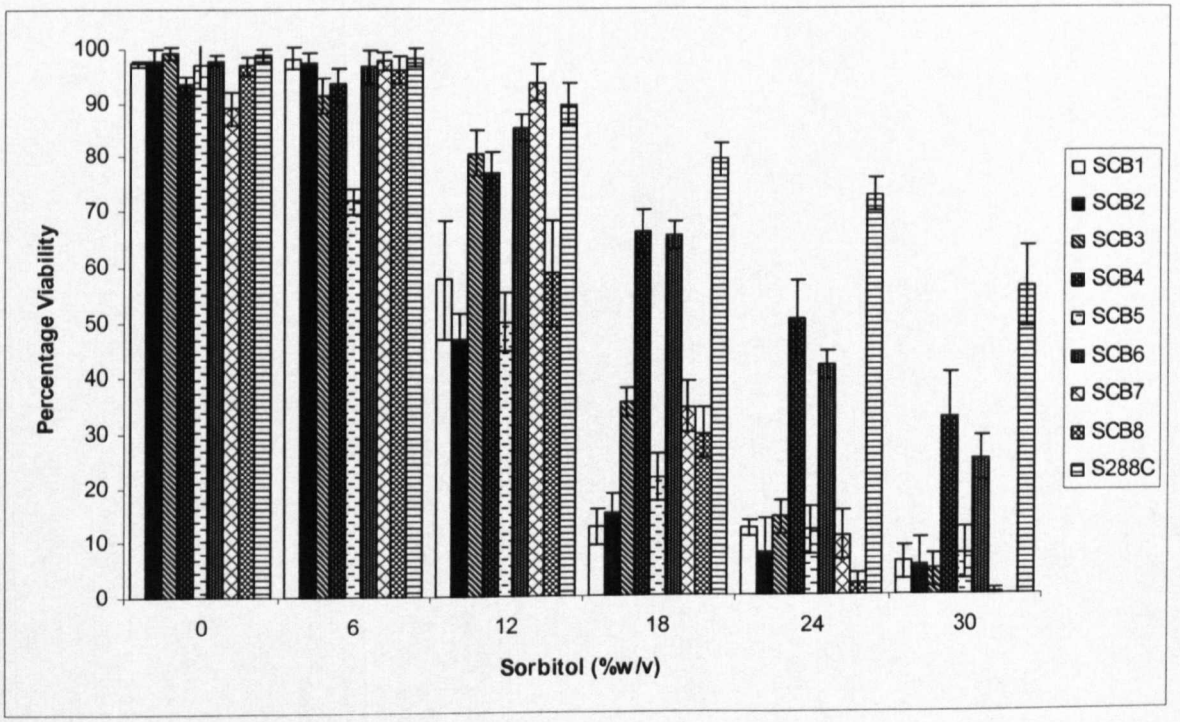
It was observed that each strain examined in this study exhibited a unique tolerance to sorbitol-induced osmotic stress (figure 3.1), yielding a strain dependent mortality profile (figure 3.2). It was noted that ale and lager strains did not exhibit group-specific tolerances to osmotic stress. Relative tolerance to sorbitol induced osmotic stress was strain dependent and could be most readily determined by two indicators: the concentration of sorbitol, at which viability decline was evident (table 3.1); and the extrapolated concentration of sorbitol required to reduce viability by 50% (table 3.2). Lager strains exhibited a greater variability in their relative tolerance to sorbitol induced stress. The lager strain SCB1 exhibited a 50% reduction in viability following exposure to *ca.* 14% (w/v) sorbitol (table 3.2), as compared with 24% sorbitol, which was required to elicit the same level of mortality in the strain SCB4 (table 3.2).



3.1(a)



3.1(b)



3.1 (c)

Figure 3.1 Tolerance of lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) strains of Saccharomyces species to sorbitol induced osmotic stress. Exponential phase cells were incubated in sorbitol (0-30% (w/v)) and incubated at 25°C for 48 hours. Triplicate samples were assessed for viability using (a) methylene violet, (b) MgANS and (c) propidium iodide. Error bars represent one standard deviation from the mean values.

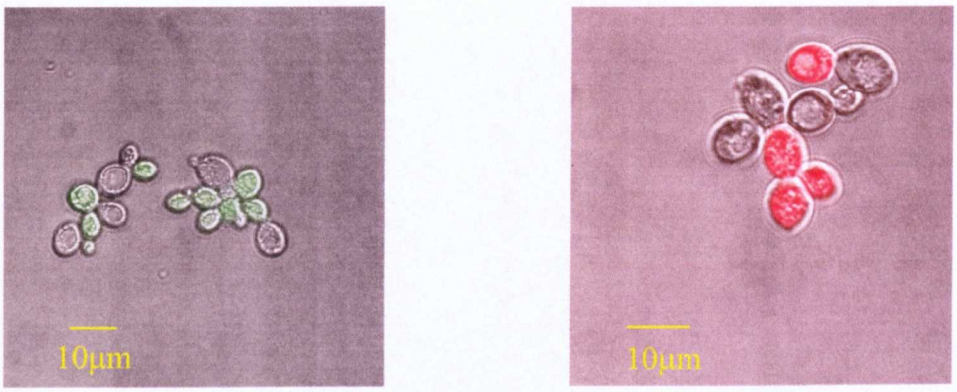
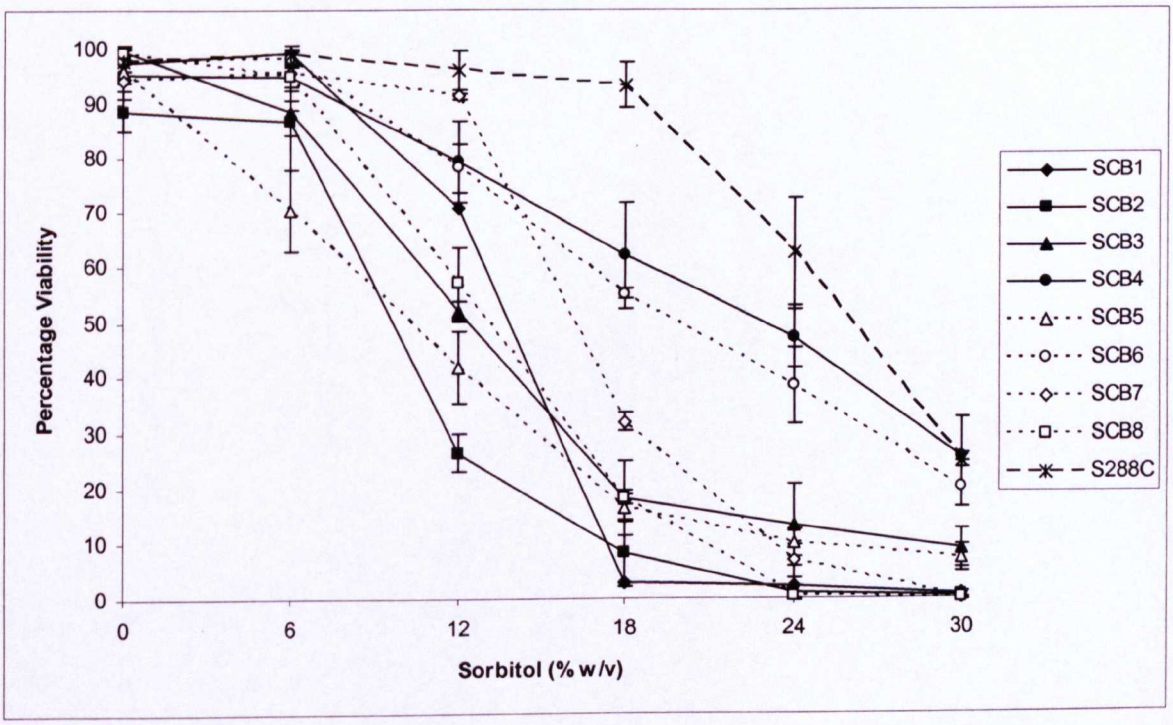
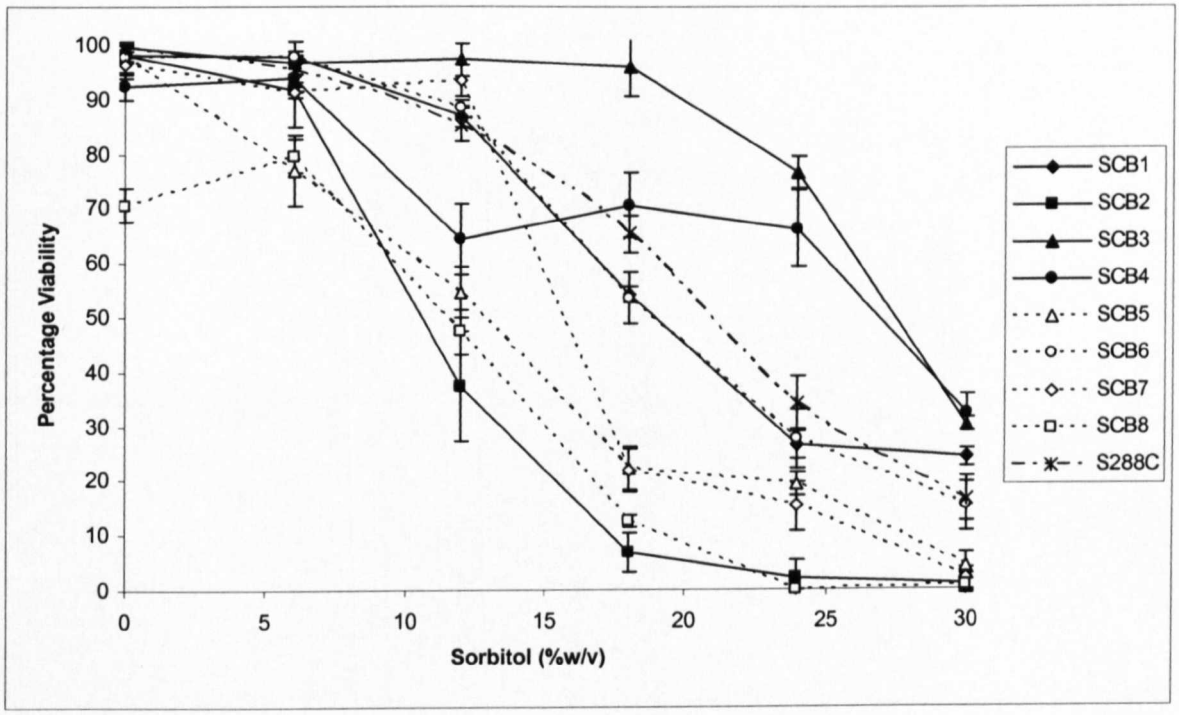


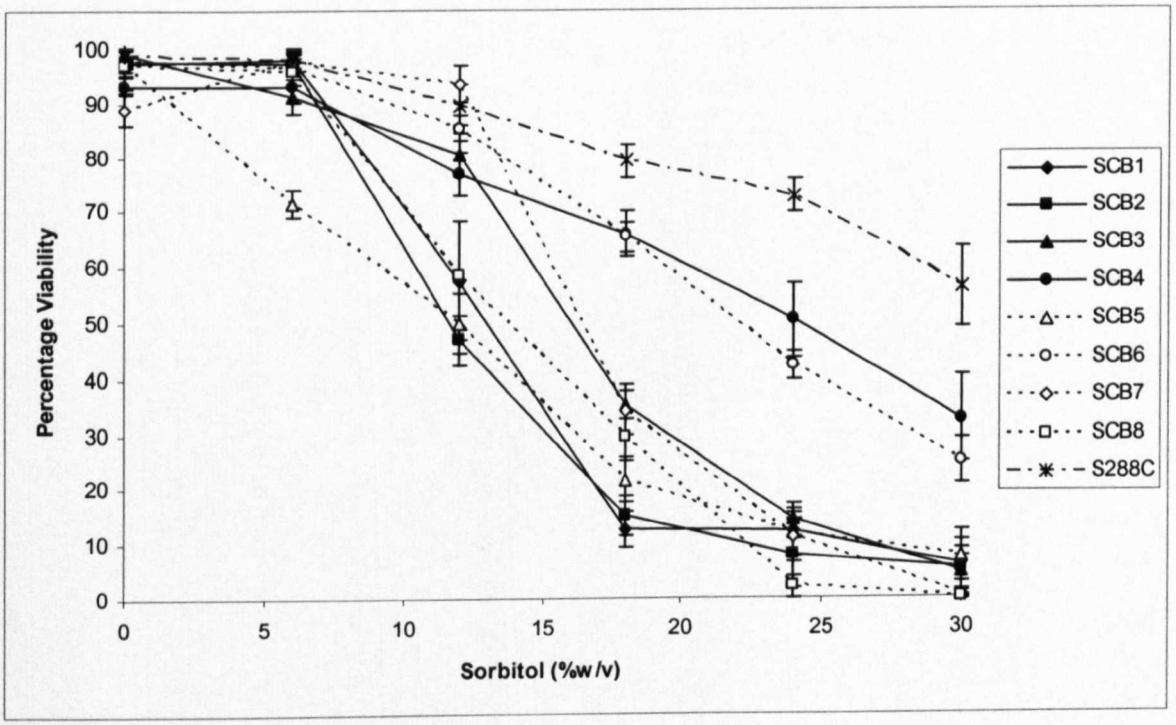
Figure 3.1.1 Examples of (a) MgANS and (b) propidium iodide viability staining in the ale strain SCB8. Cells were subject to 18% sorbitol for 48 hours and stained as described in sections 2.6.2 and 2.6.3 and examined using a Zeiss™ 510 laser scanning microscope.



3.2 (a)



3.2 (b)



3.2 (c)

Figure 3.2. Mortality profiles of lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) strains of *Saccharomyces* sp. to sorbitol-induced osmotic stress. Exponential phase cells were incubated in sorbitol (0-30% (w/v)) and incubated at 25°C for 48 hours. Triplicate samples were assessed for viability using (a) citrate buffered methylene violet (b) MgANS (c) propidium iodide. Error bars represent one standard deviation from the mean values.

Table 3.1. Relative percentages of sorbitol required to elicit a substantial (20%) reduction in viability (tolerance limit) for exponential phase lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) yeast strains determined using citrate-buffered methylene violet (CMV), MgANS, and propidium iodide (PI).

Strain	*Percentage of sorbitol required to affect viability (CMV)*	*Percentage of sorbitol required to affect viability (MgANS)*	*Percentage of sorbitol required to affect viability (PI)*
SCB1	12% (w/v)	13% (w/v)	9% (w/v)
SCB2	7% (w/v)	9% (w/v)	10% (w/v)
SCB3	12% (w/v)	23% (w/v)	13% (w/v)
SCB4	12% (w/v)	9% (w/v)	12% (w/v)
SCB5	6% (w/v)	6% (w/v)	6% (w/v)
SCB6	13% (w/v)	15% (w/v)	14% (w/v)
SCB7	14% (w/v)	14% (w/v)	14%(w/v)
SCB8	9% (w/v)	5% (w/v)	10% (w/v)
S288C	18% (w/v)	14% (w/v)	17% (w/v)

Table 3.2. Relative percentages of sorbitol required to elicit a reduction in viability by 50% (LD50) for exponential phase lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) yeast strains determined using citrate-buffered methylene violet (CMV), MgANS, and propidium iodide (PI).

Strain	*Percentage of sorbitol required to reduce viability by 50% (CMV)*	*Percentage of sorbitol required to reduce viability by 50% (MgANS)*	*Percentage of sorbitol required to reduce viability by 50% (PI)*
SCB1	14% (w/v)	19% (w/v)	13% (w/v)
SCB2	11% (w/v)	11% (w/v)	12% (w/v)
SCB3	15% (w/v)	27% (w/v)	16% (w/v)
SCB4	24% (w/v)	27% (w/v)	26% (w/v)
SCB5	12% (w/v)	12% (w/v)	12% (w/v)
SCB6	21% (w/v)	19% (w/v)	22% (w/v)
SCB7	16% (w/v)	16% (w/v)	17% (w/v)
SCB8	14% (w/v)	12% (w/v)	15% (w/v)
S288C	27% (w/v)	21% (w/v)	56% viable at 30%

In order to exclude the possibility that data variability at different sorbitol concentrations was responsible for the differences observed, F-test analysis was conducted between data sets (tables 3.3-3.8). For example, the comparison between the viabilities (determined using methylene violet) observed following exposure to 12% and 30% sorbitol revealed single tailed P-values of 0.33 and 0.34, respectively (tables 3.3 and 3.5). These results demonstrate that the statistical variances of the two groups (ale and lager) was equal (P was greater than 0.05) and thus could not be readily differentiated. Similarly, the homoscedastic two-tailed t-test yielded values of 0.27 and 0.89 for 12% and 30% sorbitol stressed cells, respectively, confirming the null hypothesis that the sample means of these data sets were statistically similar. For 30% sorbitol stressed cells values of 7.36 and 6.83 were obtained for lager and ale strains, respectively. The statistical analysis indicated that there were no significant differences in the mean values obtained for ale and lager strains, and it was therefore impossible to determine whether ale or lager strains were more osmotolerant.

Table 3.3 F test analysis of viability of ale and lager cells subjected to 12% sorbitol stress assessed using citrate buffered methylene violet.

F-Test Two-Sample for Variances	*12% Ale vs. Lager*	
	Lager	Ale
Mean	57.16	67.08
Variance	537.06	411.717
Observations	12	12
Df	11	11
F	1.30	
P(F<=f) one-tail	0.33	

Table 3.4. t-test analysis of viability of ale and lager cells subjected to 12% sorbitol stress assessed using citrate buffered methylene violet.

t-Test: Two-Sample Assuming Unequal Variances	*12% Ale vs. Lager*	
	Lager	*Ale*
Mean	57.17	67.08
Variance	537.06	411.72
Observations	12	12
Hypothesized Mean Difference	0	
Df	22	
P(T<=t) one-tail	0.13	
P(T<=t) two-tail	0.28	

Table 3.5. F test analysis of viability of ale and lager cells subjected to 30% sorbitol stress assessed using citrate buffered methylene violet.

F-Test Two-Sample for Variances	*30% Ale vs. Lager*	
	Lager	Ale
Mean	7.36	6.83
Variance	97.86	76.33
Observations	11	12
Df	10	11
F	1.28	
P(F<=f) one-tail	0.34	

Table 3.6. t-test analysis of viability of ale and lager cells subjected to 30% sorbitol stress assessed using citrate buffered methylene violet.

t-Test: Two-Sample Assuming Unequal Variances	*30% Ale vs. Lager*	
	Lager	Ale
Mean	7.36	6.83
Variance	97.86	76.33
Observations	11	12
Hypothesized Mean Difference	0	
Df	20	
P(T<=t) one-tail	0.45	
P(T<=t) two-tail	0.89	

3.2.2 Is osmotic stress tolerance growth phase dependent?

The response of haploid *S. cerevisiae* strains to stress has been demonstrated to be dependent on the physiological state of the cells (Werner-Washburne *et al.*, 1993; Hounsa *et al.*, 1998; Gasch and Werner-Washburne, 2002) with stationary phase populations exhibiting a greater resistance to a number of stresses than exponential phase cells (Hohmann, 1997, Jamieson, 1998). Steels *et al.* (1994) demonstrated that stationary phase populations of *S. cerevisiae* cells were inherently more tolerant to both heat and oxidative stress. The differences in resistance were correlated to changes in membrane composition that occur at the end of exponential growth (Werner-Washburne *et al.*, 1993), demonstrating that the established differences in the stress tolerance of stationary phase cells may be due to intrinsic physiological changes that are concomitant with the onset of stationary phase. The ability of stationary phase populations to exhibit enhanced tolerance to a range of stresses can also be accounted for by the differences in transcriptional activity that occur on entry into this growth phase. Differential expression of general stress response genes, including plasma membrane heat shock proteins (HSP), occurs between stationary and exponential phase cells (Werner-Washburne *et al.*, 1989; Panaretou and Piper, 1992) with the former exhibiting higher expression. This modification in the levels of expression of various 'stress genes' at the onset of stationary phase has been linked with the ability to survive for long periods of time during stressful conditions (Werner-Washburne *et al.*, 1996).

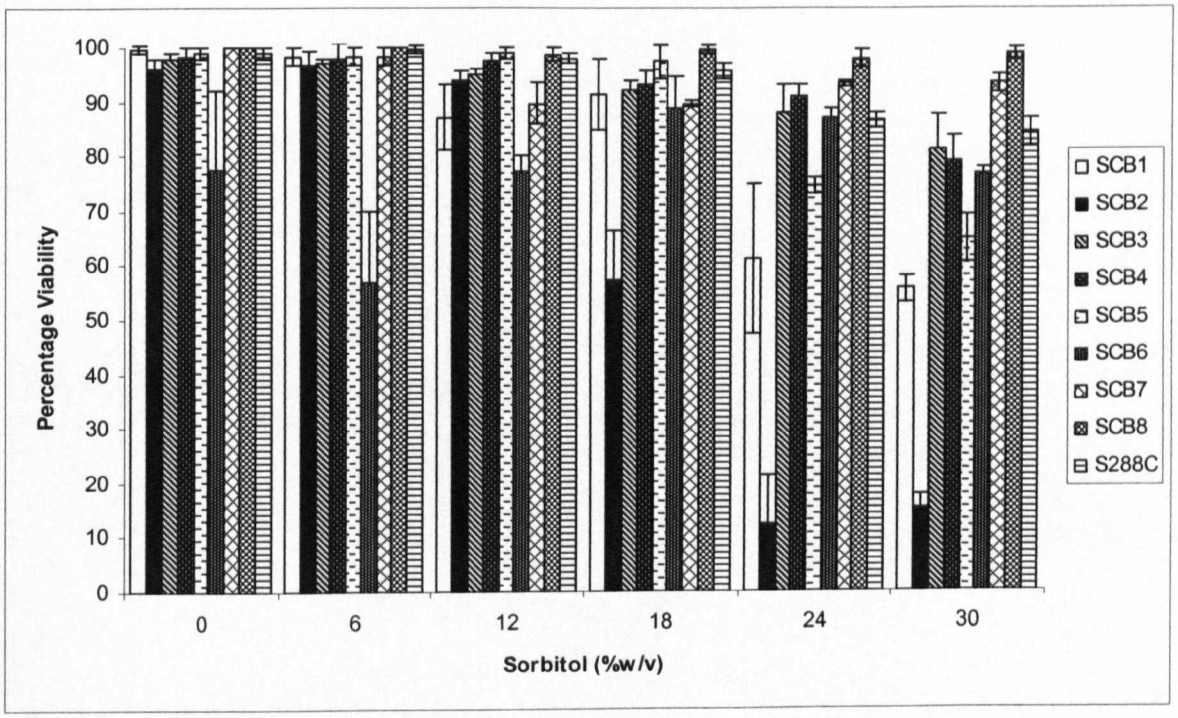
The response of brewing strains of *S. cerevisiae* to stress in the exponential and stationary phases of growth are poorly characterised to date and, although osmotic stress has been studied in both growth phases in laboratory haploid strains, little work has been done using polyploid and aneuploid brewing strains. Brewing strains were grown in YPD to obtain a stationary phase population (section 2.4.2). The optical density at 600nm was monitored and compared to a standard growth curve for each strain. Cells were subsequently harvested following the onset of stationary phase, and exposed to sorbitol as indicated in section 2.5. Viability was assessed using methylene violet (section 2.6.1), MgANS (section 2.6.2), and propidium iodide (section 2.6.3).

Stationary phase populations of ale and lager brewing yeast were more tolerant to osmotic stress than the corresponding exponential phase populations (figures 3.3 and 3.4) Within the range of sorbitol concentrations utilised (0-30% (w/v) only one strain (SCB2) exhibited a reduction in viability by 50% (figures 3.3-and 3.4). Strain SCB2

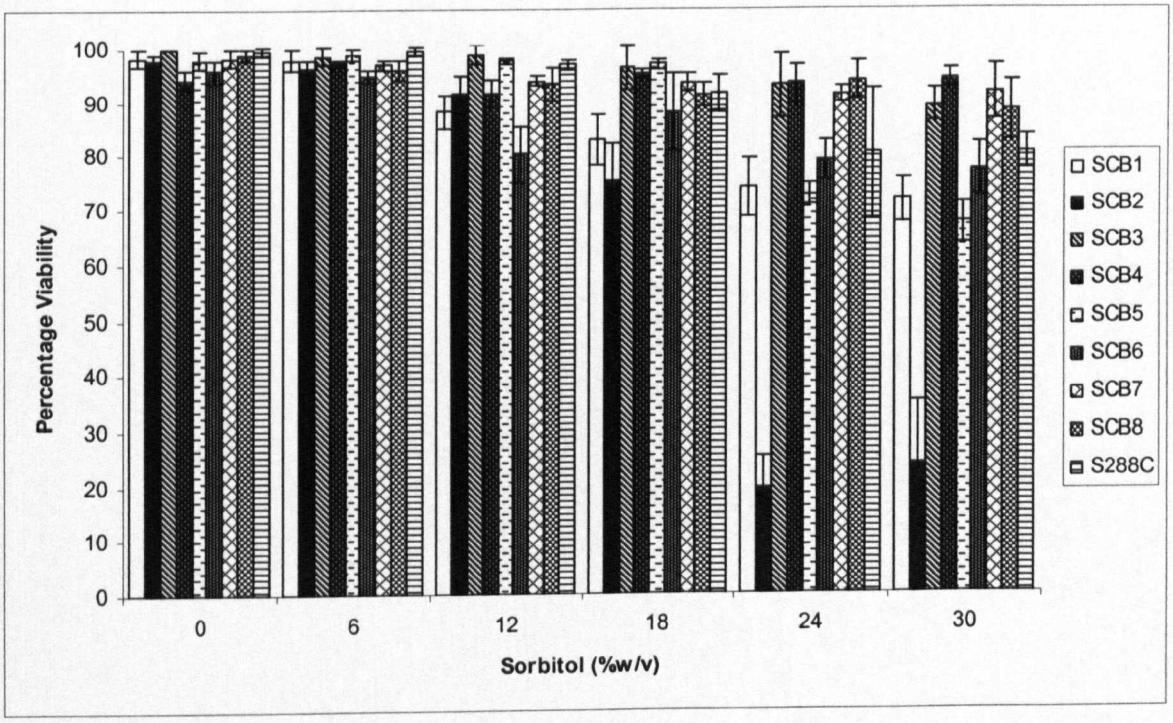
was also observed to be somewhat osmosensitive in exponential phase (figures 3.3 and 3.4). Stationary phase physiological status appeared to afford some protection against osmotic shock (compare figures 3.1 (a-c) and 3.3 (a-c)). The concentration of sorbitol required to reduce viability by 50% increased from *ca.*12% (w/v) in the exponential population to 20% (w/v) in stationary phase.

As observed for exponential phase populations, stationary phase cells exhibited a decrease in viability as exogenous sorbitol concentrations were increased, however, these viability reductions were not as marked for the latter as for the former (compare figures 3.1/3.2 and 3.3/3.4). The lager strains SCB1, SCB3 and SCB4 demonstrated a 10-30% reduction of viability for stationary phase population following exposure to 30% sorbitol (figures 3.3 and 3.4), whereas a 70-95% reduction in viability was observed for corresponding exponential phase populations in the presence of 30% (w/v) sorbitol (figures 3.1 and 3.2).

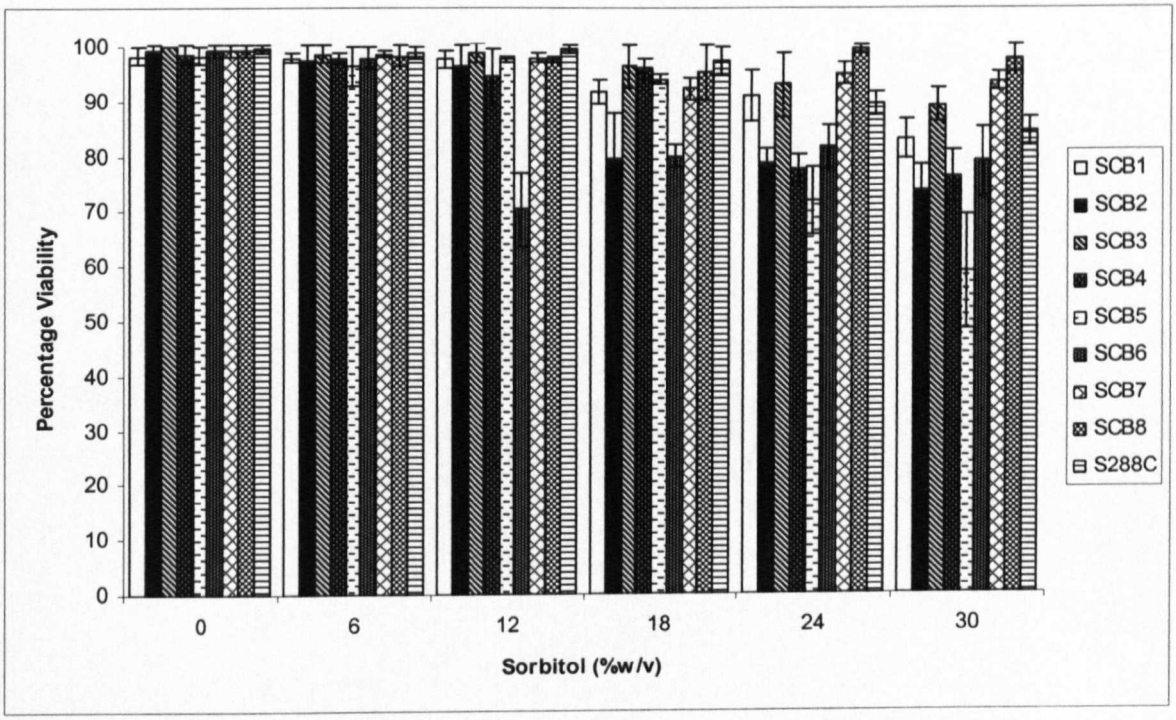
The same trend was demonstrated by the ale strains SCB7 and SCB8 (figures 3.3 and 3.4), however, strains SCB5 and SCB6 exhibited a greater osmosensitivity with a reduction of viability between 30 and 35% (figures 3.3 and 3.4). The reference laboratory haploid strain S288C exhibited a reduction in viability of 15% upon exposure to 30% (w/v) sorbitol (figures 3.3-3.4 and table 3.7), analogous to the response of lager strains, but demonstrating a greater degree of osmotolerance than ale strains. The range of sorbitol of 0-30% was sufficient to elicit a 50% reduction in viability in all exponential phase ale strains (figures 3.1 and 3.3.2 and table 3.2) and the haploid S288C strain, (figures 3.1 and 3.2), however, this was not true for any stationary phase population of ale strain examined (table 3.8).


Percentage Viability
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
Sorbitol (%w/v)
SCB1
SCB2
SCB3
SCB4
SCB5
SCB6
SCB7
SCB8
S288C

3.3 (a)

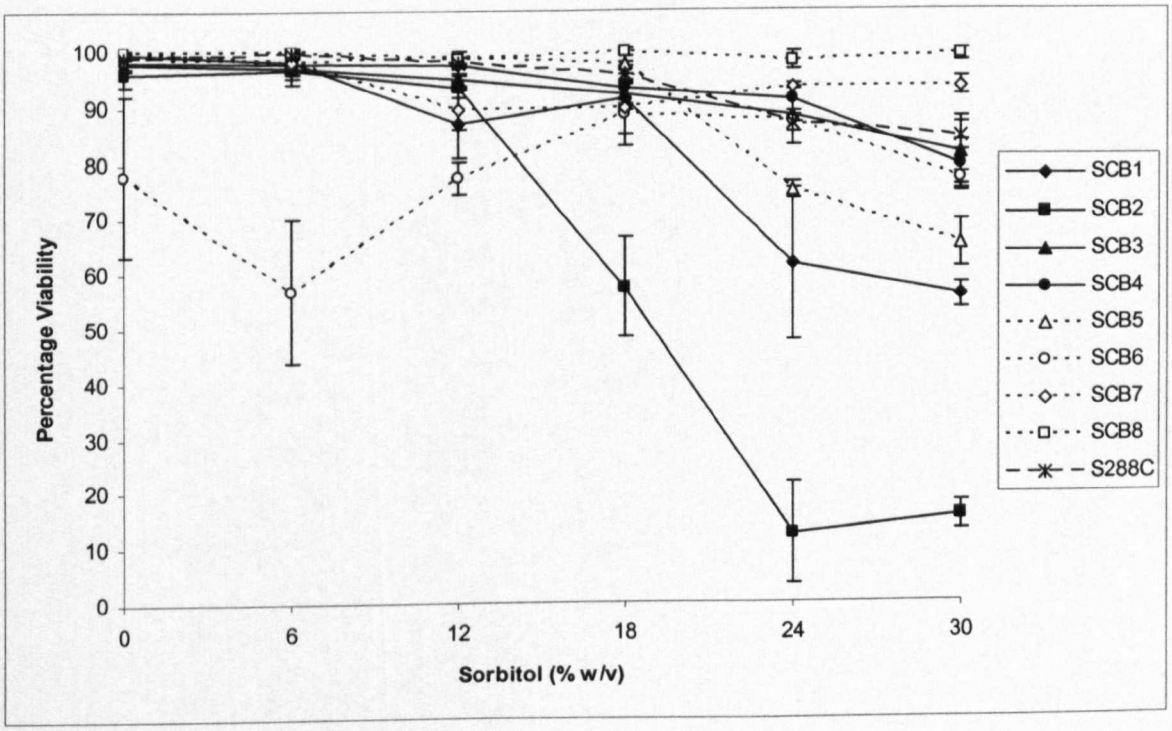

Percentage Viability
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
Sorbitol (%w/v)
SCB1
SCB2
SCB3
SCB4
SCB5
SCB6
SCB7
SCB8
S288C

3.3(b)

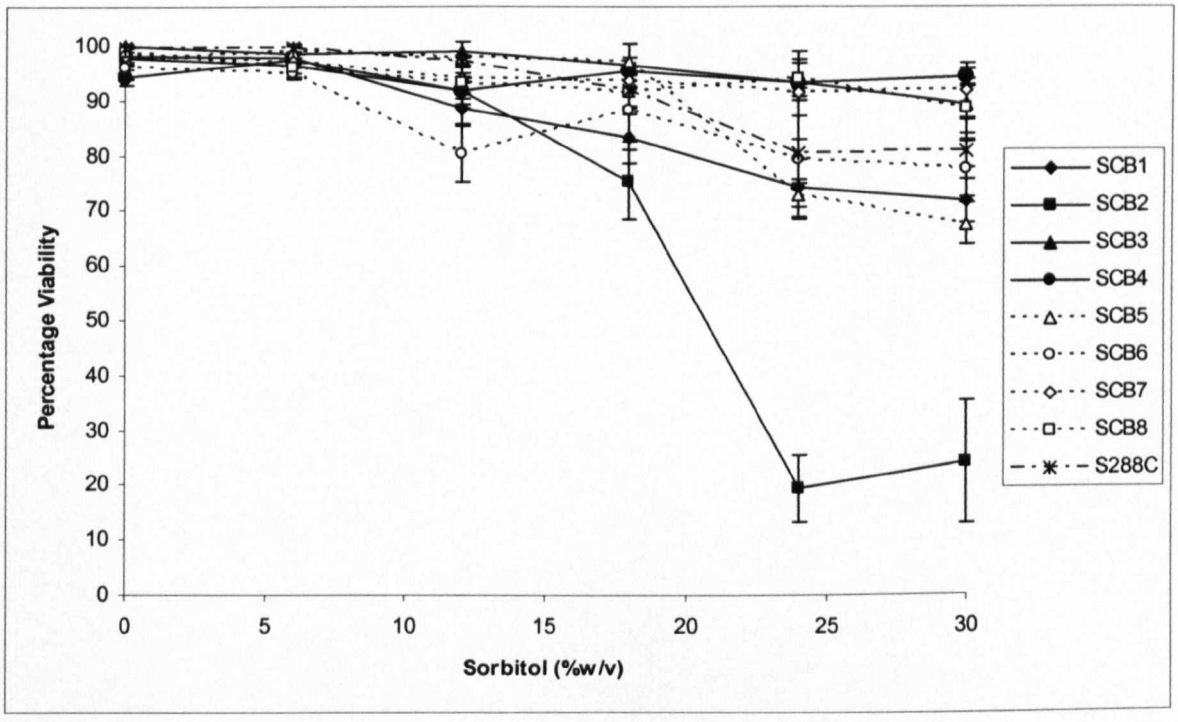


3.3 (c)

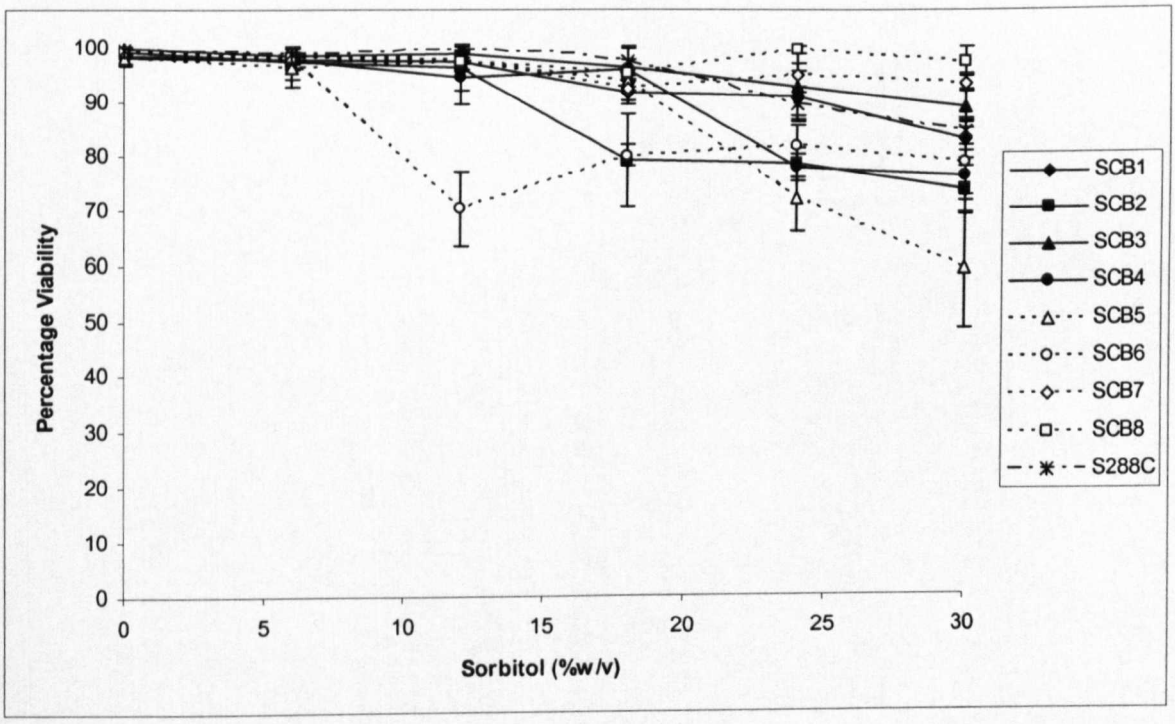
Figure 3.3. Tolerance of lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) strains of *Saccharomyces* sp. to sorbitol induced osmotic stress. Stationary phase cells were incubated in sorbitol (0-30% [w/v]) and incubated at 25°C for 48 hours. Triplicate samples were assessed for viability using (a) citrate methylene violet (b) MgANS and (c) propidium iodide. Error bars represent one standard deviation from the mean values.



3.4 (a)



3.4 (b)



3.4 (c)

Figure 3.4. Mortality profiles of lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) strains of *Saccharomyces* sp. to sorbitol induced osmotic stress. Stationary phase cells were incubated in sorbitol (0-30% [w/v]) and incubated at 25°C for 48 hours. Triplicate samples were assessed for viability using (a) citrate buffered methylene violet, (b) MgANS and (c) propidium iodide. Error bars represent one standard deviation from the mean values.

Table 3.7. Relative percentages of sorbitol required to elicit a substantial (20%) reduction in viability (tolerance limit) for stationary phase lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) yeast strains determined using citrate buffered methylene violet (CMV), MgANS, and propidium iodide (PI).

Strain	*Percentage of sorbitol required to affect viability (CMV)*	*Percentage of sorbitol required to affect viability (MgANS)*	*Percentage of sorbitol required to affect viability (PI)*
SCB1	20% (w/v)	21% (w/v)	85% viable at 30%
SCB2	14% (w/v)	17% (w/v)	24% (w/v)
SCB3	83% viable at 30%	92% viable at 30	84% viable at 30%
SCB4	30% (w/v)	94% viable at 30	24% (w/v)
SCB5	24% (w/v)	24% (w/v)	23% (w/v)
SCB6	13% (w/v)	24% (w/v)	9% (w/v)
SCB7	92% viable at 30%	91% viable at 30%	92% viable at 30%
SCB8	99% viable at 30%	89% viable at 30%	97% viable at 30%
S288C	85% viable at 30%	83% viable at 30%	85% viable at 30%

Table 3.8. Relative percentages of sorbitol required to elicit a reduction in viability by 50% (LD50) for exponential phase lager (SCB1-4), ale (SCB5-8) and laboratory haploid (S288C) yeast strains determined using citrate buffered methylene violet (CMV), MgANS, and propidium iodide (PI).

Strain	*Percentage of sorbitol required to reduce viability by 50% (CMV)*	*Percentage of sorbitol required to reduce viability by 50% (MgANS)*	*Percentage of sorbitol required to reduce viability by 50% (PI)*
SCB1	58% viable at 30%	75% viable at 30%	85% viable at 30%
SCB2	19% (w/v)	22% (w/v)	77% viable at 30%
SCB3	83% viable at 30%	92% viable at 30	84% viable at 30%
SCB4	80% viable at 30%	94% viable at 30%	78% viable at 30%
SCB5	65% viable at 30%	69% viable at 30%	57% viable at 30%
SCB6	76% viable at 30%	77% viable at 30%	77% viable at 30%
SCB7	92% viable at 30%	91% viable at 30%	92% viable at 30%
SCB8	99% viable at 30%	89% viable at 30%	97% viable at 30%
S288C	85% viable at 30%	83% viable at 30%	85% viable at 30%

F-test analysis on representative data (12% and 30% sorbitol stressed lager cells in exponential phase and stationary phase determined using methylene violet) indicated that significant differences in the variance of the two sample groups occurred. P-values of 6.1×10^{-6} and 8.3×10^{-3} for 12% and 30% sorbitol stressed cells were returned, respectively (tables 3.9 and 3.10). A heteroscedastic two-tailed T-test provided statistical evidence for the rejection of the null hypothesis of equal sample means with P-values of 1.9×10^{-3} for 12% sorbitol stressed cells, and 6.9×10^{-5} for 30% sorbitol stressed cells (tables 3.11 and 3.12). Stationary phase lager strains were therefore clearly more resistant to osmotic stress than exponential phase cells.

The same trend was demonstrated in ale strains; F-test analysis was unable to differentiate statistically between the variances of 12% and 30% sorbitol stressed cells in exponential and stationary, with P-values of 0.16 and 0.06 obtained respectively (tables 3.13 and 3.14). The subsequent homoscedastic T-test analysis, however, proved that there were significant statistical differences in the means of both sets of samples. A two-tailed P-value of 3.3×10^{-3} was obtained for the 12% stressed data set (table 3.15) and a highly significant 1.49×10^{-13} for 30% sorbitol stressed cells (table 3.16).

In summary, these data support previously published data, which suggests that stationary phase populations are more resistant to sorbitol-induced osmotic stress than exponential phase populations.

Table 3.9. F test analysis of viability of lager cells subjected to 12% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.

F-Test Two-Sample for Variances	*12% Lager Exponential vs. Stationary*	
	Exponential	Stationary
Mean	57.17	93.25
Variance	537.06	24.02
Observations	12	12
Df	11	11
F	22.36	
P(F<=f) one-tail	6.07×10^{-6}	

Table 3.10. F test analysis of viability of lager cells subjected to 30% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.

F-Test Two-Sample for Variances	*30% Lager Exponential vs. Stationary*	
	Exponential	Stationary
Mean	7.36	57.83
Variance	97.86	876.70
Observations	11	12
Df	10	11
F	0.11	
P(F<=f) one-tail	8.3×10^{-3}	

Table 3.11. t-test analysis of viability of lager cells subjected to 12% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.

t-Test: Two-Sample Assuming Unequal Variances	*12% Lager Exponential vs. Stationary*	
	Exponential	Stationary
Mean	57.17	93.25
Variance	537.06	24.02
Observations	12	12
Hypothesized Mean Difference	0	
Df	12	
P(T<=t) one-tail	9.78×10^{-5}	
P(T<=t) two-tail	1.96×10^{-3}	

Table 3.12. t-test analysis of viability of lager cells subjected to 30% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.

t-Test: Two-Sample Assuming Unequal Variances	*30% Lager Exponential vs. Stationary*	
	Exponential	Stationary
Mean	7.36	57.83
Variance	97.86	876.70
Observations	11	12
Hypothesized Mean Difference	0	
Df	14	
P(T<=t) one-tail	3.422×10^{-5}	
P(T<=t) two-tail	6.844×10^{-5}	

Table 3.13. F test analysis of viability of ale cells subjected to 12% sorbitol stress in exponential and stationary phases of growth assessed using citrate methylene violet

F-Test Two-Sample for Variances	*12% Ale Exponential vs. Stationary*	
	Exponential	Stationary
Mean	67.08	91.17
Variance	411.72	221.97
Observations	12	12
Df	11	11
F	1.86	
P(F<=f) one-tail	0.16	

Table 3.14. F test analysis of viability of ale cells subjected to 30% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.

F-Test Two-Sample for Variances	*30% Ale Exponential vs. Stationary*	
	Exponential	Stationary
Mean	6.83	83.5
Variance	76.33	202.27
Observations	12	12
Df	11	11
F	0.38	
P(F<=f) one-tail	0.06	

Table 3.15. t-test analysis of viability of ale cells subjected to 12% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.

t-Test: Two-Sample Assuming Equal Variances	*12% Ale Exponential vs. Stationary*	
	Exponential	Stationary
Mean	67.08	91.17
Variance	411.72	221.97
Observations	12	12
Hypothesized Mean Difference	0	
Df	22	
P(T<=t) one-tail	1.57×10^{-2}	
P(T<=t) two-tail	3.15×10^{-2}	

Table 3.16. t-test analysis of viability of ale cells subjected to 30% sorbitol stress in the exponential and stationary phases of growth assessed using citrate methylene violet.

t-Test: Two-Sample Assuming Equal Variances	*30% Ale Exponential vs. Stationary*	
	Exponential	Stationary
Mean	6.83	83.5
Variance	76.33	202.27
Observations	12	12
Hypothesized Mean Difference	0	
Df	22	
P(T<=t) one-tail	7.45×10^{-14}	
P(T<=t) two-tail	1.49×10^{-13}	

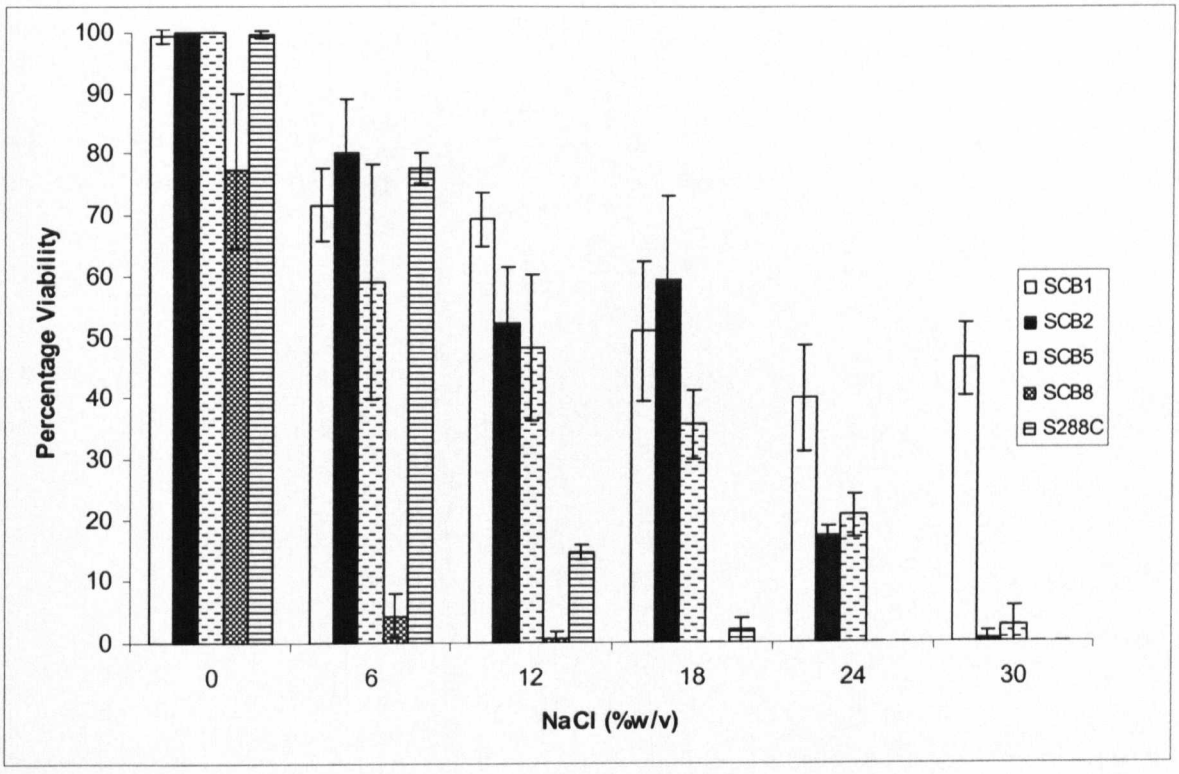
3.2.3 Is osmotic stress tolerance solute-dependent?

Osmotic stress occurs when the osmolarity of the external medium changes (section 1.6). The increase in concentration of external osmolytes induces hyperosmotic stress, and this has the consequence of imbalancing osmotic potentials. An increase in external solutes may also have the effect of inducing intracellular accumulation of the exogenous osmolyte (Serrano, 1996). The intracellular accumulation of an external osmolyte may, however, represent a challenge to cellular physiology and metabolism, where toxic ions and other solutes are involved (Yale and Bohnert, 2001). It has been previously demonstrated that laboratory haploid *S. cerevisiae* strains exhibit a greater tolerance to sorbitol stress than NaCl stress (Hounsa *et al.*, 1998), although osmotic stress resistance to either solute is also a function of the media composition, on which the cells were grown. In part, this may be explained by a reduced tolerance to salt toxicity in *Saccharomyces sp.* due to the accumulation of cellular damage as a result of dissociated Na^+ and possibly Cl^- ions (Serrano, 1996; Serrano *et al.*, 1997). It has also been suggested that an increase in intracellular monovalent cations, such as Na^+, mediate an oxidative stress, which targets various structural components of the cell (Serrano, 1996; Yale and Bonhert, 2001).

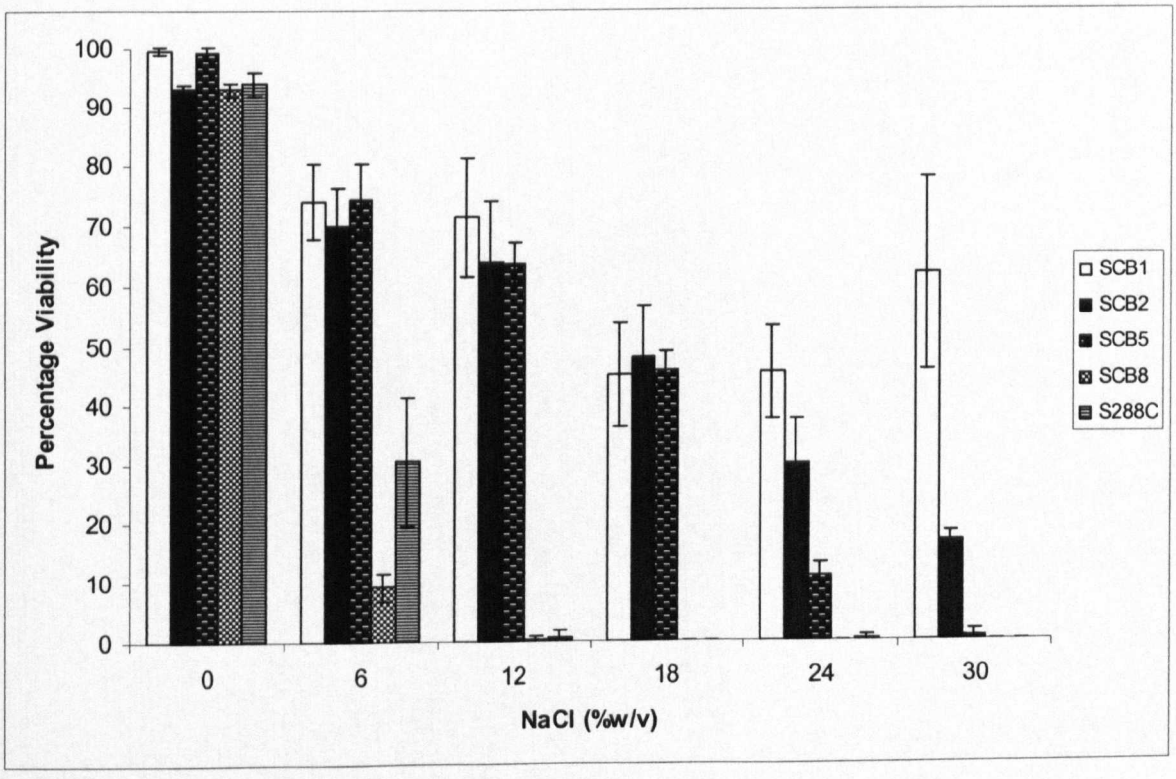
The relationship between osmotic stress tolerance and the solute used to induce this stress has not been previously reported for polyploid or aneuploid yeast strains.

Cells were grown to the required phase of growth (stationary or exponential), harvested and resuspended in NaCl at concentrations indicted and viability was determined using propidium iodide (section 2.6.1), MgANS (section 2.6.2) and methylene violet (section 2.6.3).

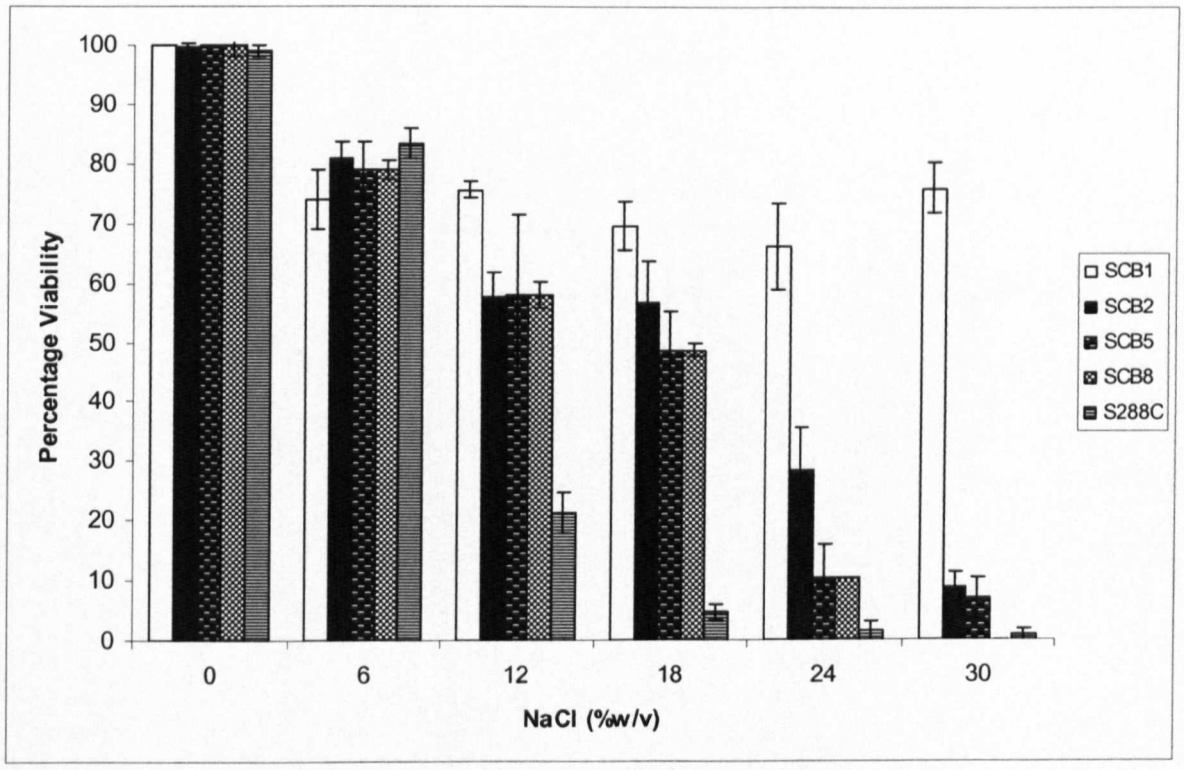
Stationary phase populations of the strains examined demonstrated a greater resistance to salt stress than exponential phase cells, supporting the previous observations that stationary phase yeast cell populations are more osmotolerant than their exponential phase counterparts. The combined effects of Na^+ toxicity and osmotic shock appear to have a greater deleterious effect upon the viability of strains utilised in this study. Concentrations of NaCl of *ca.* 14-19% were sufficient to reduce cell viability in strains SCB1 and SCB2 to 50% in stationary phase (figures 3.5 and 3.6). These data indicate that SCB2 has comparable sorbitol and salt tolerances (compare figures 3.3 and 3.5), however, the same is not true for strain SCB1. Ale strains were observed to be less tolerant to NaCl stress than lager strains in stationary phase. SCB8 demonstrated a 50% reduction in viability upon treatment with 3% NaCl, (figure 3.5). Strain SCB5 was somewhat more resistant, as a concentration of 17% NaCl was required to reduce viability to 50% (figure 3.5 and 3.6). Intermediate halotolerance between these two points was demonstrated by the laboratory haploid strain S288C (figures 3.5 and 3.6). Exponential phase cells exhibited a highly salt intolerant phenotype with viability in all strains tested (SCB1, SCB2, SCB5, SCB8 and S288C figure 3.6) reduced to approximately zero following exposure to 6% NaCl.


Percentage Viability
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
NaCl (%w/v)
SCB1
SCB2
SCB5
SCB8
S288C

3.5 (a)

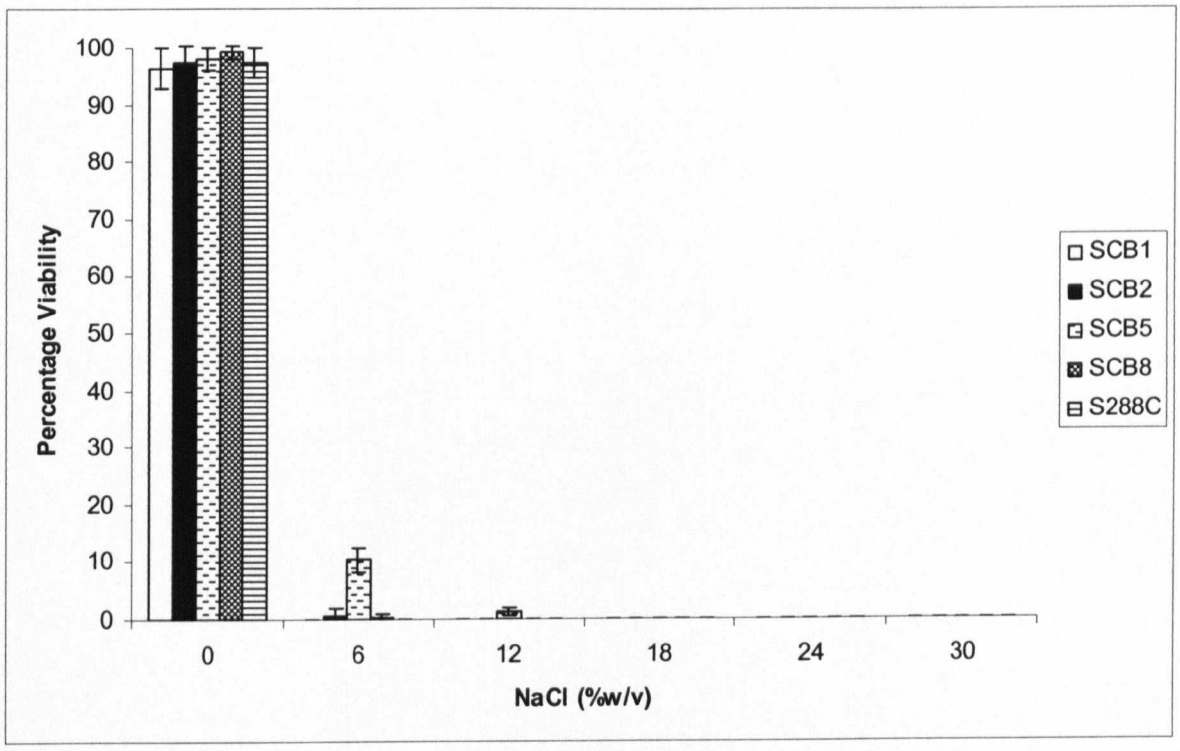

Percentage Viability
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
NaCl (%w/v)
SCB1
SCB2
SCB5
SCB8
S288C

3.5 (b)

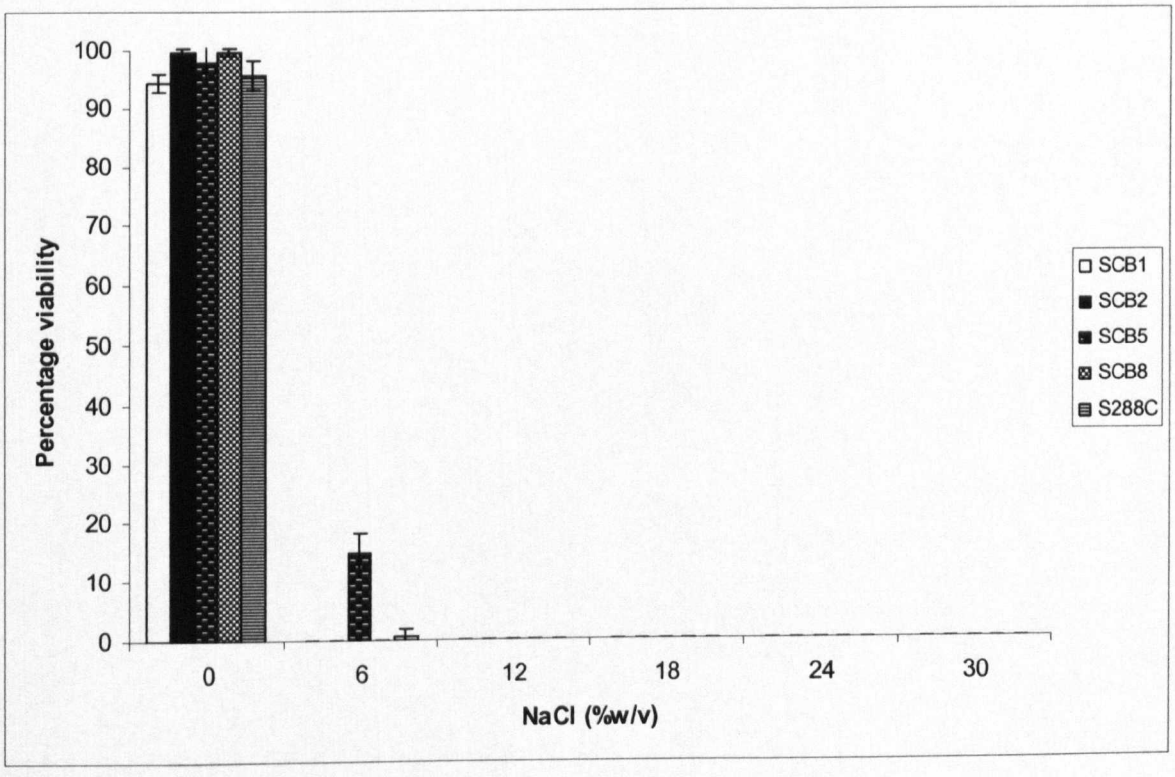


3.5 (c)

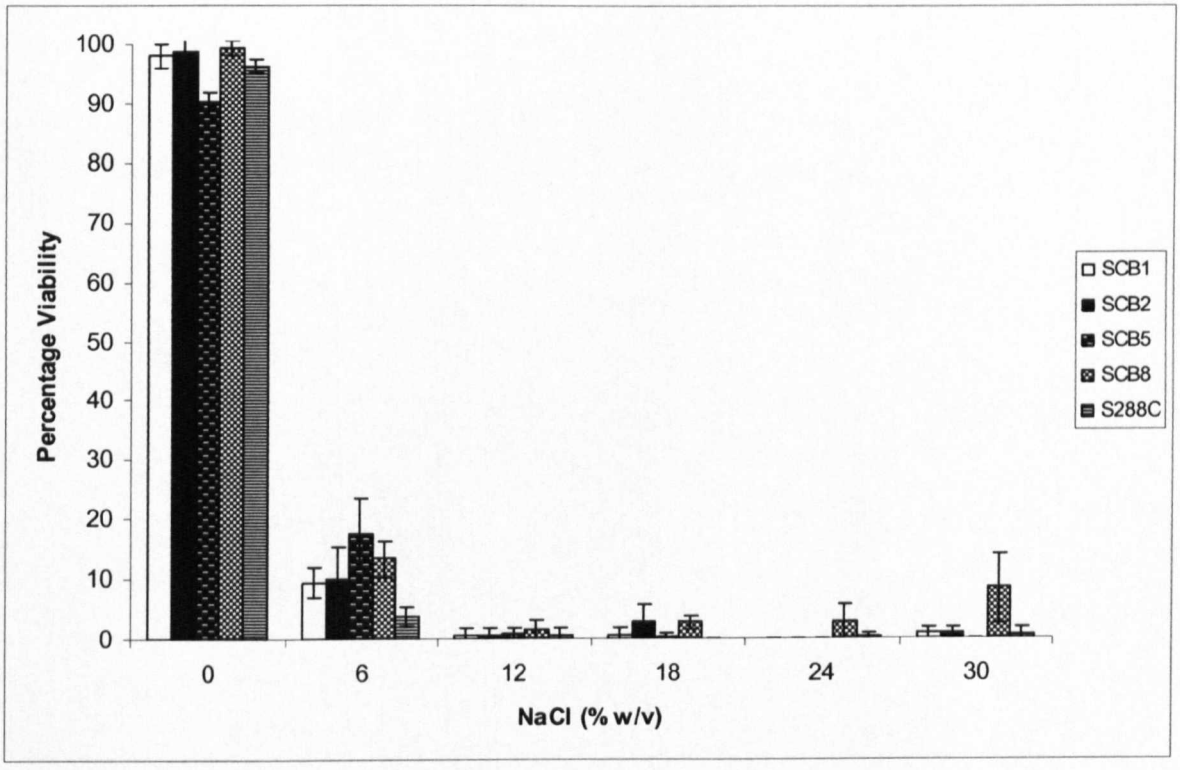
Figure 3.5. Tolerance of lager (SCB1 and 4), ale (SCB5 and 8) and laboratory haploid (S288C) strains of *Saccharomyces* sp. to NaCl induced osmotic stress. Stationary phase cells were incubated in NaCl (0-30% [w/v]) and incubated at 25°C for 48 hours. Triplicate samples were assessed for viability using (a) citrate methylene violet (b) MgANS and (c) propidium iodide. Error bars represent one standard deviation from the mean values.


Percentage Viability
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
NaCl (%w/v)
SCB1
SCB2
SCB5
SCB8
S288C

3.6 (a)


Percentage viability
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
NaCl (%w/v)
SCB1
SCB2
SCB5
SCB8
S288C

3.6 (b)



3.6 (c)

Figure 3.6. Tolerance of lager (SCB1 and 4), ale (SCB5and 8) and laboratory haploid (S288C) strains of *Saccharomyces* sp. to NaCl induced osmotic stress. Exponential phase cells were incubated in NaCl (0-30% [w/v]) and incubated at 25°C for 48 hours. Triplicate samples were assessed for viability using (a) citrate methylene violet (b) MgANS and (c) propidium iodide. Error bars represent one standard deviation from the mean values.

3.3 Discussion

Osmotic stress can be defined as any situation, where there is an imbalance of intracellular and extracellular osmolarities, sufficient to cause a deleterious change in physiology (Csonka and Hanson, 1991). Yeast is continuously subjected to changes in external osmolarity that can be extremely detrimental to cellular functioning (Hounsa *et al.,* 1998; Beney *et al.*, 2000; Tamas and Hohmann, 2003). Osmotic stress may occur when there is a low external osmotic potential, for example in deionised water, and is characterised by an influx of water into the cell resulting in hypoosmotic stress (Csonka and Hanson, 1991; Dihazi *et al.,* 2001). Conversely osmotic stress may also result from exposure to environments comprising high solute concentrations leading to hyperosmotic stress characterised by the loss of cellular water and subsequently turgor (Blomberg and Adler, 1992; Wood, 1999).

Yeast may possess an innate ability to withstand the deleterious effects of hyperosmotic pressure as a consequence of membrane structure, vacuolar functioning, residual trehalose levels and many other intrinsic factors (Latterich and Watson, 1991; Sharma *et al.,* 1996; Singer and Lindquist, 1998; Nass and Rao, 1999). In addition, a highly refined sensing and response system may also be activated. *S. cerevisiae* elicits two responses to an increase in extracellular osmolarity termed the acute and chronic responses, respectively (Nass and Rao, 1999). Nass and Rao (1999) define the chronic response (or acquired osmotolerance) as a signal transduction-mediated response to continued osmotic stress, which alters the levels of specific proteins (discussed in chapter 6). Conversely, the acute response is invoked when non-osmotically stressed cells are suddenly subject to high external osmolarity, and survival is determined by intrinsic physiological characteristics (Nass and Rao, 1999). Osmotolerance may be defined as the innate physiological resistance to osmotic stress in response to both acute and chronic environmental hyperosmotic pressure and represents the mechanisms, by which cells maintain viability in the presence of deleterious solute concentrations.

In this chapter, the effect of hyperosmotic stress on the osmotolerance of brewing and haploid *Saccharomyces* species was considered. The impact of strain, growth phase and solute type on the maintenance of viability for the haploid laboratory strain S288C, ale strains (SCB5-8) and lager strains (SCB1-4) was examined.

No inclusive study concerning the relative osmotolerances of laboratory haploid *S. cerevisiae* strains to sorbitol induced osmotic stress in liquid culture has been

published. However, the osmotolerance of a few haploid strains has been reported, including CBS1171 (Marechal *et al.*, 1995) W303-1A (Hounsa *et al.*, 1998), and K601 (Nass and Rao, 1999), to withstand osmotic stress. The extent, to which the data obtained using laboratory strains, can be extrapolated to industrial strains remains unclear, given the numerous physiological differences between haploid and polyploid production yeast strains.

Osmotolerance of ale and lager yeast to sorbitol-induced hyperosmotic stress was observed to be strain-dependent, with exponential populations of lager and ale strains demonstrating similar resistance to those of haploid strains of *S. cerevisiae,* suggesting that osmotolerance of *Saccharomyces* sp. is generally conserved. The haploid laboratory strain (S288C) used as a reference in this study, demonstrated slightly elevated osmotolerance compared to that exhibited by the production brewing strains examined. The osmotolerance of S288C could not, however, be directly compared to previous reports concerning other haploid strains (Marechal *et al.*, 1995; Hounsa *et al.*, 1998), since different growth conditions and methods of viability determination were employed in each study. The results presented in this chapter did not provide evidence for increased osmotic stress resistance in either ale or lager yeast strains, as was clearly demonstrated by comparative analysis and statistical determinations.

For commercial brewing ale and lager yeast strains assigned to the species *S. cerevisiae* and *S. cerevisiae* (syn. *S. pastorianus*), respectively, no comprehensive assessment of osmotolerance has been reported, despite the observation that the use of high gravity wort imposes additional stress on brewing yeast strains (Panchal and Stewart, 1980; Haggart *et al.*, 1999; Stewart, 1999). The results presented in this chapter clearly demonstrate that brewing yeast osmotolerance is strain-dependent and thus it is suggested that strains exhibiting greater innate resistance to hyperosmotic stress are more suitable for use in high gravity brewing. Indeed, the increase in external osmotic pressure mediated by the use of high gravity worts has been demonstrated to negatively impact upon viability, growth and fermentation performance (D'Amore, 1992). In support of this, Cahill *et al.*, (2000) have demonstrated that an increase in gravity of the propagation medium results in decreased viabilities in subsequent high gravity (17.5°P) fermentations.

The results presented in this chapter also demonstrated that osmotolerance was growth phase-dependent. Stationary phase populations of ale and lager yeast were observed to be consistently more osmotolerant than their exponential phase counterparts, when exposed to sorbitol-induced osmotic stress. This observation supports previous reports concerning the reduced sensitivity of stationary phase populations of laboratory haploid strains to osmotic and other forms of physiological stress (Werner-Washburne *et al*, 1993; Hounsa *et al*, 1998; Gasch and Werner-Washburne, 2002).

The apparent up-regulation of stress tolerance during the transition from exponential to stationary phase has been reported for several stresses (Werner-Washburne *et al.*, 1993; Blomberg 1997), and is in part due to discreet changes in physiology (Werner-Washburne *et al.*, 1993; Werner-Washburne *et al.*, 1996), including membrane composition (Steels *et al*, 1994; Sajbidor, 1997) and assimilate accumulation (Lewis and Learmonth, 1993) that occurs during the diauxic and postdiauxic phases, which mark the transition from exponential to stationary phase.

Indeed, the high level accumulation of solutes derived from metabolic processes is not a phenomenon normally associated with exponentially growing yeast cells, in which physiology is finely attuned to replication, division and thus an increase in biomass (Werner-Washburne *et al.*, 1993; Werner-Washburne *et al.*, 1996). In contrast, cells entering the diauxic shift exhibit modified metabolism allowing the accumulation of both glycogen and trehalose (Werner-Washburne *et al.*, 1993) as a result of carbon source limitation (Lillie and Pringle, 1980; Parrou *et al.*, 1999a) and nitrogen starvation (Parrou *et al.*, 1999).

Differential expression of a number of genes between the two growth phases has also been reported (Werner-Washburne *et al*, 1993; Werner-Washburne *et al*, 1996; Gasch and Werner-Washburne, 2002), specifically within the HSP70 subfamily of heat shock proteins (Werner-Washburne *et al*, 1989) and the *SNZ* ('snooze') genes (Fuge *et al*, 1994; Braun *et al*, 1996; Padilla *et al*, 1998), which are highly upregulated following the post-diauxic shift in haploid *S. cerevisiae* cells although the function of the snz proteins has yet to be assigned. Indeed, DeRisi *et al.*, (1997) have demonstrated an alteration in expression patterns of a myriad of genes during diauxic growth using DNA microarray technology.

Genetic analysis of *S. cerevisiae* during diauxic growth has revealed that there are two intrinsic changes associated with this stage of the growth phase, the mobilisation of assimilate to produce reserve carbohydrate (Parrou *et al.*, 1999a) and the general stress response as regulated by the ubiquitous stress responsive element (STRE) (Parrou *et al.*, 1999a; Parrou *et al.*, 1999b; Puig and Perez-Ortin, 2000). Thus, the enhanced osmotolerance of stationary phase populations to sorbitol-induced osmotic stress is likely to be due to the up-regulation of global stress defences and the general changes in cellular metabolism exhibited post diauxic shift.

Glycogen and trehalose accumulation are therefore biomarkers of exposure to stress and are regulated in a STRE-dependent manner. The presence of the *cis*-acting STRE core element "CCCCT" in the promoter of a range of genes induced by a number of stressful conditions demonstrates that there is a global response at the transcriptional level, which confers greater stress tolerance to cells (Parrou *et al.*, 1997; Chatterjee *et al.*, 2000). A number of genes containing STRE elements at their promoters have been identified in commercial wine yeast strains, whose expression is up-regulated to a peak at the post-diauxic shift and into the stationary phase (Puig and Perez-Ortin, 2000). These cumulative findings serve to allow a postulation to be made about stress tolerance shifts between exponential and stationary phase cells at the molecular level. Stationary phase populations are better able to withstand a number of stressful conditions due to the intrinsic induction of a wide number of 'stress genes' at the diauxic shift, the majority of which are mediated via the STREs.

The type of solute used to elicit an osmotic stress has been postulated to be an important factor in determining resistance. For all brewing strains examined, cells were less able to tolerate NaCl-induced osmotic stress than sorbitol-induced stress. This may be explained by the deleterious effects of Na^+ toxicity on normal cellular physiology and metabolism (Gaxiola *et al.*, 1992; Galinski and Trüper, 1994; Murguia *et al.*, 1996; Norbeck and Blomberg, 1997; Serrano *et al.*, 1997). An excess of Na^+ ions has been demonstrated to have deleterious effects on specific protein targets (Murguia *et al.*, 1995; Murguia *et al.*, 1996), and consequently a range of other putitatively sodium sensitive enzymes related to the 3',5'-bisphosphate nucleotidase enzyme (required for sulphate assimilation) detailed by Murguia *et al.* (1995; 1996). It has been proposed that Na^+ toxicity in *S. cerevisiae* may be due to competitive inhibition of K^+ uptake, leading to K^+ depletion in the cell (Gomez *et al.*, 1996; Prista *et al.*, 1997) and an

increased level of sodium. In some instances, the ratio of K^+ to Na^+ may result in enzyme deactivation. It is generally accepted that the cytotoxic effects of Na^+ ions are due to disruption of enzyme activity, however, there is no evidence to date that Na^+ ions disrupt structural proteins, for example of the cell membrane or cell wall of *S. cerevisiae*.

The NaCl osmotolerance of polyploid and aneuploid ale strains of *S. cerevisiae* and strains of *S. cerevisiae* (syn S. *pastorianus*) has not been previously reported. However, Blomberg (1997) detailed the resistances of the strains S288C, X2180–1A, X2180–1B and SKQ to NaCl on supplemented YPD plates. In this chapter, NaCl osmotolerance was observed to be growth phase- and strain-dependent, although no significant differences between ale and lager strain tolerance could be determined. It was clearly demonstrated that exponential phase populations of brewing strains were more sensitive to osmotic stress than stationary phase cells and this was amplified, when using the osmolyte NaCl to induce the stress. Sorbitol-induced osmotic stress tolerance was demonstrated to be strain-dependent, however, all strains examined exhibited a reduced tolerance, when sorbitol was replaced with NaCl indicating that osmotic pressure and the presence of cytotoxic monovalent cations act synergistically in reducing cell viability.

As a result of the data presented in this chapter an assessment of the utility and efficacy of a number of dyes in the determination of cell viability was explored. In general it was observed the three dyes employed yielded correlative data, however, some disparities in certain situations were apparent and it is postulated that these were due to the different modes of action of the dyes.

The hemi-magnesium salt of 1-anilinio-8-napthalene sulphonic acid (MgANS) penetrates compromised plasma membranes of non-viable yeast (King, 1981; McCaig, 1990) and binds to cytoplasmic proteins to generate fluorescence, thereby indicating the presence of non-viable cells. This dye is both easy to prepare and use, and correlates well with plate and slide count measurements of viability (McCaig, 1990).

In this study, MgANS determined viabilities were observed to correlate well with the results observed for the current recommended industry standard dye citrate methylene violet (Powell *et al.*, 2003). However, in a limited number of instances this dye appeared to either over- or underestimate viability (tables 3.1 and 3.2). A

systematic propensity for incorrect viability determination, however, was not evident and the observed disparities may well represent normal experimental error.

In contrast, the fluorescent viability dye propidium iodide demonstrated a propensity to over-estimate viability, when compared to the results obtained using both citrate methylene violet and MgANS. This phenomenon was especially noticeable, when cells were osmotically challenged with NaCl as the solute. This again indicates that the disparity in viability determination is dependent upon the mode of action of the dye utilised.

Propidium iodide penetrates cells with damaged membranes, intercalates nucleic acids, and therefore detects dead cells (Deere *et al.*, 1998; Green *et al.*, 1999). Indeed, the specificity of propidium iodide to intercalate nucleic acids has enabled this dye to be used to predict nucleic acid quantity *in situ* (Bartoletti *et al.*, 1989; Haase and Lew, 1997; Haase and Reed, 2002). More recent research using epi-fluorescence microscopy has also indicated that this dye may over-estimate cell viability in some instances (VanZanDycke *et al.*, 2003). The reasons for this have not been investigated or previously corroborated using the less subjective technique of flow cytometry (Yurkow and McKenzie, 1993; Deere *et al.*, 1998; Prudencio *et al.*, 1998; Green *et al.*, 1999; Attfield *et al.*, 2000; Attfield *et al.*, 2001; Hewitt and Nebe-Von-Caron, 2001).

From the results obtained in this study, it is suggested that propidium iodide may overestimate viability, and this may be due to the fact that although cells are metabolically inactive, they may possess membranes with sufficient integrity to reduce the ability of propidium iodide to penetrate into the cell and give a spurious result.

Osmotolerance in brewing strains is therefore dependent on strain, growth phase, solute type and solute concentrations. However, a loss of viability represents a biomarker of intense or prolonged exposure to stress and as such is a retrospective indicator of the deleterious impact of high osmotic pressure. From a brewing perspective, an indication that a cell has been exposed to sublethal levels of hyperosmotic stress may be more appropriate for assessing the suitability of brewing strains to function during high gravity fermentations.

Chapter 4

Vacuolar Morphology and Changes in Brewing Yeast

Chapter4 Vacuolar Morphology and Changes in Brewing Yeast

4.1 Introduction

It has been suggested that the yeast vacuole plays a central role in a number of diverse functions, from sequestration of cationic products and compartmentalisation of hydrolytic enzymes (Schwencke, 1977; Nishikawa *et al.*, 1990; Sporman *et al.*, 1992) to protein sorting (Banta *et al.*, 1988; Chiang, 1995) and cell division (Schwencke, 1991). Solute flux studies have shown that the vacuole provides an immediate osmoregulatory response (Latterich and Watson, 1993) and thus is implicitly associated with osmoregulation (Section 1.10.5), and therefore cellular responses to osmotic stress when the cell is challenged (Matile, 1978; Banta *et al.*, 1988).

When exposed to osmotic stress, *S. cerevisiae* cells can achieve short term osmohomeostasis via the solutes contained within the vacuole, and this organelle's capacity to release water to the cytosol with a subsequent reduction in size (Latterich and Watson, 1993; Nass and Rao, 1999). Indeed, many mutants that are defective for vacuolar function have been reported as exhibiting an extremely osmosensitive phenotype (Banta *et al.*, 1988; Latterich and Watson, 1991; 1993; Nass and Rao, 1999).

The relationship between vacuolar morphology and osmotic stress has not been fully elucidated. The yeast vacuole is a prominent and highly dynamic organelle (Klionsky, 1998). Early research demonstrated that *S. cerevisiae* cells possess a large central vacuole (Indge, 1968; Rainina *et al.*, 1979; Rainina *et al.*, 1980; Knop *et al.*, 1993), although these visualisations of yeast cells also describe the presence of 'vacuolar compartments' (Rainina *et al.*, 1979). The incidence and exact features of these "sub-vacuoles" has subsequently been demonstrated to be dependent on the physiological state of the cell (section 1.10.5; section 4.3). Indeed, vacuoles are part of the endomembrane system in *S. cerevisiae* (Banta *et al.*, 1988; Chiang, 1995) and the occurrence of entire vacuoles and derived vacuole fragments are cell cycle-dependent phenomena (Conradt *et al.*, 1992).

Elucidation of the relationship between osmotic stress and vacuolar morphology has been hindered by the lack of specific staining procedures to achieve real-time analysis. Methods for the visualisation of the vacuole appear to centre on the vacuolar membrane (tonoplast) (Vida and Emr, 1995), or the vacuolar lumen (Nedergaard *et al.*,

1990). Several stains have been reported to permit the visualisation of the tonoplast including FM4-46 (Vida and Emr, 1995), MDY-64 (Cole *et al.*, 1998), and CMAC (Stefan and Blumer, 1999) (table 4.1).

Yeast cells have been demonstrated to internalise lucifer yellow by endocytosis, which is subsequently accumulated in the vacuole. The intracellular accumulation is time-, temperature- and energy-dependent (Riezman, 1985). Although the final target of lucifer yellow is the vacuole, it has primarily been used in the elucidation of the endocytic pathway, as this is ultimately the mode of action by which the dye enters the vacuolar compartment (Chvatchko *et al.*, 1986).

Quinacrine has primarily been used in the localisation of mammalian acidic organelles, as it freely permeates membranes and actively accumulates in cellular compartments with low pH (Weisman *et al.*, 1987). The dye has been utilised in the study of the yeast vacuole (Weismann *et al.*, 1987; Guthrie and Wickner, 1988), as its low pH facilitates accumulation of the dye by ion trapping within the fungal vacuole. However, dye localisation and fluorescence has been demonstrated to be dependent on the availability of vacuolar ATP (Ohsumi and Anraku, 1983) and, therefore, is not accumulated in the vacuoles of certain deletion mutants, or populations exposed to specific environmental conditions (Nelson *et al.*, 1995).

C-DCFDA is a pH-sensitive fluorophore, which demonstrates intense fluorescence at low pH (effective pKa – 4.2) (Nedergaard *et al.*, 1990) at an emission wavelength of 529nm. This dye is a derivative of carboxyfluorescein diacetate (CFDA) and has been improved for retention in the cell (due to an increased number of negative charges). Furthermore, activation occurs at pH values which are typical of the yeast vacuole.

MDY-64 is a fairly novel tonoplast marker (Cole *et al.*, 1998), with a similar mode of action to that of FM4-64 (N-(3-triethylammoniumpropyl)-4- (p-diethylaminophenyl-hexatrienyl) pyridinium dibromide), which is a highly lipophilic styryl dye (Vida and Emr, 1995). MDY-64 has an emission maximum at 497nm and it has been demonstrated to demarcate the vacuole well (Cole *et al.*, 1998).

FUN® has an emission maximum at 530nm and is reported to become localised within the vacuole of *S. cerevisiae* cells, giving rise to refractile bodies termed cylindrical intra-vacuolar structures (CIVS) (Millard *et al.*, 1997).

LysoTracker ™ probes are a group of commercially available dyes, which are composed of a weakly basic amine conjugated to a specific fluorophore. The yellow form (dual emission maxima at 440 and 540nm) of LysoTracker™ prevents bleed-through of fluorescence that normally occurs with other lysosome dyes used. LysoTracker™ probes accumulate in organelles with low pH due to their acidotropic nature (Anderson *et al.*, 1984) and LysoTracker™ yellow may therefore be used for vacuolar visualisation due to the acidic nature of the lumen.

Table 4.1. Fluorophores used in the detection of vacuolar structure.

Dye	*Target*	*Reference*
LysoTracker Green	Acidic organelles	Anderson *et al.*, 1984
Lucifer Yellow	Endomembrane system	Riezman, 1985
Quinacrine	Vacuole lumen	Wiesman *et al.*, 1987
C-DCFDA	Vacuole lumen	Nedergaard *et al.*, 1990
FM4-64	Tonoplast	Vida and Emr, 1995
FUN®1	Vacuole lumen	Millard *et al.*, 1997
MDY-64	Tonoplast	Cole *et al.*, 1998
CMAC	Tonoplast / Plasmalemma	Stefan and Blumer, 1999

Despite the obvious possibility that vacuolar morphology may serve as a biomarker of osmotic stress, relatively little research has been done on this subject in brewing strains. The potential of this biomarker to rapidly indicate osmotic stress in strains pitched into high gravity wort has previously been suggested; however, the parameters governing the morphological characteristics have not been identified. The current understanding of brewing yeast vacuolar morphology is limited, and attempts to quantitate changes in vacuolar volume during osmotic stress have yielded variable results (Pratt-Marshall *et al.*, 2002a). Epifluorescence microscopy studies suggest that the vacuole of brewing strains increases in volume during osmotic stress (Pratt-Marshall

et al., 2002b), however, more recent research has attempted to apply image processing software to define osmotically-induced vacuolar changes. Reilly *et al.* (2003) demonstrated a decrease in vacuolar size during high gravity fermentations.

In this chapter, the potential of yeast vacuolar morphology in ale strains of *S. cerevisiae* and lager strains of *S. cerevisiae* (syn *S. pastorianus*) as a biomarker of osmotic stress was examined.

4.2 Results

Yeast cells were grown to the required phase of growth, as detailed in section 2.4.2, and assessed before and after exposure to sorbitol at the indicated concentrations. Unstressed cells were also examined. The samples were dual stained according to the protocols described in section 2.10. Cells were examined from 3 replicate samples and not less than 200 individual cells were analysed. A Zeiss® 510 laser scanning microscope was used with the appropriate settings and filters as described in section 2.10 to visualise the cells. Images obtained are representative data for a number of populations examined, and were selected for clarity and quality of staining.

4.2.1 Assessment of the utility and efficacy of yeast vacuolar dyes

Vacuolar studies have usually centred on the use of brightfield or fluorescent stains available to the investigators and no reports concerning the relative suitability of such dyes have been published. In this study, several fluorophores were assessed for their utility and efficacy in the visualisation of the vacuolar lumen and tonoplast in lager and ale strains. The stains included in this analysis were: MDY-64 (section 4.2.1.1), Lysotracker™ yellow (section 4.2.1.2), FUN® 1 (section 4.2.1.3) and C-DCFDA (section 4.2.1.4). The fluorophores were assessed for their clarity and stability during the visualisation of lager and ale brewing yeast cell vacuoles before and after exposure to osmotic stress.

4.2.1.1 MDY-64

It was demonstrated that the tonoplast was clearly and consistently stained when using MDY-64 in brewing and haploid yeast strains (figure 4.1). MDY-64 represented a negative stain for the vacuolar lumen, possibly as a consequence of dye exclusion.

The specificity of this stain was not as high as has been previously reported (Vida and Emr, 1995), but appeared to be consistent in both stationary (figure 4.2) and exponential (figure 4.3) phase cell populations. Furthermore staining of other cellular membranes, such as the nuclear envelope, and components of the endomembrane system appeared to occur with MDY-64 (figures 4.2 and 4.3).

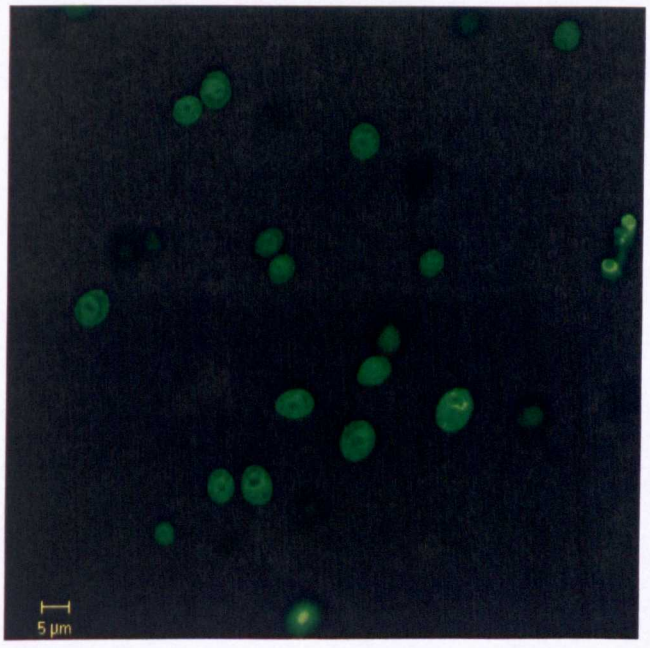


Figure 4.1. Assessment of MDY-64 as a tonoplast-specific marker. Stationary phase populations of S288C (laboratory haploid) were stained with MDY-64 and visualised using confocal microscopy.

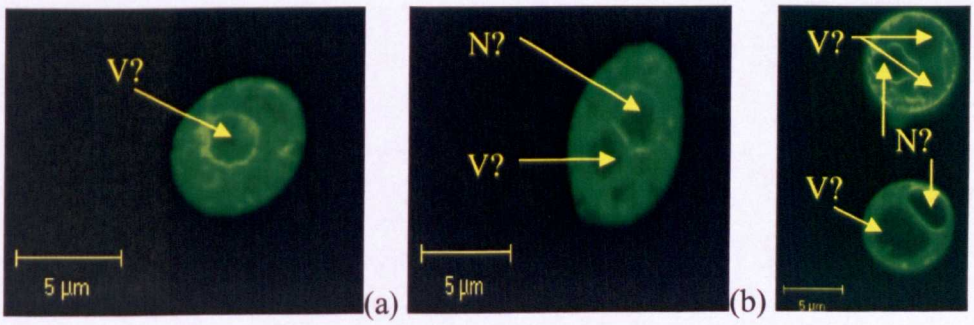
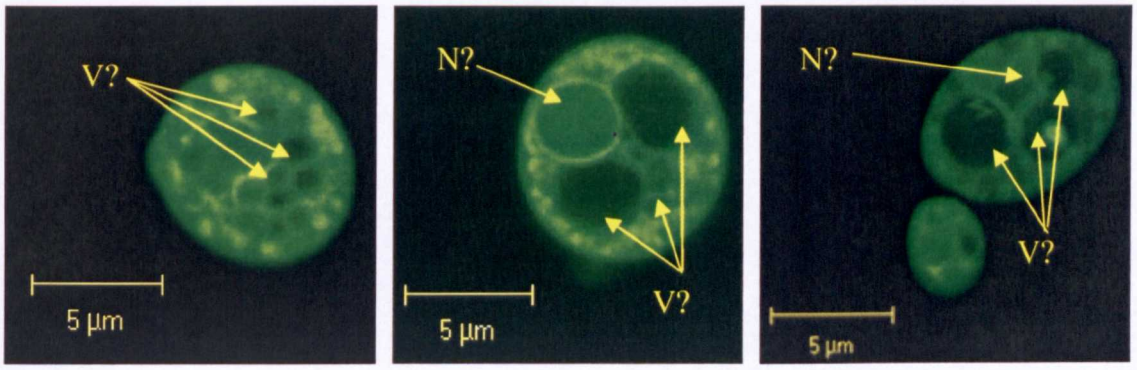


Figure 4.2. Assessment of MDY-64 as a tonoplast-specific marker. Stationary phase populations of (a) S288C (laboratory haploid) (b) S288C (laboratory haploid) and (c) SCB8 (ale) brewing yeast strains were stained with MDY-64 and visualised using confocal microscopy putitatively showing the vacuole (V?) the nucleus (N?).



(a) (b) (c)

Figure 4.3. Assessment of MDY-64 as a tonoplast-specific marker. Exponential phase populations of (a) SCB2 (lager) (b) SCB8 (ale) and (c) S288C (laboratory haploid) yeast strains were stained with MDY-64 and visualised using confocal microscopy putitatively showing the vacuole (V?) the nucleus (N?).

4.2.1.2 LysoTracker™ yellow

The acidotropic dye LysoTracker™ has not previously been used as a marker for yeast vacuoles. This dye is commercially available for the visualisation of mammalian lysosomes (McIntosh *et al.*, 2003). It was postulated that LysoTracker™ yellow would enable the visualisation of the vacuolar compartment, but photobleaching and dye bleed-through yielded variable results. Adequate visualisation of the vacuole with LysoTracker™ yellow required the use of a transmitted light overlay (figures 4.4 and 4.5)

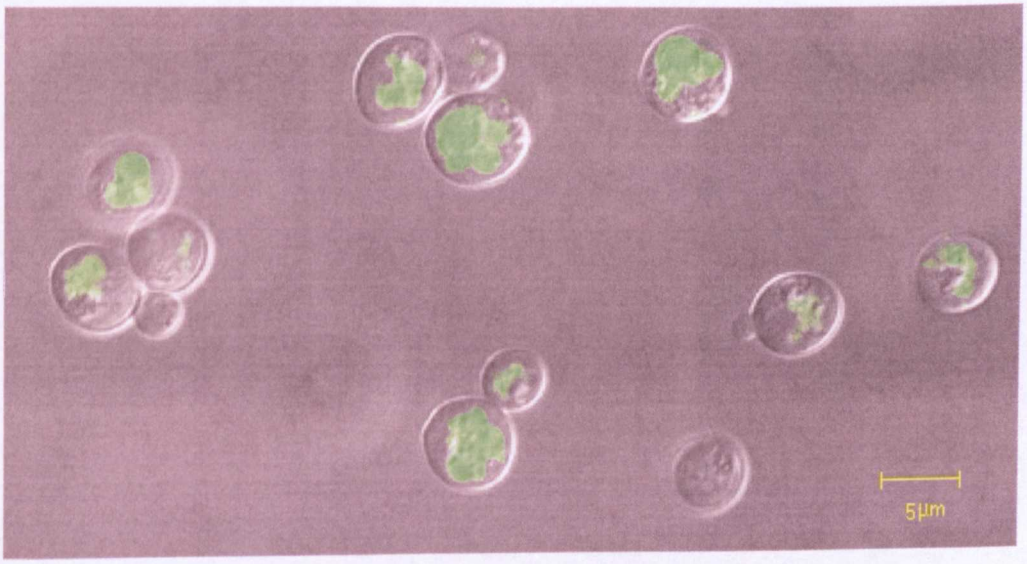


Figure 4.4. Assessment of LysoTracker ™ yellow as a vacuole-specific marker. Exponential phase populations of SCB5 (ale) brewing yeast were stained with LysoTracker ™ yellow and visualised using confocal microscopy and a transmitted light overlay.

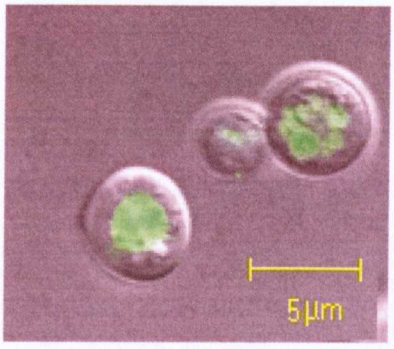


Figure 4.5. Assessment of LysoTracker ™ yellow as a vacuole-specific marker. Exponential phase populations of SCB5 (ale) brewing yeast were stained with LysoTracker ™ yellow and visualised using confocal microscopy and a transmitted light overlay.

4.2.1.3 FUN®1

FUN®1 has previously been described as a potential vitality dye in yeast (Van Zandycke *et al.*, 2003). The dye has been shown to negatively stain the vacuole, however, when a vital cell is stained, the dye appears to be incorporated into the vacuole and forms so-called cylindrical intravacuolar structures (CIVs). In this study, CIVs were not detected irrespective of the physiological state of the cells, however, this may be due to the intrinsic difficulties associated with the correct incubation/buffer regime required to visualise these structures *in vivo* (S.M Van Zandycke, *pers. comm.*). Furthermore, FUN®1 demonstrated consistently poor staining, required the use of transmitted light overlays (figure 4.6) and proved to be of limited use for vacuole visualisation.

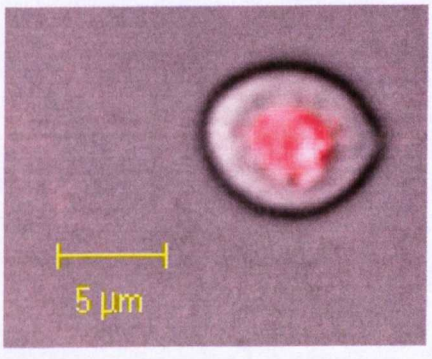


Figure 4.6. Assessment of FUN®1 as a vacuole-specific marker. Exponential phase populations of S288C (laboratory haploid) yeast were stained with FUN®1 and visualised using confocal microscopy.

4.2.1.4 C-DCFDA

C-DCFDA has been demonstrated to be actively localised in the yeast vacuole and is metabolised to a fluorescent derivative, putitatively catalysed by an array of vacuolar hydrolases (Nedergaard *et al.*, 1990). Despite its potential, C-DCFDA has received only limited attention, and no comprehensive report exists concerning the efficacy of this dye in the visualisation of yeast vacuoles. This study demonstrated that C-DCFDA yielded consistent visualisation of the vacuolar lumen, although demarcation of the lumen and remainder of the cell was not obvious, since no plasma membrane staining was apparent (figures 4.7, 4.8, 4.9). The use of this dye alone was unsuitable for vacuolar visualisation; however, the use of transmitted light overlays or a second fluorophore would have circumvented these problems (section 4.2.1.7).

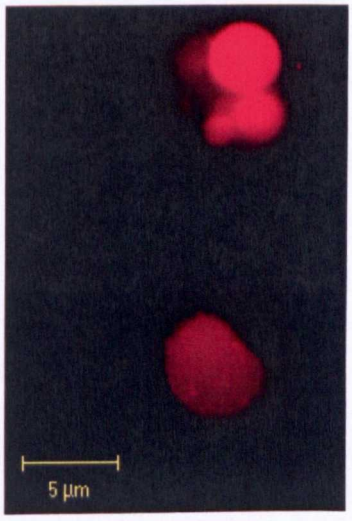


Figure 4.7. Assessment of C-DCFDA as a vacuole-specific marker. Stationary phase populations of SCB8 (ale) brewing yeast were stained with C-DCFDA and visualised using confocal microscopy.

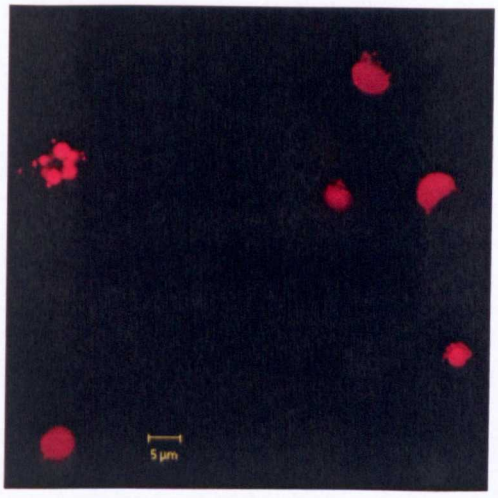


Figure 4.8. Assessment of C-DCFDA as a vacuole-specific marker. Late exponential phase populations of SCB8 (ale) brewing yeast were stained with C-DCFDA and visualised using confocal microscopy.

Figure 4.9. Assessment of C-DCFDA as a vacuole-specific marker. Exponential phase populations of SCB8 (ale) brewing yeast were stained with C-DCFDA and visualised using confocal microscopy.

4.2.1.5. Co-localisation using multiple stains

The problems associated with the use of the individual dyes MDY-64 (section 4.2.1.1), LysoTracker™ yellow (section 4.2.1.2), FUN®1 (section 4.2.1.3) and C-DCFDA (4.2.1.4) in isolation demonstrated a requirement for a dual staining procedure to permit adequate staining of both the tonoplast and the lumen. Furthermore, co-localisation without the use of transmitted light was desirable. Experimental analysis of the utility of the combination of the dyes Cell Tracker™ blue CMAC and C-DCFDA (section 4.2.1.5.1), FUN®1 and MDY-64 (section 4.2.1.5.2) and MDY-64 and C-DCFDA (section 4.2.1.5.3) was performed.

4.2.1.5.1 Cell Tracker™ blue CMAC and C-DCFDA

In order to identify a useful combination of dyes for co-localisation studies, the commercially available membrane marker dye CellTracker™ was assessed for its ability to demarcate the cell periphery in combination with the vacuole lumen marker C-DCFDA. This combination of dyes yielded poor clarity of the vacuole and a relatively low fluorescence intensity even after extensive optimisation (figure 4.10).

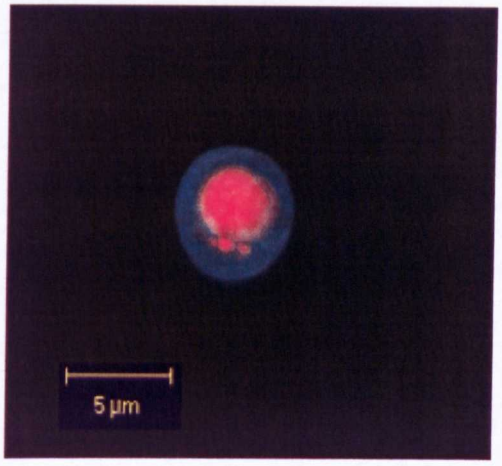


Figure 4.10. Assessment of the ability of C-DCFDA and CellTracker™ Blue CMAC to co-localise to the vacuole. Stationary phase populations of SCB2 (lager) brewing yeast were stained with C-DCFDA and CellTracker™ Blue CMAC and visualised using confocal microscopy.

4.2.1.5.2 FUN®1 and MDY-64

The dyes FUN®1 (section 4.2.1.3) and MDY-64 (section 4.2.1.1) do not demonstrate bleed-through of fluorescence, therefore despite the limitations experienced in the use of FUN®1 as described in section 4.2.1.3, this dye pair was examined for their ability to co-localise to the yeast vacuole. As demonstrated in figure 4.11, the fluorescence intensity of FUN®1 remained low, and appeared to affect the visualisation of staining of MDY-64. The fluorescence intensity was detected at very low levels, which necessitated the use of transmitted light overlays in order to demarcate the cell periphery.

Figure 4.11. Assessment of the ability of FUN®1 and MDY-64 to co-localise to the vacuole. Exponential phase populations of SCB1 (lager) brewing yeast were stained with C-DCFDA and CellTracker™ Blue CMAC and visualised using confocal microscopy using a transmitted light overlay

4.2.1.5.3 MDY-64 and C-DCFDA

To examine the potential of dual staining to visualise the vacuole, the lumen marker, C-DCFDA, and the membrane marker, MDY-64, were utilised and provided a reproducible and clear demarcation of vacuoles within brewing yeast cells (figure 4.12). This permitted the localisation of the vacuole, distinguishing it from other MDY-64-labelled membrane-bounded organelles within the cell. As indicated in figure 4.12, the nuclear membrane was clearly visible, however, further experimental analysis would be required in order to conclusively localise this organelle. It is also possible that organelles involved in the endomembrane system (endoplasmic reticulum and associated vesicles) are stained by MDY-64, but again this supposition cannot be supported from the results presented here. The plasma membrane is clearly visible, and permits the demarcation of the cell periphery, allowing for greater clarity of interpretation of images without the use of transmitted light overlays.

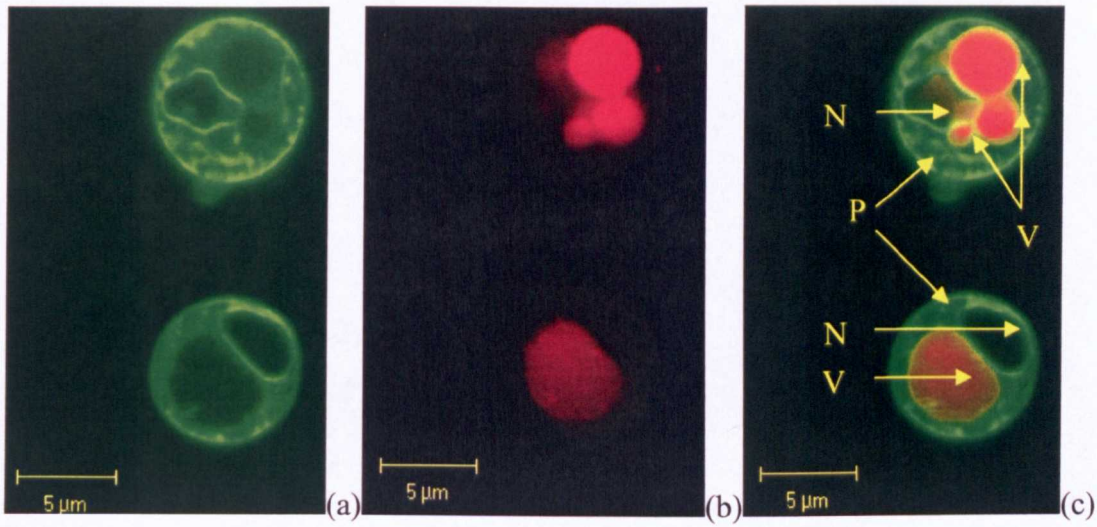


Figure 4.12. Early stationary phase SCB8 (ale) cells stained with (a) MDY-64 (b) C-DCFDA and (c) merged image of (a) and (b) showing the vacuole (V) and nucleus (N) and Plasma membrane (P)

4.2.2 Vacuolar morphology in non-stressed cells

Confocal microscopical analysis of stationary phase, YPD-grown cells revealed that in many cases a large central vacuole was evident, although this was sometimes accompanied by a number of smaller fragments (figures 4.10, 4.12 , 4.13). Further analysis of stationary phase populations, however, revealed the occurrence of an altogether different morphology. Figures 4.10, 4.12 and 4.13 demonstrate the appearance of a number of small vacuoles; however, this morphology was limited to a relatively small proportion of individuals within the cohort of cells examined. The occurrence of a proportion of cells with fragmented vacuoles demonstrated a lack of consistency in vacuole ultrastructure of brewing yeast cells exhibiting stationary phase morphology.

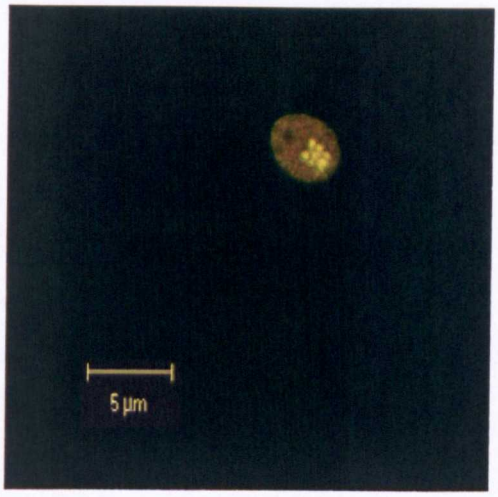


Figure 4.13. SCB2 (lager) stationary phase YPD-grown cell stained with C-DCFDA and MDY-64 and visualised using confocal microscopy.

4.2.3 Vacuolar morphology in sorbitol-stressed cells

The supposition that vacuolar morphology may be affected by osmotic stress is largely derived from experimental data concerning the osmotic sensitivity of haploid laboratory *S. cerevisiae* strains, defective in vacuolar morphology (Banta *et al.*, 1988; Srinivasan *et al.*, 1997). Furthermore, deletion mutants deficient in the Na^+/H^+ exchanger (*Δnhx1*) and vacuolar functioning appeared to be extremely osmosensitive. More recently, Bonangelino *et al.*, (2002b) have demonstrated that osmotic stress appeared to induce vacuolar fragmentation in wild-type laboratory haploid *S. cerevisiae* cells. However, Nass and Rao (1999) have reported that an immediate response to osmotic stress involved vacuolar shrinkage. Furthermore, it has been demonstrated that the phospholipid phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2) has a role in the control of vacuolar morphology (Weisman, 2003). Indeed, PtdIns (3,5)P_2 levels have been shown to be regulated by osmotic stress (Dove *et al.*, 1997; Bonangelino *et al.*, 2002a; Dove *et al.*, 2002).

In order to assess the impact of sorbitol-induced osmotic stress on vacuolar morphology, cells were grown to the required phase (section 2.4.2), washed in phosphate buffer and exposed aerobically to a range of sorbitol concentrations (dH_2O, 6%, 12%, 18%, 24%, 30%) for 24 hours (section 2.4.3) to ascertain whether or not this had any impact on vacuolar morphology.

Figure 4.14 demonstrates that cells exposed to osmotic shock, as induced by 30% (w/v) sorbitol, did not exhibit any clear differences as compared with non-stressed

(YPD-grown) cells (figures 4.11and 4.13). The same fragmentation pattern was observed. when other strains were examined as shown in figure 4.15.

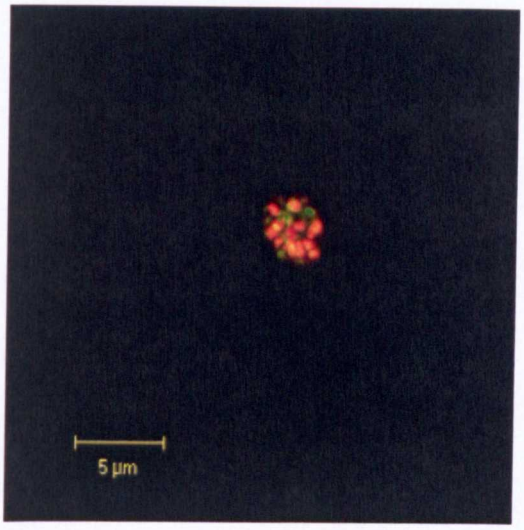


Figure 4.14. SCB1 (lager) stationary phase 30% sorbitol stressed cell dual stained with C-DCFDA and MDY-64.

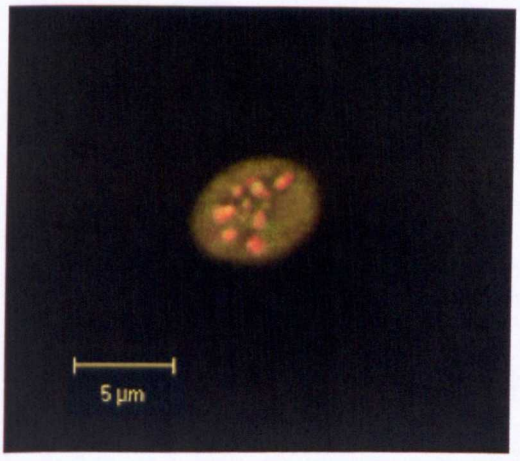


Figure 4.15. SCB5 (ale) stationary phase 30% sorbitol stressed cell stained with C-DCFDA and MDY-64.

4.2.4 Growth phase- and cell cycle-dependent changes in vacuolar morphology

In an attempt to understand the physiological processes governing the changes observed in vacuolar morphology, the supposition that vacuolar dynamics are linked to the cell cycle as suggested by Matile and Wiemken (1967), and Schwencke (1977) (Section 4.3) was investigated. Exponentially growing cells were examined for vacuolar morphology and compared to cells in the stationary phase of growth. Figure 4.16 shows that vacuolar fragmentation was observed during exponential growth. Moreover, figure 4.16 demonstrated the differences in vacuolar morphology (vacuolar fragmentation) between replicating and non-replicating cells (large central vacuoles).

The large central vacuolar morphology of non-replicating cells was also clearly demonstrated in figure 4.17.

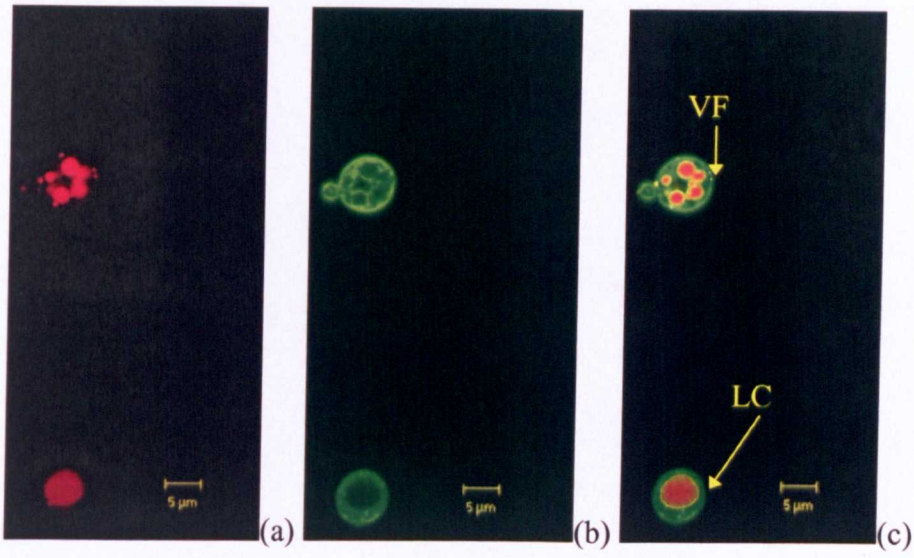


Figure 4.16. Late exponential phase SCB8 (ale) cells stained with (a) C-DCFDA and (b) MDY-64, demonstrating large central vacuoles (LC) and vacuolar fragments (VF) in the composite image (c).

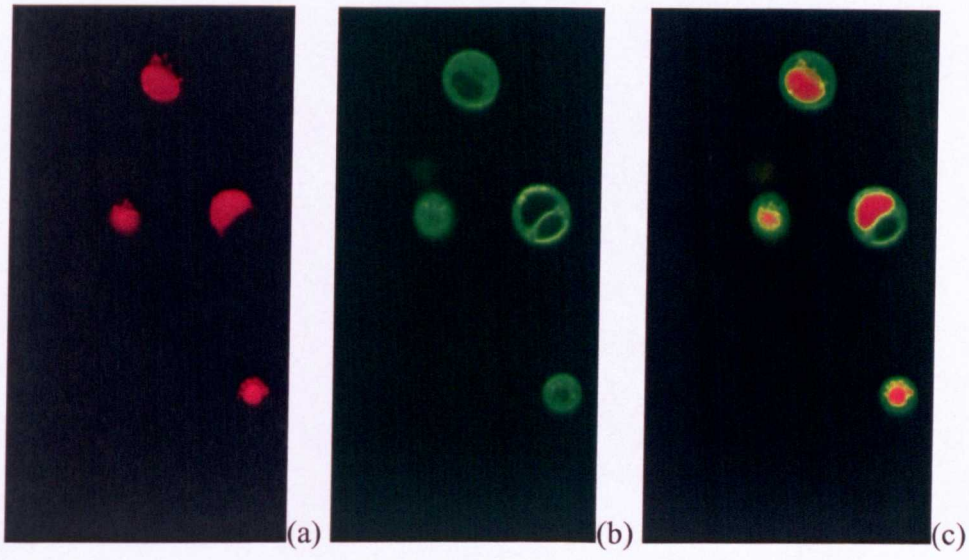


Figure 4.17. Late exponential phase SCB8 (ale) cells stained with (a) C-DCFDA and (b) MDY-64, composite image (c).

Further analysis of actively replicating cells demonstrated that the propensity of a cell to undergo vacuolar fragmentation was intrinsically linked to the replication cycle. The highest number of vacuolar fragments was recorded in a

budding cell (figure 4.11). Furthermore, it was observed that vacuolar morphology alternated according to the point of the cell cycle, in which the cell was examined. Figures 4.18-4.20 represent the different vacuolar morphologies associated with replication in the production brewing ale strain SCB8.

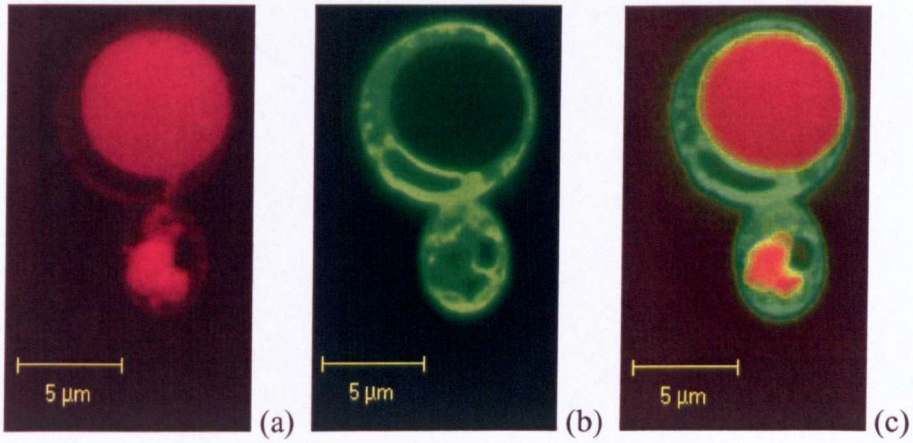


Figure 4.18. Exponential phase SCB8 (ale) cells stained with (a) C-DCFDA (b) MDY-64 and (c) a composite image of (a) and (b).

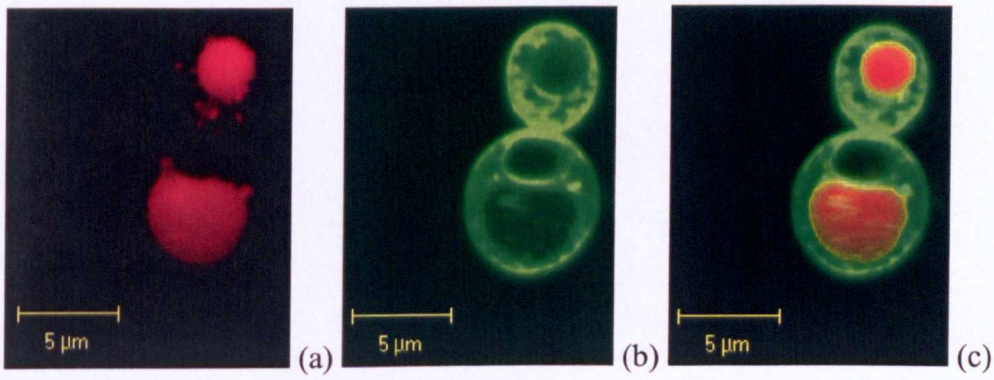
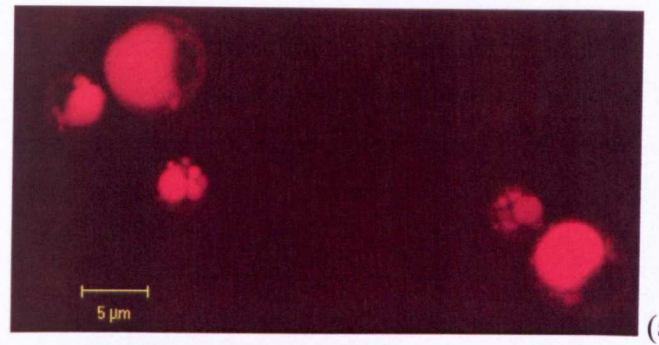
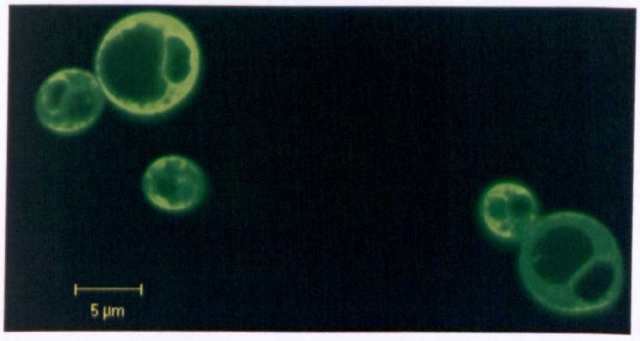


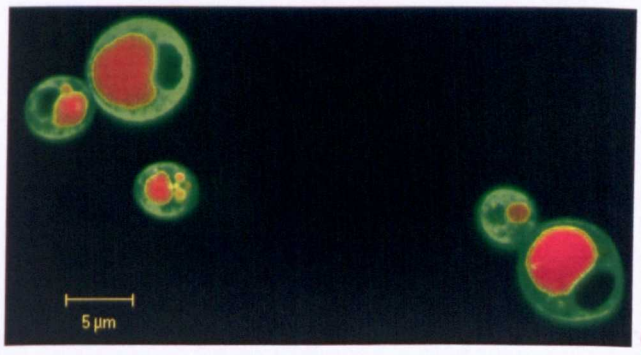
Figure 4.19. Exponential phase SCB8 (ale) cells stained with (a) C-DCFDA (b) MDY-64 and (c) a composite image of (a) and (b).



(a)



(b)



(c)

Figure 4.20. Exponential phase SCB8 (ale) cells stained with (a) C-DCFDA (b) MDY-64 and a composite image of (a) and (b).

In order to corroborate the hypothesis that vacuolar fragmentation is dependent on the phase of the replication cycle exhibited by the cell, very late (96 hours) stationary phase SCB8 (ale) cells were stained and subjected to confocal microscope analysis. Cells examined in this late stationary phase exhibited predominantly large vacuoles, and the incidence of vacuolar fragmentation was markedly decreased (figures 4.21-4.23). The results presented in this study demonstrated that vacuolar fragmentation does occur during progression through the cell cycle.

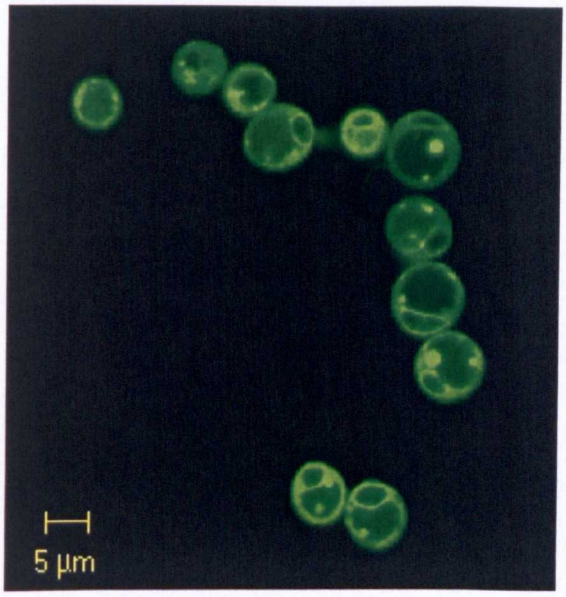


Figure 4.21. SCB8 (ale) late stationary phase cells stained with MDY-64.

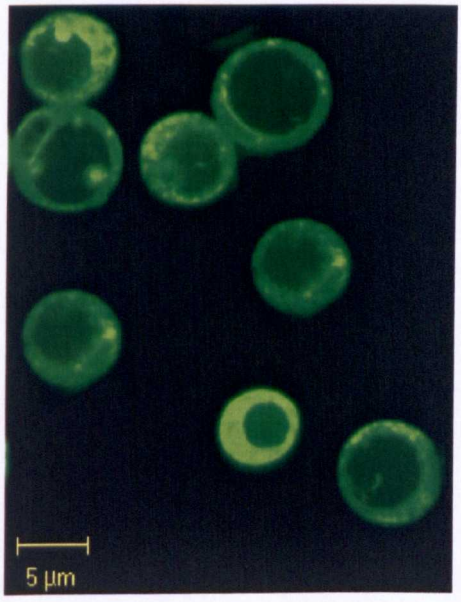


Figure 4.22. SCB8 (ale) late stationary phase cells stained with MDY-64.

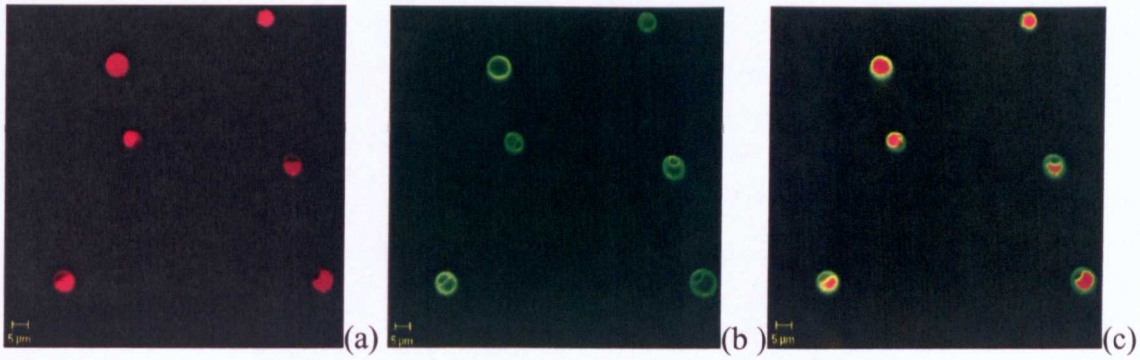


Figure 4.23. SCB8 late stationary phase cells stained with (a) C-DCFDA (b) MDY-64 and a composite image (c).

A comparison of vacuolar morphologies in non-stressed stationary phase cells (figures 4.18-4.20), and exponentially growing cells (figures4.21-4.23) demonstrated the clear distinction between the fragmented form of this organelle and the intact large central morphology. A composite diagram of images collected from exponentially growing (budding) cells suggested the means by which the vacuole is inherited from mother to daughter cell during mitotic growth (figure 4.24).

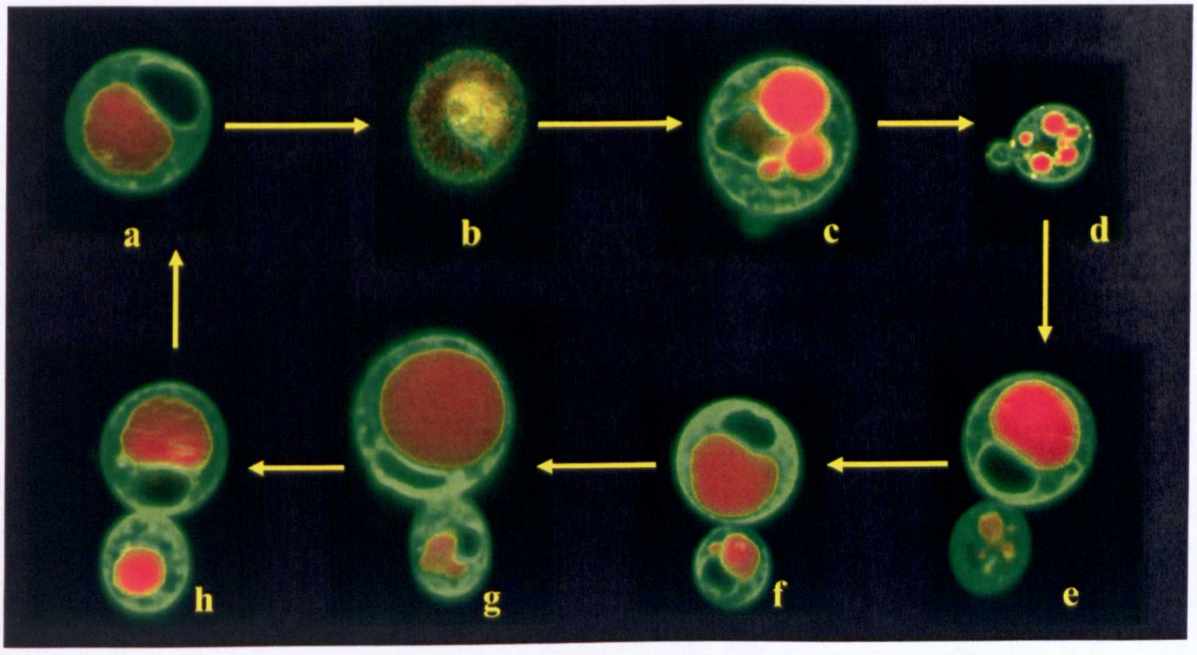


Figure 4.24 A composite diagram composed from images of actively replicating ale (SCB8) cells indicating the changes in vacuolar morphology from an entire central vacuole (a), through the process of fragmentation (b-d) and eventually to reformation of the entire vacuole (e-g) and the formation of a single vacuole from the maternally-derived inherited vacuole fragments before cytokinesis (h). Cells stained using MDY-64 and C-DCFDA.

4.3 Discussion

The objectives of this study were to demonstrate the potential of vacuolar morphology as a biomarker of osmotic stress in ale and lager brewing yeast strains. The vacuolar compartment in production brewing yeast strains has received relatively little attention, and certainly a comprehensive attempt to correlate vacuolar morphology and osmotic stress is not evident from the literature. The work completed by Pratt-Marshall and co-workers (2002a) remains the only published research on this subject, and has shown that there is an increase in vacuolar size during hyperosmotic stress in high gravity fermentations. These investigations, however, have shown that there may be some variability in vacuolar morphology, and did not include a comparison of vacuole stains with different modes of action.

More recently, Reilly *et al.*, (2003) have reported that it may be possible to apply image analysis software to the quantification of vacuolar morphology during osmotic stress in brewing yeast strains. However, the findings of Reilly *et al.*, (2003) demonstrated that although yeast vacuoles were less fragmented during high gravity fermentations, the total size of the vacuole did not appear to be discernibly larger. Both the studies by Pratt-Marshall *et al.*, (2002a) and Reilly *et al.*, (2003) demonstrate that vacuolar morphology in brewing yeast is constantly given to fluctuations, although in both studies, only the putative tonoplast marker MDY-64 was utilised.

Stress-induced changes in vacuolar morphology are not well understood. It has been demonstrated that correct vacuolar functioning and morphology is dependent upon a number of vacuolar proteins (Fab1p, Vac7p and Vac14p, to name a few) (Bonangelino *et al.*, 1997; Gary *et al.*, 1998; Bonangelino *et al.*, 2002a; Bonangelino *et al.*, 2002b). The phospholipid phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2) has also been shown to play a central role. It has been demonstrated that PtdIns(3,5)P_2 is regulated by Fac1p and Vac7p, deficiencies of which have been shown to be manifested as "an enlarged unlobed vacuole,e defective in vacuolar inheritance and acidification" (Bonangelino *et al.*, 2002a). Furthermore, a genome-wide screen undertaken by Bonangelino *et al.*, (2002b) identified a number of vacuolar protein sorting genes whose absence conferred an aberrant (mostly fragmented) vacuolar morphology. The findings that PtdIns(3,5)P_2 levels increase during osmotic stress (Dove *et al.*, 1997) indicate strongly a role for this compound in vacuolar morphological changes during osmotic stress, as mediated by a number of vacuolar proteins. Bonangelino *et al.*, (2002a) have

identified a vacuolar protein, Vac14p, which activates the lipid kinase Fab1p, regulates PtdIns(3,5)P_2 levels and subsequently vacuolar morphology during osmotic stress. Vacuolar fragmentation is therefore observed to occur as a result of osmotically-induced increases in PtdIns(3,5)P_2 mediated by Vac14p, supporting the hypothesis that there are morphological changes in brewing yeast vacuoles as a function of osmotic stress. An examination of the literature pertaining to vacuolar morphology suggested that although indirect evidence for osmotically-induced vacuolar changes has been previously reported (Srinivasan *et al.*, 1997; Banta *et al.*, 1998; Cooke *et al.*, 1998; Bonangelino *et al.*, 2002a; Bonangelino *et al.*, 2002b), the osmohomeostatic capabilities of the vacuole indicated that an attempt to correlate vacuolar morphology (more specifically vacuolar size) and water activity changes would prove fruitful (Serrano *et al.*, 1996; Bone *et al.*, 1998; Nass and Rao, 1999). Moreover, the involvement of a number of ancillary proteins and phospholipids related to vacuolar morphology have also been demonstrated to be regulated by osmotic stress (Cooke *et al.*, 1998; Bonangelino *et al.*, 2002a; Bonangelino *et al.*, 2002b).

Several vacuolar staining protocols have been reported (Riezman, 1985; Wiesman *et al.*, 1987; Nedergaard *et al.*, 1990; Lum and Wright, 1995; Vida and Emr, 1995; Millard *et al.*, 1997; Cole *et al.*, 1998; Stefan and Blumer, 1999; Eitzen *et al.*, 2002; Hyde *et al.*, 2002). In order to effectively demonstrate changes in vacuolar morphology in this study, the suitability of several staining procedures was assessed.

It was demonstrated that the yeast tonoplast marker MDY-64 required a short incubation time and, upon incorporation into the vacuolar membrane, exhibited intense fluorescence without photobleaching, enabling prolonged confocal analysis, where appropriate. However, contrary to the findings of Cole *et al.* (1998) MDY-64 was not tonoplast-specific and appeared to stain multiple membranes, including membrane-bounded vesicles and the nuclear envelope. Despite this potential disadvantage the dye did not permeate the vacuolar lumen and could subsequently be used to demarcate the boundary of the vacuole by a combination of membrane binding and dye exclusion.

The lack of specificity of MDY-64 for the tonoplast may reflect the mode of action of this dye as it is reported as being strongly lipophilic (Vida and Emr, 1995). Furthermore, MDY-64 belongs to the styryl group of lipophilic dyes, which include FM4-64 and FM1-43, which have previously been demonstrated to stain the yeast tonoplast (Vida and Emr, 1995) and motor nerve terminal membranes (Ribchester *et al.*,

1994), respectively. This group of dyes have been used in a wide range of biological systems as membrane markers due to their low toxicity, high solubility in aqueous solution and intense fluorescence, when incorporated in lipid-rich membrane systems (Mycielska *et al.*, 2000). In addition, styryl dyes are reported to remain membrane bound and do not become internalised in endosomes during recycling, as demonstrated by FM1-43 (Murty and Stevens, 1998), a finding supported in this study by the lack of staining of the vacuolar lumen.

LysoTracker™ yellow also permitted the visualisation of the vacuole in brewing yeast, but with more variable results. Photobleaching of the fluorophore resulted in variable fluorescence intensities, which made confocal analysis with this dye problematic. The commercially available Lysotracker™ probes are a group of dyes, which comprise a fluorophore conjugated with a weak base and are believed to accumulate in acidic organelles (Griffiths *et al.*, 1988). The exact mechanism of retention of these acidotropic dyes is currently unknown, however, it is suggested by the manufacturer (Molecular Probes Inc., the Netherlands) that protonation of the dye occurs with a resultant retention of the dye within the membrane of the organelle.

FUN®1 is a membrane-permeant, halogenated cyanine compound (Millard *et al.*, 1997) and as is suggested to bind nucleic acids. However, when utilised in yeast, FUN®1 appears to bind to proteins with a resultant increase in fluorescence intensity and the formation of cylindrical intravacuolar structures (CIVs) (Millard *et al.*, 1997). FUN®1 was found to be an ineffective and highly variable dye for the visualisation of the yeast vacuolar lumen. Moreover, FUN®1-stained cells did not exhibit the previously reported CIVs, which have been suggested to form as a result of the dye's interaction with the vacuolar lumen proteins (Millard *et al.*, 1997).

The dye 5-(and6-)carboxy-2',7'-dichlorofluoroscein (CFDA) has been previously utilised as a useful marker in the elucidation of the endocytic pathway (McLaurin *et al.*, 1998). However, CFDA is not able to permeate cells with an intact plasma membrane, an attribute that has proven useful for viability assessment (Breeuwer *et al.*, 1994). The application of a carboxyl dichlorinated derivative of CFDA alters the chemistry of this molecule sufficiently to allow penetration of lipid bilayers. C-DCFDA was utilised in this study as it has previously been demonstrated to localise to the vacuolar lumen (Roberts *et al.*, 1991), and other acidic organelles (Nedergaard *et al.*, 1990). C-DCFDA was observed to clearly and consistently stain the

vacuolar lumen, and exhibited a high level of fluorescence emission, when examined using laser scanning confocal microscopy. Photobleaching did not occur with this dye, however, as C-DCFDA is a lumen marker, which is converted to a fluorescent form by vacuolar hydrolases, it could not be used singly in order to demonstrate gross morphological changes in the vacuole.

To circumvent the problems associated with single dye usage, a number of experiments were undertaken to ascertain the efficacy and utility of specific dye combinations. Prevention of cross-over of fluorescence emission (='bleed-through') was achieved by utilising pairs of dyes with distinct emission spectra for these co-localisation experiments. An additional membrane dye Cell Tracker™ blue was used as a putative marker for the cellular periphery, however, this dye yielded poor results, specifically due to low fluorescence emission and, even when used in combination with C-DCFDA, yielded images of poor clarity and quality. FUN®1 was used in conjunction with MDY-64, as this dye pair did not demonstrate bleed-through of fluorescence; however, experimental analysis showed that fluorescence emission energy was very low, and therefore transmitted light images were required to achieve an appropriate image.

It was established that co-staining with the vacuolar lumen marker C-DCFDA and the membrane stain MDY-64 was a useful tool in visualisation of the yeast vacuole. Co-localisation of these dyes occurred readily and it was also possible in many cases to determine the morphology, location and orientation of the nucleus with reference to the vacuole. Without dual staining experiments for the nucleus however (e.g RNase treatment and staining with propidium iodide) and other cellular components (Mito-Tracker™ for Mitochondria) the exact nature of staining of MDY-64/C-DCFDA was impossible to determine. Analysis of the yeast vacuole by confocal microscopy therefore revealed that the utilisation of a number of commercially available fluorescent probes were highly effective in identification and demarcation of the yeast vacuole *in vivo*. The identification of suitable dyes for the elucidation of the morphology of brewing yeast vacuoles aided the assessment of vacuolar changes during normal physiology and also during stress.

Early work on the yeast vacuolar system was unable to attribute a specific mode of action, or method of vacuolar morphological changes. The changes in the yeast vacuole were initially thought to be minimal in non-stressed populations, although

Schwencke suggested in 1977 that yeast vacuolar fragmentation occurred as part of the normal replication cycle. This model proposed that the vacuolar compartment is highly dynamic and is readily given to flux, between an aggregated, large central morphology, and a number of small vacuolar fragments, dependent upon the stage of the cell cycle attained.

This model was subsequently supported by research by Conradt *et al.* (1992), who demonstrated that *S. cerevisiae* cells inherit their vacuoles from their mothers during cell division. The vacuoles were observed to pass to the daughter cell from the mother cell in a targeted motion via tubular or vesicular structures (Conradt *et al.*, 1992), however, it is also thought that vacuolar fragmentation may drive vacuolar segregation (Hill *et al.*, 1996). The process has been reconstituted *in vitro*, and was demonstrated to be dependent on physiological temperature, cytosolic factors, and ATP. The resulting daughter cell undergoes a physiological change which mediates the fusion of the inherited vacuolar material (Haas *et al.*, 1994). A number of specific and general protein factors including N-ethylmaleimide-sensitive factor (NSF) (Haas and Wickner, 1996) also appeared to be involved. It has been suggested that the vacuoles to be fused complete a priming event, which prepares them for fusion, mediated by SNARE proteins, which allow for the targeted docking of membranes (Haas and Wickner, 1996). The observation that *de novo* synthesis does not normally account for vacuole formation in daughter cells, suggests that there is an exquisitely refined process governing the multiplication and replication of vacuoles.

It has been demonstrated that vacuolar movement is achieved by interactions between the cytoskeleton (more specifically actin), the myosin V motor protein Myo2p and the vacuole (Hill *et al.*, 1996). Indeed, the abolition of Myo2p function results in a cessation of replication and concurrently the accumulation of small vacuolar fragments in the cell (Johnson *et al.*, 1991; Govindan *et al.*, 1995). More recently, Ishikawa *et al.*,(2003) have identified a specific region on the globular region of the Myosin-V COOH terminal domain that binds vesicular cargo.

The role of the yeast vacuole cannot be underestimated as it is essential that *S. cerevisiae* cells possess a functional vacuole in order to remain viable (Weisman, 2003). However, it has been shown that *S. cerevisiae* haploid strains that are defective for vacuolar inheritance (Δ*vac1-1* (*pep7*), and Δvps3) remain viable and are able to undergo cellular replication and therefore proliferate (Weisman *et al.*, 1987; Raymond *et al.*,

1990). In strains where vacuolar inheritance is blocked, daughter cells are observed to rapidly generate vacuoles *de novo* (Gomes de Mesquita, 1997); however, this is an extremely rare and ultimately contrived phenomenon.

In this study, vacuoles of ale and lager yeast strains demonstrated two distinct forms, with some cells exhibiting prominent large central vacuoles, and others exhibiting highly fragmented (or pro-) vacuoles. The reasons for this were not immediately understood, however, further examination of populations of cells in the exponential and stationary phases of growth indicated that vacuolar fragmentation was more readily detectable in exponentially growing (budding) cells, than in stationary phase populations. Indeed, vacuolar fragmentation was more apparent in populations, where replication was observed by virtue of the production of daughter cells through budding. To a large extent this research concurs with the findings reported for vacuolar changes in laboratory haploid cells.

Evidence was presented in this chapter for the involvement of a vacuolar fission/fusion-mediated inheritance of this organelle in production brewing yeast strains, supporting the model proposed by Schwencke (1977), and to some extent the observations of Hill and his co-workers (1996). It is suggested that specific staining for cytoskeletal proteins would enable an assessment of whether the mechanism proposed by Hill *et al* (1996) is conserved in ale and lager brewing yeast strains.

In this chapter, vacuolar fragmentation was observed during replication, and vacuolar fusion appeared to coincide with the entry into stationary phase. However, the exact mechanism of vacuolar inheritance in the production brewing yeast strains examined in this study remains somewhat unclear. Despite this, the model of vacuolar fragmentation and fusion as the driving force behind vacuole inheritance in brewing strains is supported in this study. At no point were "tubular vesicular segregation structures" (Conradt *et al.*, 1992) observed, however, it is suggested that this should be further investigated.

The novel staining regime utilised in conjunction with confocal microscopy established a degree of clarity and specificity of vacuole visualisation hitherto undemonstrated. The co-localisation of the dyes MDY-64 and C-DCFDA enabled the physiological response of the yeast vacuole to osmotic stress to be investigated but did not corroborate the findings of Pratt-Marshall *et al.*, (2002a) that vacuolar size increased during osmotic stress, as induced by high gravity fermentation. In this study, vacuolar

fragmentation was observed to occur irrespective of the osmotic stress applied. Although fragmentation has been demonstrated to occur as a result of osmotically-induced PtdIns(3,5)P_2 levels, as detailed by Dove *et al.*, (1997), it was impossible to differentiate between vacuolar fission as a function of replication, or fragmentation mediated by high osmolarity.

In the brewing context, inoculation (pitching) into hyperosmotic media (high gravity worts) may result in vacuolar morphological changes, however, the differentiation of such changes from those incurred as a result of the transition from stationary to exponential phase that marks the commencement of brewing fermentation could not be readily achieved. Furthermore, it is suggested that vacuolar 'size' is not a good correlative parameter for osmotic stress due to the fringe effects of fragmentation and cannot be used as a biomarker of osmotic stress in production brewing yeast strains.

Chapter 5

Compatible Solute Accumulation in Osmotically Stressed *Saccharomyces* species.

Chapter 5 Compatible Solute Accumulation in Osmotically Stressed *Saccharomyces* Species

5.1 Introduction

The physiological response of a range of organisms to osmotic stress in many cases involves the accumulation of compatible solutes (section 1.5.4). The fundamental requirement of biological molecules that act as compatible solutes is that they are so-called order-forming or kosmotropic molecules (from the Greek κόσμος (kosmos, order)). Kosmotropes are molecules or ions that can stabilise water, and consequently proteins, thereby acting as osmoprotectants during water stress (Galinski *et al.*, 1997). Conversely, chaotropes (from the Greek χαος (chaos, disorder)), are molecules or ions, which actively disrupt the structure of water and give rise to protein denaturation (e.g. guanidinium salts and urea) (Eggers and Valentine, 2001).

The major benefit in accumulating compatible solutes to an organism subjected to osmotic stress is the circumvention of a deleterious effect on physiology. Compatible solutes, by their very definition may be accumulated at very high levels to balance extracellular and intracellular osmolarities without seriously impairing protein functionality and a range of other physiological characteristics (Gilbert *et al.*, 1998; section 1.5.4). The major benefits of accumulating organic osmolytes appears to be two-fold, firstly these molecules are able to affect an increase in intracellular osmotic potential without the need for the production of stabilising proteins within the cell (Yancey *et al.*, 1982), and secondly they can be readily assimilated following the removal of the osmotic stress (Kempf and Bremner, 1998).

The most highly conserved feature of compatible solutes is that they are usually uncharged, or zwitterionic (Poolman and Glaasker, 1998). Plant cells preferentially accumulate the imino acid proline during drought, or salt stress (Serrano, 1996), and amino acids and their derivatives, such as the quaternary zwitterionic ammonium compounds, glycine betaine, alanine betaine, and proline/hydroxyproline betaine (Yancey *et al.*, 1982; Hanson *et al.*, 1994) (Figure 5.1) have also been observed to accumulate in osmotically stressed plants.

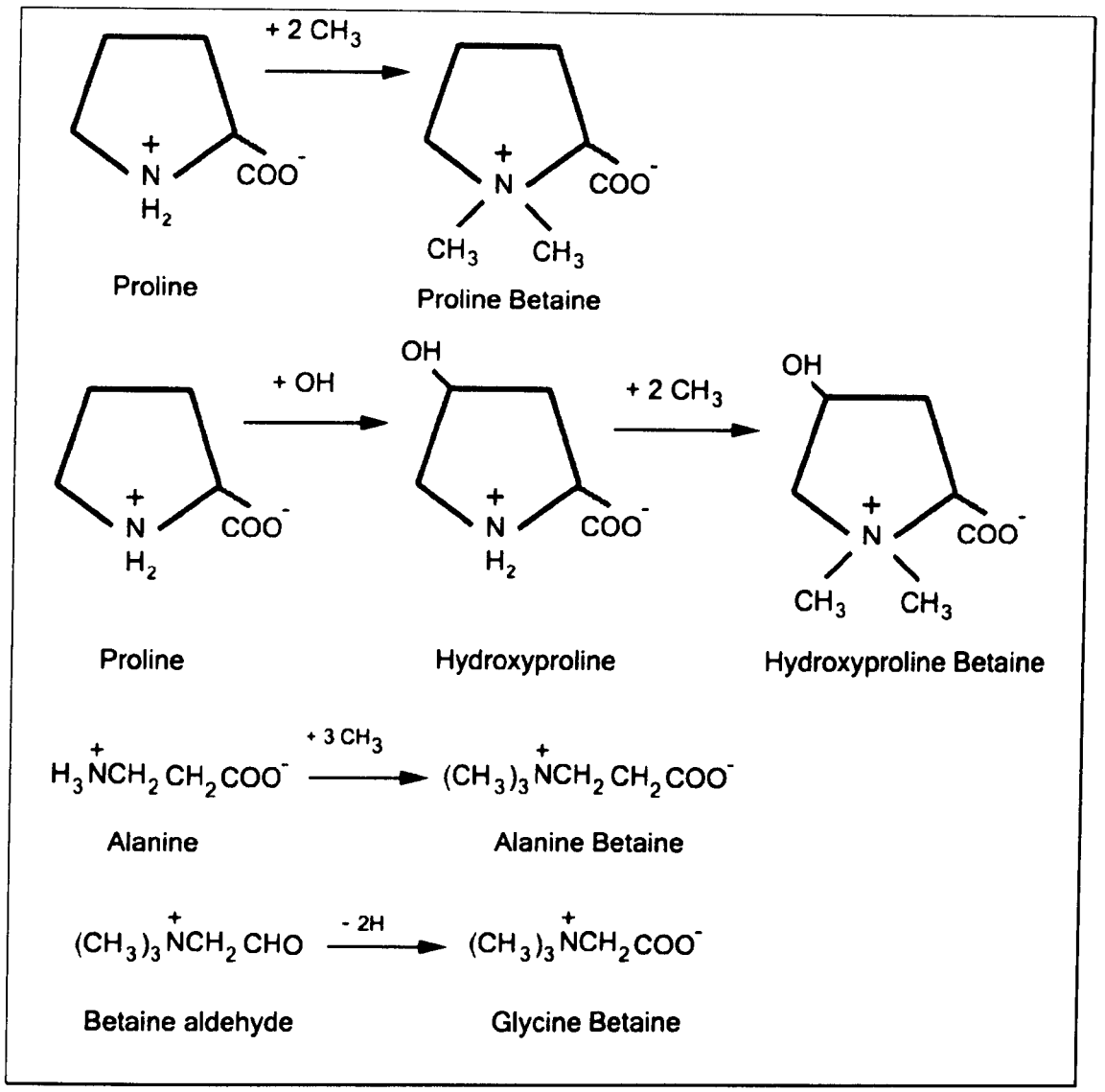


Figure 5.1. Zwitterionic quaternary ammonium compounds and their betaine derivatives utilised as compatible solutes in a range of organisms. The processes and order of the hydroxylation and methylation steps remains unclear in many cases. Adapted from Hanson *et al.*, (1994).

In *S. cerevisiae* it is generally accepted that the polyhydric alcohol glycerol is accumulated and has a major role to play in osmoregulation (Brown, 1978; Brown *et al.*, 1986; Blomberg and Adler, 1989; Blomberg and Adler, 1992; Albertyn *et al.*, 1994; Hohmann, 1997) (section 1.5.4). The use of a series of gene deletion (knockout) mutants, deficient in enzymes in the glycerol biosynthetic pathway have been utilised to study osmosensitive phenotypes (Albertyn *et al.*, 1994; Eriksson *et al.*,1995; Liden *et al.*, 1996; Ansell *et al.*, 1997; Hounsa *et al.*, 1998). Glycerol has therefore been demonstrated to be an important player in the osmotic stress response of *S. cerevisiae*. The abolition of the ability to produce glycerol is thus extremely deleterious and cells

are unable to survive hyperosmotic conditions. Furthermore, glycerol has an important role to play in anaerobic stress as the requirement of NAD^+ in its production enables glycerol to serve as the final product in a 'redox-dump' pathway (Ansell *et al.*, 1997).

High levels of intracellular glycerol appear to confer the capacity to withstand high extracellular osmolarity. Glycerol has also been demonstrated to be quickly accumulated, following exposure to unfavourably high osmotic potential (Albertyn *et al.*, 1994; Norbeck and Blomberg, 1996; Rep *et al.*, 2003). Upregulation of the genes involved in glycerol production occurs during osmotic stress, the most notable of these being *GPD1* (Norbeck and Blomberg, 1996). *GPD1* encodes a cytoplasmic NAD^+ dependent glycerol-3-phosphate dehydrogenase (Albertyn *et al.*, 1994). The subsequent increase in intracellular glycerol can therefore be correlated to osmotic stress in haploid yeast strains of the W303a and S288C genetic backgrounds (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995; Liden *et al.*, 1996; Ansell *et al.*, 1997; Hounsa *et al.*, 1998).

The importance of retention of glycerol during osmotic stress was further demonstrated by the findings that a specific glycerol transport channel (Fps1p) is effectively closed, when *S. cerevisiae* cells are exposed to hyperosmolar conditions (Luyten *et al.*,.1995; Tamas *et al.*, 2000). It has also been shown that in laboratory haploid *S. cerevisiae* cells the Fps1p channel is essential during osmotic stress and due to the surprisingly high impermeability of the plasma membrane to glycerol it can almost exclusively regulate glycerol efflux (Luyten *et al.*, 1995; Tamas *et al.*, 2000).

Polyhydric alcohols (polyols) are a conserved group of molecules with a basic sugar backbone, and multiple hydroxyl groups; they include sorbitol, xylitol and mannitol, the latter of which is also utilised by a number of bacterial (Kets *et al.*, 1996) and plant (Vera-Estrella *et al.*, 1999) species. There is increasing evidence for the evolutionary conservation of this group of molecules as compatible solutes (Yancey *et al.*, 1982). However, their production in many cases as a bi-product of normal physiology has served to complicate matters (Brown, 1978; Brown *et al.*, 1986; Hohmann, 1997; Galinski *et al.*, 1997; Poolman and Glaasker, 1998) (sections 1.5.4 and 1.5.8).

The intracellular accumulation of compatible solutes is therefore central to the tolerance of *S. cerevisiae* to high concentrations of solutes. The response of haploid laboratory strains has been studied extensively and glycerol has been demonstrated to be an important molecule in mediating water potential balance, however, the similarities

that these results have with production brewing strains remain unclear. Furthermore, it is unknown if solutes other than glycerol are utilised as organic osmolytes during hyperosmotic stress. The possibility that *S. cerevisiae* cells can accumulate amino acids for example as a compatible solute remains untested.

5.2 Results

Yeast cells were grown to the required growth phase as indicated in section 2.4.2, enumerated, washed and resuspended in either sorbitol or NaCl as required (section 2.5). Cells were exposed to osmotic stress for 48 hours to permit adequate exposure to this stress; previous studies using haploid *S. cerevisiae* cells have used exposure times up to 12 hours, however, production brewing strains are typically exposed to stress for longer periods. Water (dH_2O) was used as a negative control for stress experiments. Although dH_2O imparts a hypertonic stress to yeast cells it was identified as the only means of retaining cells in corresponding conditions without the influence of extracellular osmolytes. Glycerol determinations were conducted on triplicate samples of 1×10^9 cells. Variances of the data sets were determined by F-test analysis, at the 95% confidence interval. T-test analyses were performed using either heteroscedastic or homoscedastic tests dependent upon the spread of data sets according to the F-test analysis. Standard deviations from the normal distribution were calculated and represented graphically as error bars, where appropriate.

5.2.1 Intracellular glycerol time course

The dynamic metabolic machinery of *S. cerevisiae* enables the re-routing of assimilates according to environmental conditions (Dickinson, 1999). During osmotic stress, cells may also experience starvation, and this may be manifested in the mobilisation of another endogenous carbon source to produce compatible solutes. It is also entirely possible that intracellularly accumulated glycerol could be mobilised to allow survival of the organism during starvation (Lillie and Pringle, 1980). It has been demonstrated that the stress protectant molecule trehalose (section 1.5.2) can act as a reserve carbohydrate (Lillie and Pringle, 1980); this has led to the supposition that this molecule could be used as an endogenous carbon source precursor for the formation of glycerol during prolonged exposure to osmotic stress (Hounsa *et al.*, 1998). This theory has been conclusively disproved by Hounsa *et al.*, (1998); however the utilisation of

trehalose during prolonged exposure to osmotic stress by brewing yeast strains has not been ascertained. The findings that stress protectant molecules such as trehalose can be mobilised during osmotic stress leads to the supposition that ale and lager *S. cerevisiae* cells exposed to sorbitol induced osmotic stress utilise glycerol as an endogenous carbon source as a function of starvation. To test this hypothesis a time course of glycerol accumulation was performed using SCB1 (lager) cells that were exposed to 24% (w/v) sorbitol for 48 hours. Sorbitol (24% w/v) was utilised in this study since in previous experiments it represented a sufficiently high enough concentration to elicit an osmotic stress response without causing a deleterious affect on the viability of stationary phase SCB1 cells (section 3.2.2). The intracellular content of glycerol was assayed hourly as described in section 2.7 in triplicate over a period of 48hours.

Figure 5.2 demonstrates that intracellular glycerol concentration in SCB1 stationary phase cells exposed to sorbitol osmotic stress decreased during the first two hours, and then increased for a further 6 hours until a relatively stable intracellular concentration was reached.

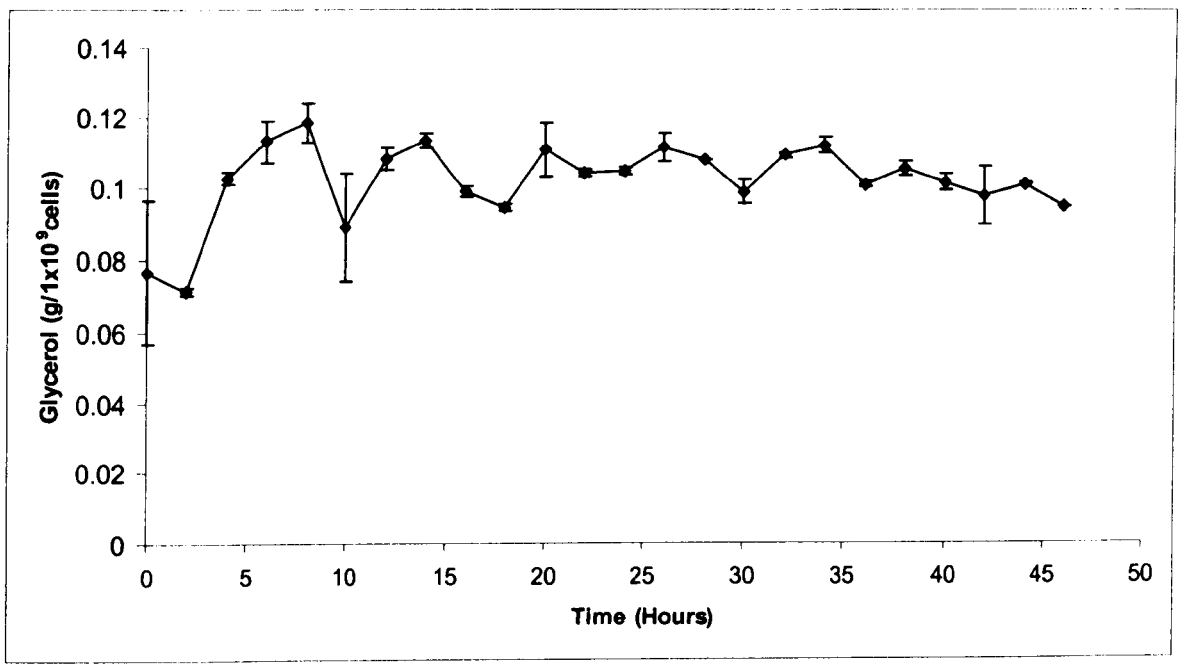


Figure 5.2. SCB1 (lager) strain intracellular glycerol concentrations following exposure to 24% (w/v) sorbitol at 25°C for 48 hours. Data represent the mean of triplicate samples, error bars are the standard deviation of a normal distribution of the samples.

5.2.2 Do brewing yeast strains accumulate glycerol during osmotic stress?

The role of glycerol in the osmotic stress response of *Saccharomyces cerevisiae* has been reviewed by Hohmann (2002). The induction of the glycerol biosynthetic pathway involves the HOG pathway, and is regulated by multiple stimuli (section 1.5.6). Intracellular glycerol accumulation during osmotic stress is suggested to be the main stratagem utilised by *S. cerevisiae* in order to counteract an increase in extracellular osmolarity, however the exact levels of accumulation of this solute in brewing strains has received relatively little attention (Panchal and Stewart, 1980, Hounsa *et al.*, 1998). The complex nature of intracellular glycerol accumulation may be dependent upon various factors, including differential expression of genes involved in glycerol production, dissimilation and export (Albertyn *et al.*, 1994; Eriksson *et al.*,1995; Liden *et al.*, 1996; Ansell *et al.*, 1997; Hounsa *et al.*, 1998) (chapter 6) (sections 1.5.9 and 1.9.3).

In order to examine the impact of strain on brewing yeast glycerol accumulation during exposure to hyperosmotic shock, lager (SCB1-4) and ale (SCB5-8) brewing yeast strains were exposed to 48 hour incubation in various concentrations of sorbitol.

The data presented here demonstrate that intracellular accumulation of glycerol across a sorbitol concentration range appeared to follow a strain-dependent pattern. No correlation between exogenous sorbitol concentration, and intracellular glycerol accumulation was apparent. In many instances, yeast strains demonstrated an initial increase in intracellular glycerol concentration with increased sorbitol concentration followed by a subsequent reduction in intracellular glycerol at higher solute concentrations (figures 5.3 and 5.4) (table 5.1). However, in all cases there appeared to be some variation in the levels of glycerol observed.

The hypothesis that solute concentration did not correlate to glycerol accumulation was confirmed by the observation that some strains (SCB2, SCB3 and SCB7) exhibited a decrease in intracellular glycerol levels with increased solute concentration (figures 5.3 and 5.4). Furthermore, the intracellular glycerol accumulation in the haploid laboratory strain S288C did not appear to modify in response to increasing osmotic stress (figure 5.4 and table 5.1). F-test analysis of indicative groups (12% and 30% sorbitol stressed cells) provided P-values of 0.011 for 12% sorbitol stressed ale versus lager values and 0.09 for 30% sorbitol stressed ale versus lager. These values indicated that there was a significant difference in variance

($P<0.05$) in 12% sorbitol stressed cells (between ale and lager cells), however, the variances were statistically similar during 30% sorbitol stress.

Two-tailed T-test analyses (assuming unequal variances for 12% sorbitol stress populations, and equal variances for 30% sorbitol stressed cells) yielded P-values of 0.004 for 12% sorbitol stressed and 0.838 for 30% sorbitol stressed cells. These data indicate that there were significant differences between the levels of intracellular glycerol accumulated by ale and lager cells when stressed with 12% sorbitol, however significant differences were not apparent following exposure to 30% sorbitol. No significant differences for ale and lager yeast could be determined for concentrations exceeding 30% sorbitol. Statistical analyses indicated that there were significant differences between the variances of ale and lager strains when exposed to 12% sorbitol, although the variances were statistically similar when cells were treated with 30% sorbitol.

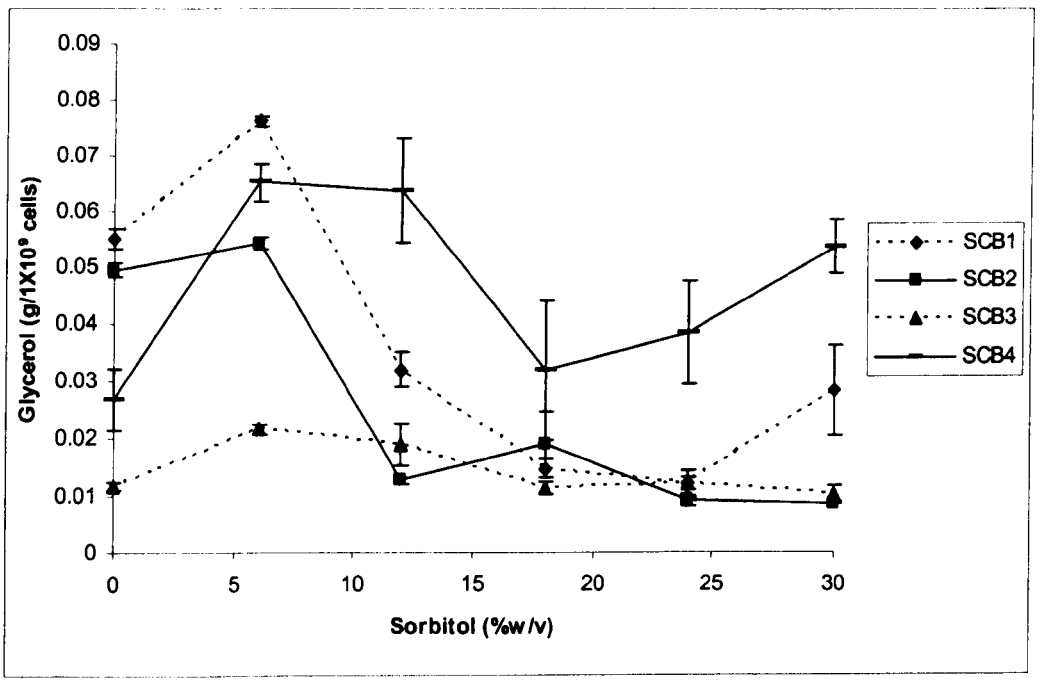


Figure 5.3. Intracellular glycerol levels in lager (SCB1-SCB4) strains exponentially grown populations. Populations were exposed to sorbitol or distilled water (0%) for 48 hours at 25°C. Triplicate samples were assayed for intracellular glycerol content; error bars represent one standard deviation of a normal distribution.

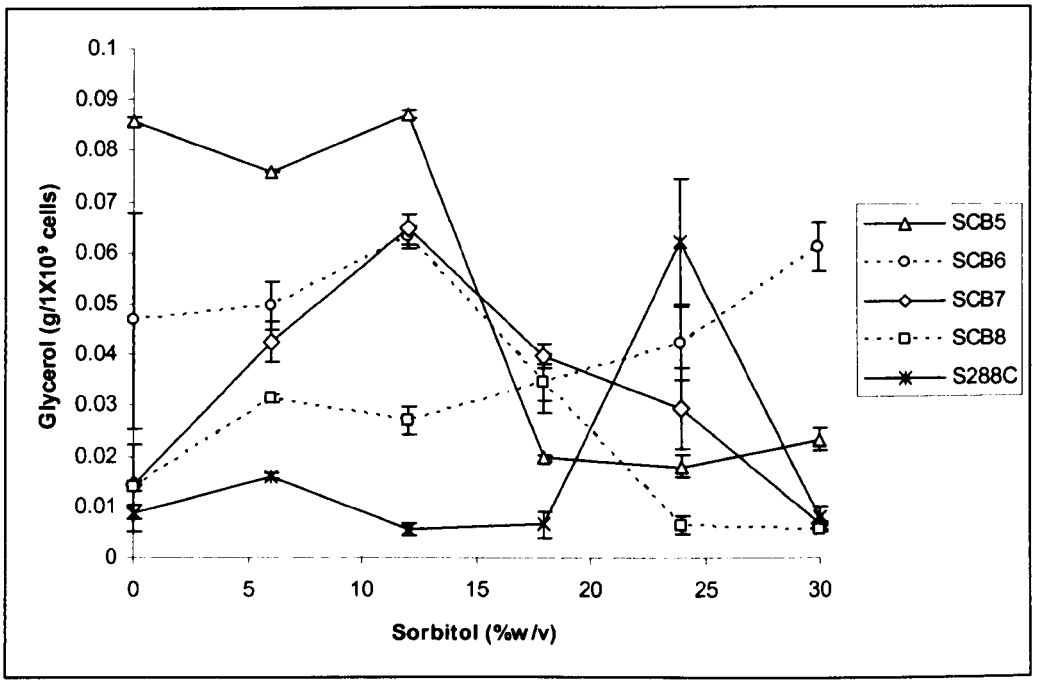


Figure 5.4. Intracellular glycerol levels in ale (SCB5-SCB8) and laboratory haploid (S288C) strains exponentially grown populations. Populations were exposed to sorbitol or distilled water (0%) for 48 hours at 25°C. Triplicate samples were assayed for intracellular glycerol content; error bars represent one standard deviation of a normal distribution

Table 5.1. Intracellular glycerol accumulation (mg /1×10^9 cells) for sorbitol-stressed exponential phase lager (SCB1-4) ale (SCB5-8) and laboratory haploid (S288C) cell populations. Cells were stressed for 48 hours at 25°C; values represent the mean of independent triplicate samples

Strain	*Sorbitol (%w/v)*		
	0	**12**	**24**
SCB1	55.1	31.9	12.2
SCB2	49.5	12.8	9.2
SCB3	11.8	19.0	12.1
SCB4	26.9	63.7	38.3
SCB5	85.7	86.9	18.0
SCB6	46.7	63.0	42.0
SCB7	14.1	64.5	29.4
SCB8	13.8	27.1	6.5
S288C	8.9	5.5	60

5.2.3 Intracellular glycerol accumulation and growth phase.

Hounsa *et al.*, (1998) have reported that intracellular glycerol levels during osmotic stress demonstrate a clear upshift following the transition between exponential and stationary phases of growth in laboratory haploid *S. cerevisiae* cells (strain W303-1A). These findings, however, are only relevant for 'moderately' osmotically-stressed cells (0.61 molal (3.5% w/v) or 0.91 (5.3% w/v) molal NaCl) and were only observed over a four-hour stress period in a yeast nitrogen base (YNB) growth medium, where the water activity was adjusted. The response of laboratory haploid *S. cerevisiae* cells to severe osmotic stress over a 48 hour stress period has not been previously reported. Furthermore, the relationship between intracellular glycerol levels and growth phase for this stress period is not known. Some limited data exist concerning intracellular glycerol accumulation in brewing yeast strains during osmotic stress; however comparisons between the stationary and exponential phases of growth have not been made (Panchal and Stewart, 1980).

In order to assess the impact of growth phase on glycerol accumulation following exposure to sorbitol induced stress, exponential and stationary phase populations of the lager (SCB1-4), ale (SCB5-8) and laboratory haploid strain (S288C) were harvested and exposed to sorbitol induced osmotic stress (section 2.5). Glycerol concentration was determined using the method outlined in section 2.7.

Differences between the levels of intracellular glycerol for exponential and stationary phase populations were apparent (compare figures 5.3/5.4 and 5.5/5.6). The lager strain SCB2 demonstrated an increase in intracellular glycerol level as exogenous sorbitol is increased (figure 5.5), the same phenomenon was displayed by the ale strain SCB5 (figure 5.6), although to a lesser extent. Interestingly, exponential phase populations for both strains SCB2 and SCB5 demonstrated a decrease in intracellularly accumulated glycerol with a comparable increase in sorbitol induced osmotic stress (figures 5.3 and 5.4).

Exponential and stationary phase populations of strain SCB3 did not appear to exhibit modified intracellular glycerol concentrations irrespective of the level of osmotic stress applied (figures 5.3 and 5.5). The intracellular glycerol content of stationary phase populations of the lager strain SCB4 was unaffected by osmotic stress (figure 5.5), however, corresponding exponential phase populations exhibited a clear dual peak of glycerol accumulation (figure 5.3). A dual peak of intracellular glycerol

was also demonstrated by exponential phase ale strain SCB6 cells (figure 5.4), and this was mirrored by corresponding stationary phase populations (figure 5.6). Stationary phase populations of the ale strains SCB7 and SCB8 demonstrated an initial increase in intracellular glycerol, (figure 5.6.) and then a decrease as sorbitol concentration increased.

The ale strain SCB8 clearly demonstrated an increased level of intracellularly accumulated glycerol in stationary phase (figure 5.6), compared to exponential phase (figure 5.4). Statistical analyses did not indicate any significant differences between the variances of two groups of indicative data (12% sorbitol stressed, and 30% sorbitol stressed cells). F-test analysis yielded P-values of 0.409 and 0.087 between exponential and stationary phase populations for 12% and 30% sorbitol stressed cells respectively. The resultant t-test analysis, assuming equal variances returned a two-tailed P-value of 0.608 between exponential and stationary phase cells when stressed with 12 % sorbitol; this indicates that at this lower concentration there is no statistical evidence of an alternation in glycerol levels. However, t-test analysis upon 30% sorbitol stressed cells data between exponential and stationary phase populations gives a two-tailed p-value of 4.07×10^{-5}, a highly significant indication that differences in the means of 0.0571g glycerol /1×10^{9} cells (stationary) and 0.0239g glycerol /1×10^{9} cells (exponential) is not attributable to random sampling errors.

The data analysis presented here indicates that at the lower concentration of sorbitol there were no significant differences in either the variances or mean values between exponential and stationary phase populations. Comparing the data analysis for 30% sorbitol stressed cells one can establish that the distribution of the data points is uniform between exponential and stationary phase, however the two sample means demonstrated a highly significant difference, demonstrating that exponential and stationary phase populations responded differently to high solute challenge (sorbitol 30% w/v). Therefore, it was demonstrated that although there were some conserved responses to osmotic stress for stationary phase populations (table 5.2) there was considerable strain dependent variation.

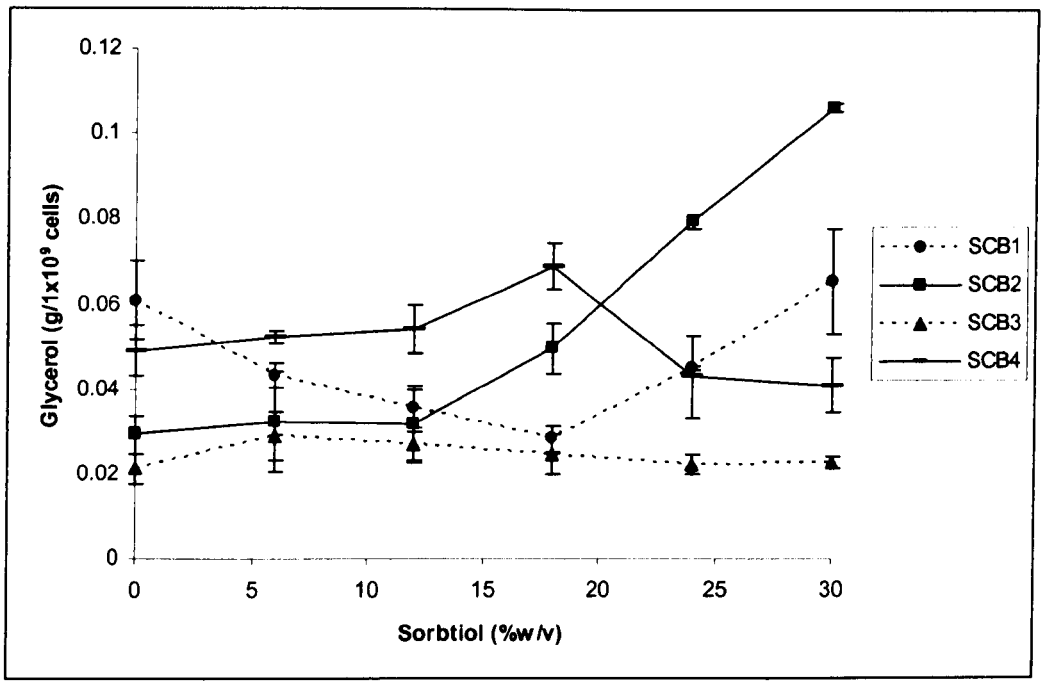


Figure 5.5. Intracellular glycerol levels in lager (SCB1-SCB4) strain stationary grown populations. Populations were exposed to sorbitol or distilled water (0%) for 48 hours at 25°C. Triplicate samples were assayed for intracellular glycerol content; error bars represent one standard deviation of a normal distribution.

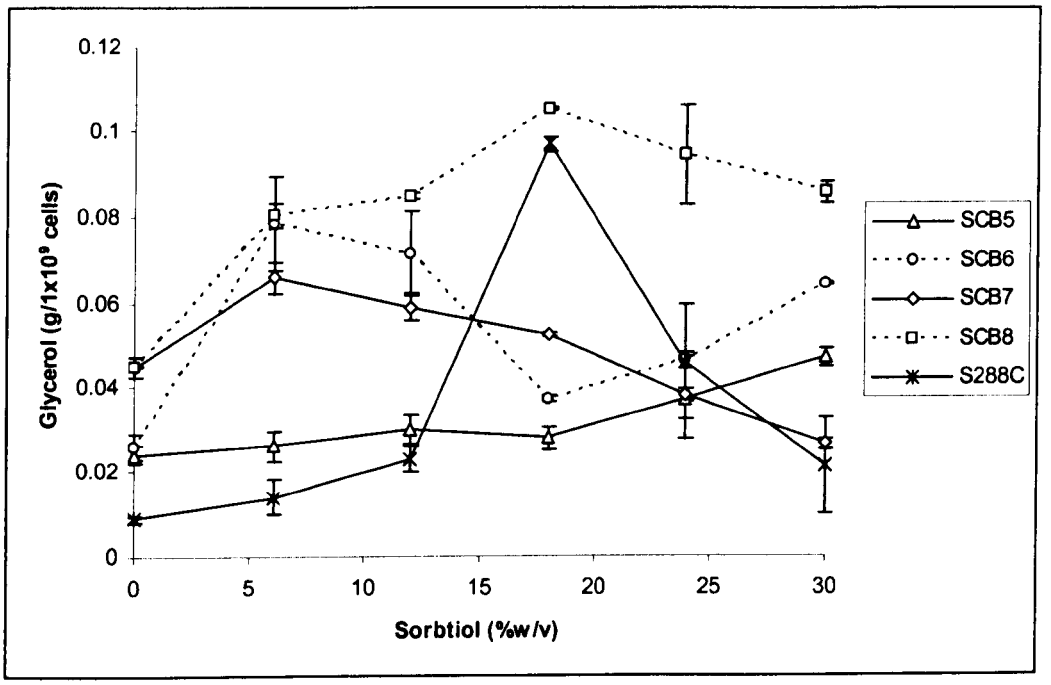


Figure 5.6. Intracellular glycerol levels in ale (SCB5-SCB8) and laboratory haploid (S288C) strains stationary grown populations. Populations were exposed to sorbitol or distilled water (0%) for 48 hours at 25°C. Triplicate samples were assayed for intracellular glycerol content; error bars represent one standard deviation of a normal distribution.

Table 5.2. Intracellular glycerol levels in lager (SCB1-4) ale (SCB5-8) and laboratory haploid (S288C)strains stationary grown populations. Populations were exposed to sorbitol or distilled water (0%) for 48 hours at 25°C. Triplicate samples were assayed for intracellular glycerol content (mg /1×10^9 cells).

Strain	*Sorbitol (%w/v)*		
	0	**12**	**24**
SCB1	60.9	35.4	45.1
SCB2	29.2	31.7	79.6
SCB3	21.1	26.8	22.4
SCB4	49.0	54.0	43.0
SCB5	24.0	30.1	37.4
SCB6	26.0	71.4	46.3
SCB7	45.1	59.0	38.3
SCB8	45.1	84.7	94.2
S288C	9.0	23.0	45.7

5.2.5 Intracellular glycerol accumulation and solute type.

The cellular response to NaCl-induced extracellular osmolarity involves the activation of the calcineurin/calmodulin signalling pathway (Gaxiola *et al.*, 1992; Murgia *et al.*, 1995, Murgia *et al.*, 1996; Ganster *et al.*, 1998) (section 1.5.7), which upregulates the expression and activity of transmembrane ion proton pumps (Mendoza *et al.*, 1994, Mendoza *et al.*, 1996; Matsumoto *et al.*, 2002). The consequence of this regulation is that potentially damaging monovalent cations can be removed from the cell, with the resultant increase in tolerance to salt stress. The impact that osmotic stress has on brewing yeast strains is not well understood, in addition the effects that different types of solute have on cellular responses in brewing yeast are unknown. The ramifications of this dearth of knowledge to the brewing process are wide-ranging as brewers' wort is a highly complex medium composed of a myriad of solute types (section 1.4.2.1). Furthermore, evidence for an alternation in response to solute type from the laboratory haploid strains is evident, and it has been demonstrated that expression of key enzymes in glycerol biosynthesis are activately differently in sorbitol and NaCl-induced osmotic stress (Rep *et al.*, 1999).

All strains examined in this study clearly demonstrated lower levels of intracellular glycerol when exposed to NaCl as compared to the corresponding concentrations of sorbitol in stationary phase (figure 5.7). Stationary phase populations

of cells demonstrated a lower level of intracellularly accumulated glycerol and less variation in this lower level was demonstrated (figure 5.7). Exponential phase cells exposed to salt induced osmotic stress clearly demonstrated an initial decrease in intracellular glycerol (figures 5.8) and the subsequent residual level remained unchanged by increasing concentrations of exogenous salt.

Statistical analyses of the data sets provided evidence for a difference in response to different solute types. The variance of representative groups (12% sorbitol and salt stressed, and 30% sorbitol and salt stressed stationary phase cells) was demonstrated to be significantly different at the 95% confidence level with P-values of 1.47×10^{-2} and 3.2×10^{-3} for 12% solute stressed, and 30% solute stressed cells respectively. T-test analysis returned two-tailed P-values of 3.04×10^{-5} and 1.77×10^{-7} for 12% sorbitol versus salt stressed stationary phase cells, and 30% sorbitol versus salt stressed cells respectively.

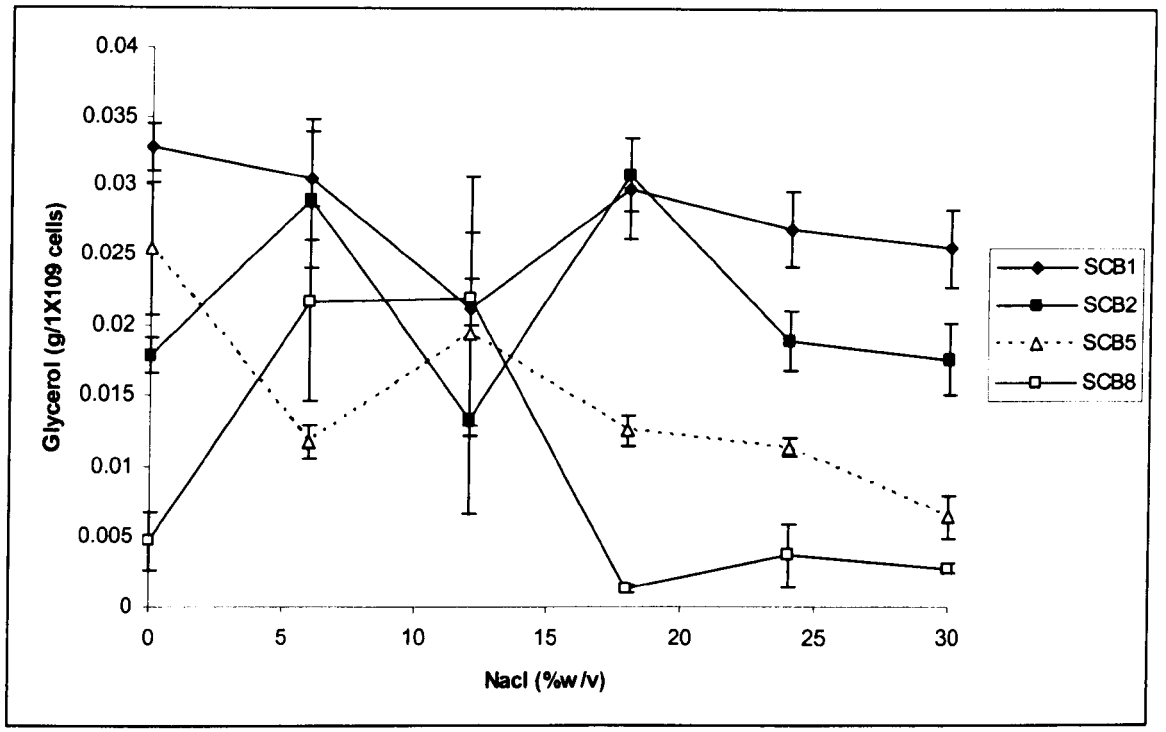


Figure 5.7. Intracellular glycerol levels in lager (SCB1 and SCB2) and ale (SCB5 and SCB8) strains stationary grown populations. Populations were exposed to NaCl or distilled water (0%) for 48 hours at 25°C. Triplicate samples were assayed for intracellular glycerol content; error bars represent one standard deviation of a normal distribution.

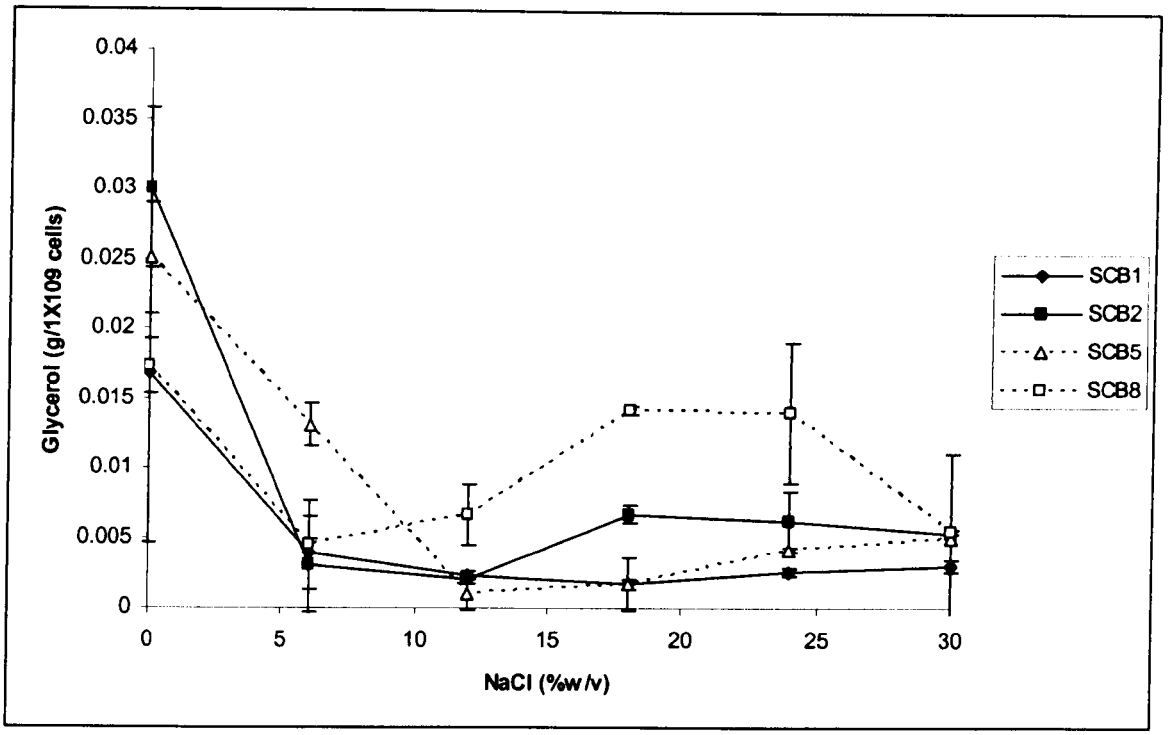


Figure 5.8. Intracellular glycerol concentrations following exposure to NaCl for lager (SCB1 and SCB2) and ale (SCB5 and SCB8) cells in the exponential phase of growth. Cells exposed for 48 hours to a range of NaCl concentration and distilled H_2O (0%). Triplicate samples were assayed for intracellular glycerol content; error bars represent one standard deviation of a normal distribution.

5.2.6 Intracellular amino acid accumulation in brewing yeast.

In many organisms a number of compatible solutes are utilised in order that the cumulative osmotic potential of these molecules can counteract high external osmolarities. In *S. cerevisiae* this has not been demonstrated, however given that glycerol accumulation did not correspond to the profile normally observed in haploid laboratory yeast (Brown, 1978; Reed *et al.*, 1987; Bellinger and Larher, 1987; Meikle *et al.*, 1991; Blomberg and Adler, 1992), it was suggested that alternative compatible solutes may also be accumulated to assist in the defence against the hyperosmotic stress imposed.

Compatible solutes are a highly conserved group of molecules with a specific set of physical properties (Galinski *et al.*, 1997; Gilbert *et al.*, 1998). Some amino acids serve as compatible solutes, most notably glycine (in the form of glycine betaine) in bacteria, and proline in plants (Poolman and Glaasker, 1998; Welsh and Herbert, 1999) (section 1.5.4).

Evidence for the potential role of proline during osmotic stress in *S. cerevisiae* comes from work on sporulation and the subsequent thermotolerance and osmotolerance of ascospores. Ho and Miller (1978) demonstrated a high level of accumulation of free proline in ascospores, which was correlated to an increase in desiccation tolerance. However, proline accumulation during osmotic stress has not been studied for vegetative cells of *Saccharomyces sp.*, but its potential to serve as a compatible solute has been revealed by its ability to stabilise water and act a cryopreserving agent (Tagaki *et al.*, 1997).

In order to investigate the hypothesis that brewing yeast strains employ alternative compatible solutes in the defence against osmotic stress, the occurrence of proline and other amino acids were examined. Cellular extracts obtained using the methods outlined in section 2.9 were examined by TLC and a ninhydrin based assay as described in section 2.8. Cells were exposed to sorbitol at the indicated concentrations, and the cellular contents extracted by boiling in water for ten minutes. Standard solutions were prepared as 1mM concentrations in water, and 2μl of these standards were added to a thin layer chromatography plate as detailed in section 2.9. A 2μl aliquot of the cellular extract was added to the TLC plate, which was subsequently developed as described in section 2.9.

Figures 5.9a and b show representative TLC plates developed using ninhydrin. Each amino acid can be differentiated in the sample extract by its R_f (retention factor) value and the characteristic colour produced when complexed with ninhydrin to form variations of Ruhemann's purple. The most clearly visible and easily identifiable of all of the amino acids is proline, as a characteristic yellow product is formed. The R_f values for each of the amino acids and spots on the extract sample were calculated according to the following equation:

$$R_f = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

In order to avoid complications due to experimental variations, and small differences in solvent composition, individual R_f values were calculated for each standard on each plate. Experimentally, this was more accurate than referring to an R_f average value for each standard, and limits errors due to variations in experimental procedures.

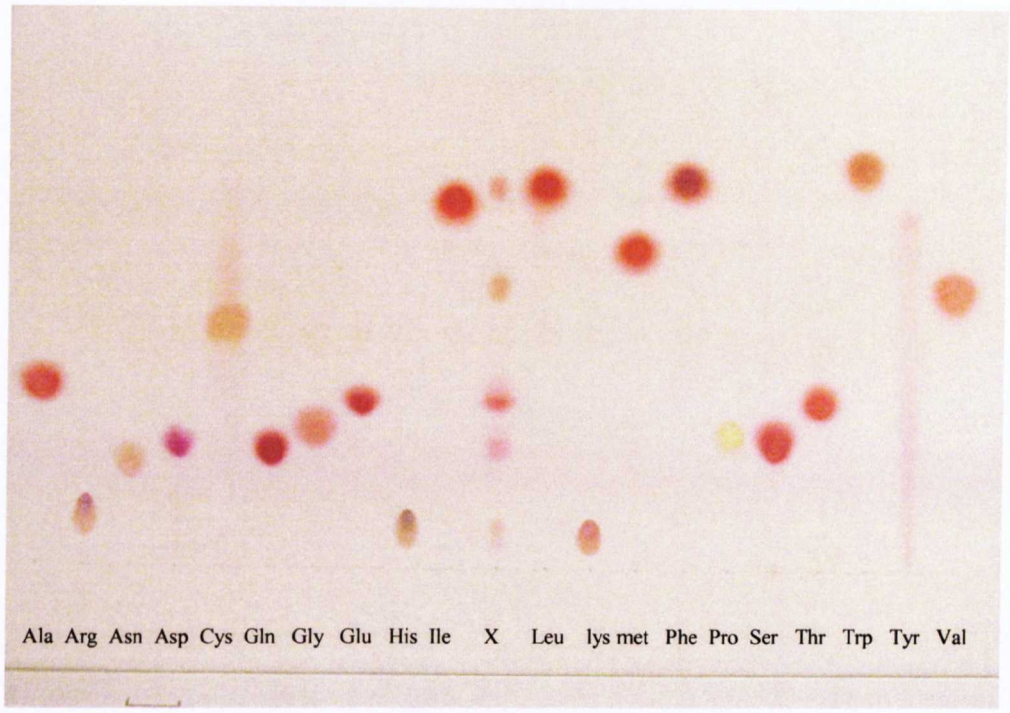


Figure 5.9a. Silica TLC plate with standard amino acid solutions and extract of SCB1 12% sorbitol-stressed cells. The characteristic variations of Ruhemann's purple shifted to yellow are demonstrated by proline and cysteine.

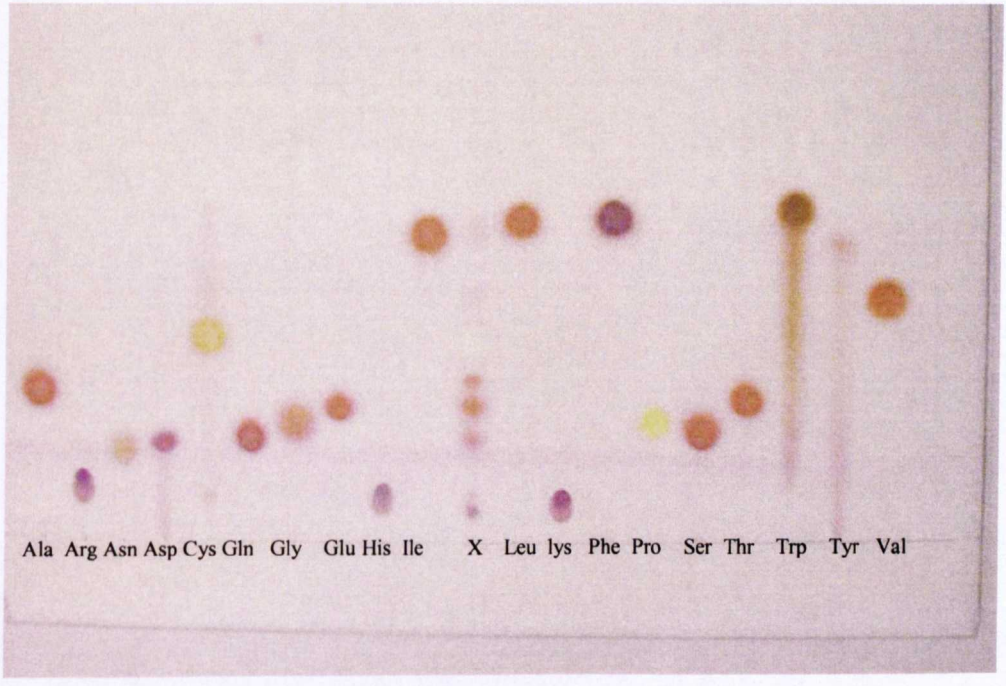


Figure 5.9b. Silica TLC plate with standard amino acid solutions and extract of SCB8 12% sorbitol-stressed cells. The characteristic variations of Ruhemann's purple shifted to yellow are demonstrated by proline and cysteine.

A qualitative examination of the composition of the amino acid pool accumulated during osmotic stress indicated that this phenomenon was strain-dependent (figures 5.9a and b). Closer examination of the composition of the amino acid pool of brewing yeast cells when subjected to osmotic stress reveals that there are clear alternations, however in many cases this compositional change does not appear to follow a specific pattern. In the case of SCB1 (table 5.3), the amino acids alanine and leucine were observed in non-stressed cells, however, these were not detected in extracts from sorbitol stressed cells. Conversely, in this strain a number of amino acids were apparent in extracts of stressed cells, although these amino acids were not detected in non-stressed samples. The amino acids arginine, cysteine, glutamine, glycine, isoleucine, methionine, serine and threonine were all detected at some point during osmotic stress, but not in the control situation. Furthermore, it was demonstrated that glycine was detected in extracts from SCB1 cells upon exposure to concentrations of sorbitol of 18% and above. Two amino acids, valine and glutamine were present in all conditions, which may represent a requirement for these compounds at all times, otherwise this may be due to increased ability to resolve these two amino acids using this rather crude approach. The results from the ale strain SCB8 are somewhat different, the data indicate that there is a compositional change between the intracellular amino acid pool during osmotic stress and as a consequence the amino acids detected were different. SCB8 (table 5.1) demonstrates the presence of glycine, lysine, and serine when not osmotically stressed, however during sorbitol stress these amino acids are not detected. Furthermore during osmotic stress, glutamate and valine were detected but these were not observed in the control situation. A number of other amino acids were detected at varying degrees of osmotic stress, however a consistent pattern was not observed.

Table 5.3. Amino acids detected by TLC during a series of stress experiments on exponential lager (SCB1) and ale (SCB8) cells subject to a range of sorbitol concentrations for 48 hours. Presence of amino acid as determined by colour identification and Rf value indicated by black triangle (▲). Undetected amino acid by TLC indicated by dashed line (-).

	SCB1						*SCB8*					
	Control	*6%*	*12%*	*18%*	*24%*	*30%*	*Control*	*6%*	*12%*	*18%*	*24%*	*30%*
Ala	▲	-	-	-	-	-	▲	▲	▲	▲	▲	▲
Arg	-	▲	-	▲	-	-	-	▲	-	-	▲	-
Asn	-	-	-	-	-	-	-	-	-	▲		-
Asp	▲	▲	-	▲	▲	-	▲	-	▲	-	▲	-
Cys	-	-	▲	-	▲	-	▲	-	-	▲	-	▲
Gln	-	-	▲	-	-	-	-	-	-	-	-	▲
Glu	▲	▲	▲	▲	▲	▲	-	▲	▲	▲	▲	▲
Gly	-	-	-	▲	▲	▲	▲	-	-	-	-	-
His	-	-	-	-	-	-	-	-	▲	-	-	▲
Ile	-	-	▲	-	▲	-	-	▲	-	▲	-	-
Leu	▲	-	-	-	-	-	▲	-	▲	▲	▲	-
Lys	▲	-	-	-	-	▲	▲	-	-	-	-	-
Met	-	-	-	-	▲	▲	-	-	-	-	-	-
Phe	-	-	-	-	-	-	-	-	-	▲	-	▲
Pro	-	-	-	-	-	-	-	-	-	-	-	-
Ser	-	▲	-	▲	▲	-	▲	-	-	-	-	-
Thr	-	-	▲	▲	-	-	-	-	-	-	-	-
Trp	-	-	-	-	-	-	-	-	-	-	-	-
Tyr	-	-	-	-	-	-	▲	-	▲	-	-	-
Val	▲	▲	▲	▲	▲	▲	-	-	▲	▲	▲	▲

Significantly, proline was absent from all TLC plates examined in this study (for examples see figures 5.9a and b), as previously stated this amino acid is easily identified using ninhydrin-based assays as the colour shift of the dye complex to yellow is conspicuous.

To further confirm the presence or absence of proline in cellular extracts of osmotically stressed cells, the lager strain SCB1 was exposed to a range of sorbitol concentrations as previously described (section 2.5), and proline was extracted into toluene, and assayed against a standard curve according to the methods outlined in section 2.8 (figure 5.9). The profile was compared to a baseline value for proline assayed from non stressed cells of the same strain (figure 5.11).

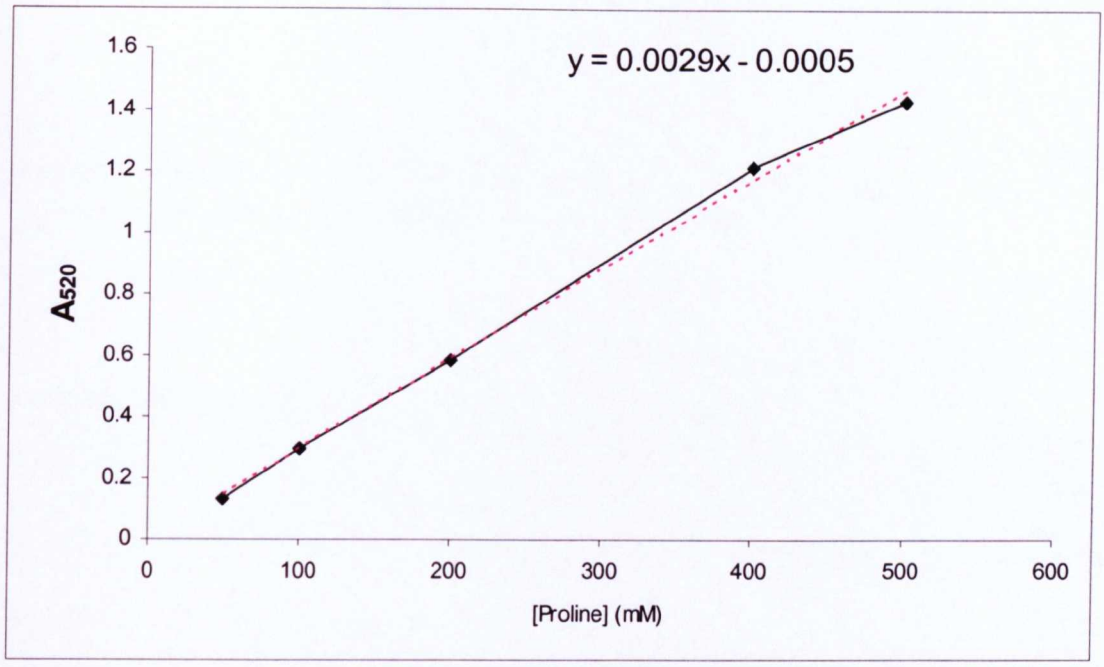


Figure 5.10. Standard curve for proline absorbance at 520nm. Standard solutions of L-proline (Sigma) were prepared by serial dilution and extracted into toluene as described in methods.

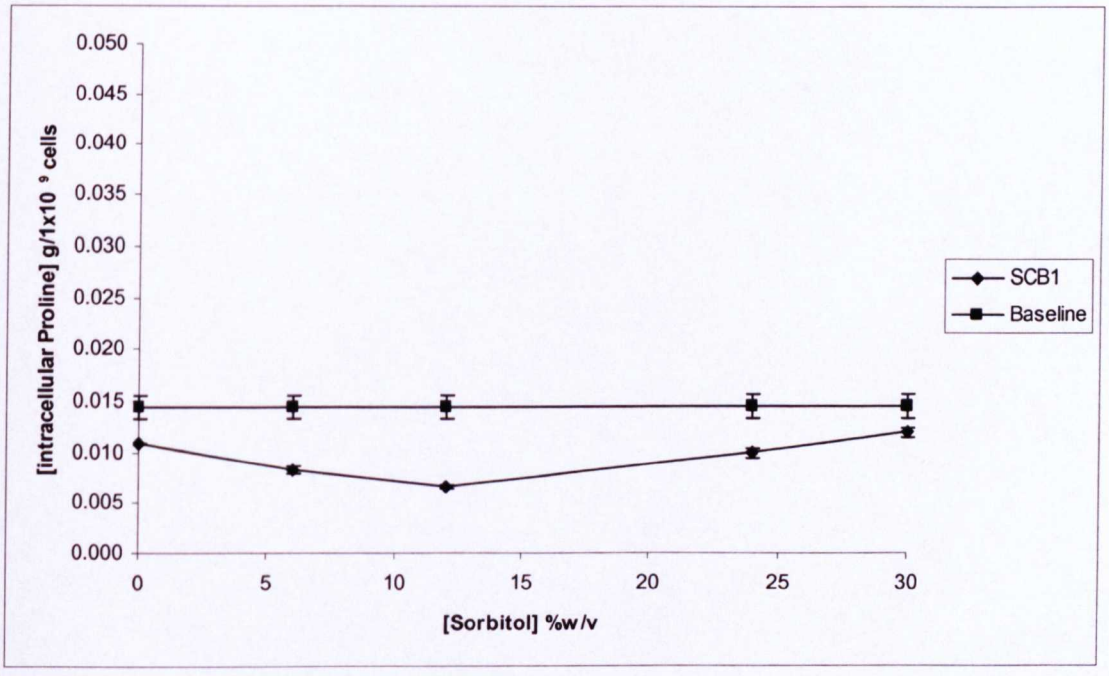


Figure 5.11. Intracellular proline accumulation in exponential phase SCB1 (lager) cells exposed to sorbitol stress, compared to a baseline of intracellular proline exhibited by non-stressed cells. Triplicate samples of cells were exposed to sorbitol for 48 hours at the indicated concentrations and assayed for proline.

Proline appeared to be present at very low concentrations during osmotic stress (below the levels observed for non stressed cells) (figure 5.10), thus quantitatively confirming the results obtained using TLC.

5.3 Discussion

Osmoadaption is the culmination of a series of sensing mechanisms in *S. cerevisiae* and represents the major stratagem employed by this organism in counteracting the deleterious effects of an unfavourable water potential in order to survive (reviewed by Hohmann, 1997; Nevoigt and Stahl, 1997) (section 1.5.3). In most cases, osmoadaption involves the preferential accumulation of one or more solutes in order to affect an increase in internal osmolarity and consequently balance osmotic potentials (Brown, 1978; Brown *et al.*, 1986; Poolman and Glaasker, 1998,). *S. cerevisiae* cells demonstrate a difference in membrane fluidity upon exposure to osmotic stress, possibly as an osmoadaptive mechanism to allow for stretching and contraction of the plasmalemma (Tunblad-Johansson and Adler, 1987; Sajbidor, 1997).

Aside from some minor structural rearrangements, most organisms adapt to hyperosmolarity by altering their intracellular water potential with the addition of solutes (section 1.5.3). The accumulation of a number of metabolic products within the yeast cell during osmotic stress may therefore be an indication of the degree of stress experienced.

Intracellular glycerol levels has been postulated to be a putative biomarker of osmotic stress in *S. cerevisiae* and *S. cerevisiae* (syn. S. *pastorianus*) brewing strains (section 1.6.4) (Panchal and Stewart, 1980). The rationale for the investigation into this hypothesis comes from a wealth of information previously elucidated by the investigation of the response of haploid laboratory *S. cerevisiae* strains at the physiological, biochemical and molecular level (Brown, 1978; Reed *et al.*, 1987; Bellinger and Larher, 1987; Meikle *et al.*, 1991; Blomberg and Adler, 1992; Albertyn *et al.*, 1994; Hohmann, 1997; Pahlman *et al.*, 2001; Remize *et al.*, 2001).

Glycerol accumulation was observed to occur following exposure to osmotic stress, although the accumulation pattern did not conform to that normally attributed to a "true" compatible solute (Brown, 1978; Galinski and Trüper, 1994; Kempf and Bremner, 1998). There may be a number of reasons for this behaviour of non-

conformity, including increased glycerol efflux or dissimilation, or a reduction in the activity of a number of genes/enzymes involved in its production.

Intracellular glycerol levels in brewing strains was observed to be strain-dependent, however, statistical analyses were unable to differentiate between the relative responses of *S. cerevisiae* ale and *S. cerevisiae* (syn. S. *pastorianus*) lager brewing strains, when exposed to the upper limits of sorbitol stress, suggesting that the patterns of accumulation are conserved for *Saccharomyces* sp. Glycerol accumulation in response to osmotic stress was also shown to be partially dependent on growth phase. When exposed to 30% sorbitol, exponential and stationary phase populations exhibited highly significant differences in their intracellular glycerol levels, the same response however, was not evident at lower concentrations. This observation has not been previously reported and the reasons for this differential response require further elucidation.

The accumulation of glycerol was also significantly affected by the solute used to elicit the osmotic stress. Sorbitol stressed cells demonstrated higher levels of glycerol than their salt stressed counterparts, although this may in part be due to the reduced level of viability demonstrated by salt stressed cohorts compared with sorbitol stressed populations (section 3.2.3). The different responses to salt and sorbitol stress are not unanticipated as the mechanisms for sensing external osmolarity and salt stress are well defined and distinct in *S. cerevisiae*. Sorbitol stress activates the HOG pathway (section 1.5.6) (Brewster *et al.*, 1993; Maeda *et al.*, 1994; Maeda *et al.*, 1995), although it is not sorbitol *per se* that induces this signal transduction pathway, rather it is a function of osmotic stress sensing as a result of cell membrane or turgor pressure changes (Posas *et al.*, 1996; Posas and Saito, 1997).

It has been demonstrated that other solutes, both assimilable and non-assimilable induce this specific pathway which culminates in the upregulation of glycerol biosynthesis (Brewster *et al.*, 1993; Albertyn *et al.*, 1994; Maeda *et al.*, 1994). Salt stress, however, is an entirely different proposition from the point of view of sensing and signalling. Halotolerance is dependent on the removal of toxic monovalent cations. Upon exposure to hypersaline conditions there is a Ca^{2+} mediated response, which involves the ubiquitous protein calmodulin, which activates a calcineurin (protein phosphatase)-mediated signalling pathway (Gaxiola *et al.*, 1992, Murgia *et al.*, 1995; murgia *et al.*, 1996; Ganster *et al.*, 1998) (section 1.6.7).

The activation of the calcineurin pathway results in the upregulation of the activation of transmembrane ion pumps with the eventual aim of detoxifying the cell from monovalent cations. It has not been demonstrated that the calcineurin pathway mediates the accumulation of any compatible solutes; however, it is possible that salt stress may co-activate both the calcineurin pathway and the HOG pathway via mutual surface receptors (Serrano, 1996).

The glycerol accumulation profiles observed for brewing yeast strains did not correspond to those normally encountered in haploid strains of *S. cerevisiae* (Brown, 1978; Reed *et al.*, 1987; Bellinger and Larher, 1987; Meikle *et al.*, 1991; Blomberg and Adler, 1992). The lack of glycerol accumulation in the manner of a compatible solute may be partially explained by either the intracellular degradation or extracellular export of this compatible solute.

The reasons for the reduced level in intracellular glycerol may also be explained as a function of cell viability, especially in the exponential phase of growth, since this parameter markedly decreased. However, intracellular glycerol levels in stationary phase cells cannot be explained in terms of viability, as the reduced level of intracellular glycerol is more likely to be due to an alternative physiological response to salt stress.

Intracellular glycerol degradation has not been previously demonstrated to occur during osmotic stress in *Saccharomyces* species. However, two routes of utilisation can occur, (section 1.5.8) and it is suggested that one possible reason for the occurrence of an atypical glycerol accumulation pattern may reflect the up-regulation of both the synthetic and degradative pathways resulting in a "futile cycle", in which no net increase in intracellular glycerol occurs.

An alternative hypothesis for the atypical profiles observed involves the action of the glyceroaquaporin protein channel, Fps1p, which permits the gated passive export of glycerol. An examination of the function of the closely related aquaporin, *AQY,* family of proteins has suggested a role in control of osmotically induced water loss (Bonhivers *et al.*, 1999), however it appears that the proteins are not a prerequisite for the maintenance of viability. Furthermore, the high degree of genetic variability and polymorphisms within the aquaporin genes *AQY1* and *AQY2* in *S. cerevisiae* hints at the seemingly dispensable nature of these proteins (Bonhivers *et al.*, 1999; Laize *et al.*, 2000). In production and wild yeast strains, the aquaporin *AQY2* is missing or non-functional and only *AQY1* is observed to mediate water fluxes (Laize *et al.*, 2000).

More recently Tanghe *et al.*, (2002) have correlated *AQY1* expression to the ability of industrial bakers' yeast to withstand freezing, by facilitating rapid efflux of water, a preventative measure against ice crystal formation. Recent research suggests that the yeast aquaporins are unable to transport glycerol, and act simply to provide a channel for water movement (Stefan Hohmann, *pers. comm.*)

In some organisms the accumulation of amino acids is extensively utilised as means of surviving unfavourable external water potentials (sections 1.5.3, 1.5.4, 5.1). Amino acids and their closely related allies (the betaine derivatives) represent a number of molecules with the correct properties which allow for hyperaccumulation without deleterious effects upon normal physiology and metabolism (Galinski and Trüper, 1994; Welsh and Herbert, 1999). These molecules are highly stabilising, and can be assimilated following the removal of an increase in external osmolarity (Poolman and Glaasker, 1998).

In plants, it has been observed that proline confers osmotic tolerance following an osmoadaptive response (Yancey *et al.*, 1982; Hanson *et al.*, 1994), however, in this study this imino acid, and powerful osmoprotectant is not visible in any of the extracts analysed by thin layer chromatography. Subsequent quantitative analysis of cellular extracts could not determine a peak of intracellular proline accumulation, indeed the level of intracellular proline detected during osmotic stress was seen to be below that of the baseline level accumulated by non-stressed cells.

Osmotic stress does however influence the composition of the amino acid pools exhibited by *S. cerevisiae* and *S. cerevisiae* (syn. S. *pastorianus*) strains. The reasons for this change in abundance of a number of amino acids is not clear and has not been previously reported, however it can be hypothesised that the differential expression of one, or a number of genes involved in stress adaptation, and the resultant stress proteins would necessitate a requirement for specific amino acids (reviewed by Mager and Krujiff, 1995).

The results presented in this chapter demonstrate that industrial production strains of brewing yeast did not accumulate glycerol in response to osmotic stress in a typical manner. The lack of correlation to external osmolarity suggested that there were inherently dissimilar physiological or biochemical regulation of glycerol accumulation in brewing yeast strains compared to laboratory haploid strains. Furthermore, it is

suggested that these differences may involve modifications in the regulation of synthesis, degradation or export of glycerol in these strains.

Chapter 6

Osmotically-Induced Gene Expression in Brewing Yeast Strains

Chapter 6 Osmotically-induced gene expression in brewing yeast strains

6.1 Introduction

The publication of the entire genome of *Saccharomyces cerevisiae* in 1996 (Goffeau *et al*, 1996) following the efforts of some 600 researchers worldwide ushered in a new era in molecular biology. As the first eukaryotic organism to have its' genome sequenced, *S. cerevisiae* occupies a unique position in genomic history. The S288C laboratory haploid strain utilised in this study has subsequently been analysed by a consortium of 134 European laboratories in the highly ambitious European functional analysis network (EUROFAN) project, in order to assign function to approximately 1000 (orphan) genes of no known function (Dujon, 1998). The first stage of the EUROFAN project (EUROFAN I) was completed in 1998, and phase two of the project (EUROFAN II) was initiated as part of a worldwide collaboration to determine the function of all of the ca.6200 yeast genes in the *S. cerevisiae* genome (Planta *et al.*, 1999; Tuller *et al.*, 1999; Lucau-Danila *et al.*, 2000; Willer *et al.*, 2000; Castrillo *et al.*, 2003). The collaborative projects briefly described here demonstrate how information about the genetic structure of *S. cerevisiae* is becoming more widely available.

Although post-genomic data availability has important ramifications in many areas of *S. cerevisiae* biology, sequencing and functional analysis techniques have been applied to the strain S288C and strains isogenic to it (Winston *et al.*, 1995; Goffeau *et al.*, 1996; Lucau-Danila *et al.*, 2000) and therefore further research is required to ascertain how applicable this information is to production brewing strains of ambiguous genetic backgrounds.

Many areas of research have profited directly from this information, none more so than in the areas of signaling and stress tolerance. The steps in the signal transduction (MAPK cascade) (section 1.5.5) pathway response to hyperosmolarity, and the subsequent stress-induced gene expression are well understood. However, the current availability of genomic data has aided in the elucidation of other participants in these refined processes (Marquez *et al.*, 1998; Ketela *et al.*, 1998; Nelson *et al.*, 2004). Furthermore, the high-throughput screening of the physiological response of knock-out mutants have identified proteins putatively involved in osmotic stress sensing, signal transduction and osmotic adjustment (Ahktar *et al.*, 1997; Reynolds *et al.*, 1998).

An increase in external osmolarity in *S. cerevisiae* results in the induction of the high osmolarity (HOG) pathway leading to the upregulation of the biosynthesis and retention of intracellular glycerol (Maeda *et al*, 1994; Albertyn *et al*, 1994; Maeda *et al*, 1995). The process of signal transduction has been extensively researched in *S. cerevisiae* haploid laboratory strains (Gustin *et al*, 1998; Madhani *et al*, 1998; Posas *et al*, 1998; Alberts *et al*, 2002), however, it has not been studied in the genetically similar polyploid and aneuploid brewing yeast strains. The eventual fate of the final protein in the HOG signal transduction pathway, Hog1p, involves the regulation of the expression of various genes, including those involved in the production of enzymes concerned with glycerol biosynthesis (Albertyn *et al*, 1994; Remize *et al*, 2001; Pahlman *et al*, 2001). The most clearly defined interaction is between the HOG pathway and *GPD1* (Albertyn *et al*, 1994), which encodes cytoplasmic glycerol 3-phosphate dehydrogenase, and *GPD2,* which encodes its isoform. *GPD1* has been described as the rate-limiting step in glycerol biosynthesis (Albertyn *et al*, 1994). The sequence and function of this pair of genes is well detailed, and it has been observed that laboratory haploid Δ*GPD1* deletion mutant strains demonstrate both an osmosensitive phenotype, and a reduction in the levels of intracellular glycerol. Δ*gpd2* mutants do not exhibit a detectable reduction in osmotolerance; however, the double knockout mutant Δ*gpd1*/Δ*gpd2* is a conditional-lethal for high osmolarity (Ansell *et al.*, 1997). In addition, during osmotic stress it has been demonstrated that although *GPD1* mRNA levels increase up to 50-fold (Albertyn *et al.*, 1994; Rep *et al.*, 1999) the level of transcripton of *GPD2* decreases in hyperosmotic stress (Ansell *et al.*, 1997). The differential expression of the *GPD1* and *GPD2* isoenzyme genes, therefore, demonstrates distinct and alternative biological activities. Upon closer inspection, it is observed that *GPD2* has an important role to play in redox balance, and is highly expressed during anaerobiosis (Ansell *et al.*, 1997).

Glycerol dissimilation in *S. cerevisiae* involves a number of typified genes (table 6.1 and section 1.6.8). Glycerol can be degraded in a retrograde manner via glyerol-3-phosphate involving the genes *GUT1* and *GUT2* (Pavlik *et al.*, 1993; Ronnow and Kielland-Brandt, 1993). Conversely, glycerol degradation may occur via dihydroxyacetone phosphate catalysed by two putative glycerol dehydrogenases encoded by *GCY1* and *YPR1,* respectively (Norbeck and Blomberg, 1997) and subsequently to dihydroxyacetone under the action of dihydroxyacetone kinase, which is encoded by *DAK1* (Norbeck and Blomberg, 1997). This alternative retrograde

pathway is not well characterised, however, Norbeck and Blomberg (1997) have demonstrated that *GCY1* expression is strongly upregulated during hypersaline (1.4M NaCl) stress, whereas *YPR1* mRNA levels are only slightly increased in the same salt stress conditions. The contribution that this glycerol catabolic pathway makes to overall glycerol breakdown is unknown; indeed it is unclear if *S. cerevisiae* degrades glycerol via *GCY1* and *YPR1*. In addition it has been postulated that this metabolic pathway is primarily involved in redox balance (Costenoble *et al.*, 2000).

The interaction of the HOG pathway with all of the genes involved in the production of specific enzymes that catalyse glycerol biosynthesis or degeneration are not clearly understood. Elucidation of these interactions will undoubtedly confirm the metabolic regulation of osmotolerance.

In this chapter, the occurrence and degree of sequence similarity of the gene *GPD1* in *S. cerevisiae* (S288C) and in production ale and lager brewing yeast strains has been investigated. Furthermore the expression of this gene has been examined in brewing yeast strains to elucidate the relationship between expression and the glycerol accumulation profiles previously observed (chapter 5).

The realisation that glycerol accumulation profiles were aberrant for the production brewing ale and lager strains examined in this thesis (chapter 5), has lead to the suggestion that there is an alternative mechanism of glycerol accumulation related to glycerol production, dissimilation or export. In this chapter, *GPD1* expression and intracellular and extracellular glycerol accumulation has been investigated.

Table 6.1 Genes encoding proteins involved in glycerol production and dissimilation

ORF	*Standard name*	*Length (bp)*	*Function*	*Location*	*Reference*
YIL053W	*GPP1*	816	Isoform 1 of glycerol 3-phosphatase. Induced during hyperosmotic stress.	Cytoplasm, Nucleus	Norbeck *et al.*, 1996
YER062C	*GPP2*	753	Isoform 2 of glycerol 3-phosphatase. Induced during hyperosmotic stress.	Cytoplasm, Nucleus	Norbeck *et al.*, 1996
YDL022W	*GPD1*	1176	Glycerol 3-phosphate dehydrogenase.	Cytoplasm	Albertyn *et al.*, 1994
YOL059W	*GPD2*	1323	Glycerol production via conversion of glyerol-3-phosphate and NAD+ to glycerol phosphate and NADH.	Cytoplasm	Ansell *et al.*, 1997
YHL032C	*GUT1*	2130	Glycerol kinase, converts glycerol to glycerol-3-phosphate.	Cytoplasm	Pavlik *et al.*, 1993
YIL155C	*GUT2*	1950	Glycerol-3-phosphate dehydrogenase	Mitochondria	Ronnow and Kielland-Brandt, 1993
YML070W	*DAK1*	1755	Dihydroxyacetone kinase	Cytoplasm	Norbeck and Blomberg, (1997)
YOR120W	*GCY1*	939	aldo-keto reductase/ putative glycerol, xylitol and arabitol dehydrogenase	Cytoplasm, Nucleus	Norbeck and Blomberg, (1997)
YDR368W	*YPR1*	939	aldo-keto reductase/ putative glycerol dehydrogenase	Cytoplasm, Nucleus	Norbeck and Blomberg, (1997)

6.2 Results

6.2.1 Detection of glycerol biosynthesis genes in haploid and brewing strains

6.2.1.1 PCR primer design

The Stanford university *Saccharomyces* genome database (http://www.yeastgenome.org) is an online resource containing information concerning the molecular biology and genetics of *S. cerevisiae* (Cherry *et al.*, 1998). The full genome sequence is available for inspection and information concerning assigned and putative functions of ORFs is also supplied. Using this resource, the sequences for genes involved in glycerol biosynthesis were retrieved, and specific primers were designed to facilitate PCR detection of these genes in four brewing yeast strains (lager strains SCB1 and SCB2, and ale strains SCB5 and SCB8) (table 6.2). PCR primers were designed with different restriction enzyme sites in order to aid later cloning of PCR products. The Webcutter algorithm (http://www.firstmarket.com/cutter/cut2.html) was utilised to produce a restriction enzyme map for each of the genes. Restriction sites not present within each gene were selected for incorporation into the primers. The laboratory haploid strain S288C was used as a positive control for the PCR detection of glycerol biosynthesis genes, and a gradient of annealing temperatures was used in order to obtain the optimum results.

Table 6.2 Oligonucleotide (a) forward and (b) reverse primers utilised in this study, indicating restriction enzyme sites (in bold typeface).

Gene	*Forward (5'-3') Primer*
GUT1	GCG**GGATCC**ATGTTTCCCTCTCTCTTCCGACTTGTAAG
GUT2	GCG**GGATCC**ATGTTTTCGGTAACGAGAAGAAGAGC
GPD1	GCG**GGATCC**ATGTCTGCTGCTGCTGATAGTTAAACTTAAC
GPD2	GC**GGATCC**ATGCTTGCTGTCAGAAGATTAACAAG
GPP1	GCG**GGATCC**ATGAAACGTTTCAATGTTTTAAAATATATCAG
GPP2	GCG**GGATCC**ATGGGATTGACTACTAAACCCCTATC
DAK1	GCG**AGATCT**ATGTCCGCTAAATCGTTTGAAGTCAC

(a)

Gene	*Reverse (3'-5') Primer*
GUT1	GCG**GAATTC**TTATTGGAAGTTTTCTAGAACCTGTTCGTG
GUT2	GCG**CTCGAG**TTAGACACCAAACGTCTTGATGAAG
GPD1	CCC**GCGGCCGC**CTAATCTCATGTAGATCAATTC
GPD2	GCG**AAGCTT**CTATTCGTCATCGATGTCTAGCTCTTC
GPP1	GCG**TCTAGA**TTACCATTTCAACGCGTCTA
GPP2	GCG**TCTAGA**TTACCATTTCAACAGATCG
DAK1	GCG**GAATTC**TTACAAGGCGCTTTGAACCCCCTTC

(b)

6.2.1.2 PCR detection of genes

Although the genes involved in the biosynthesis and degradation of glycerol have been characterised in *S. cerevisiae* haploid laboratory strains, their presence and sequence in aneuploid and polyploid ale (*S. cerevisiae*) and lager (*S cerevisiae* (syn. *S. pastorianus*) strains have not been previously reported. Genomic DNA was extracted from ale (SCB5, 8), lager (SCB1, 2) and laboratory haploid (S288C) strains according to the methods outlined in section 2.12. Using the primers outlined in table 6.2, the occurrence of genes involved in the biosynthesis and degradation of glycerol were examined.

All genes examined in this study were detected in the lager strains SCB1 and SCB2, the ale strains SCB5 and SCB8 and, unsurprisingly, the laboratory haploid reference strain S288C (table 6.3). Figure 6.1 shows a representative agarose electrophoresis gel with clear bands *circa* 1200 base pairs, as would be expected for *GPD1* (1176 bp). The results of this PCR were particularly clear, as each of the four annealing temperatures utilised yielded a positive result without non-specific binding.

Table 6.3. Genes involved in glycerol biosynthesis screened for in this study using PCR in ale (SCB5, SCB8), lager (SCB1, SCB2) and laboratory haploid (S288C) strains. (Y) indicates that the gene of interest was detected.

	Gene						
	GPP1	*GPP2*	*GPD1*	*GPD2*	*GUT1*	*GUT2*	*DAK1*
Strain	Y	Y	Y	Y	Y	Y	Y
SCB1	Y	Y	Y	Y	Y	Y	Y
SCB2	Y	Y	Y	Y	Y	Y	Y
SCB5	Y	Y	Y	Y	Y	Y	Y
SCB8	Y	Y	Y	Y	Y	Y	Y
S288C	Y	Y	Y	Y	Y	Y	Y

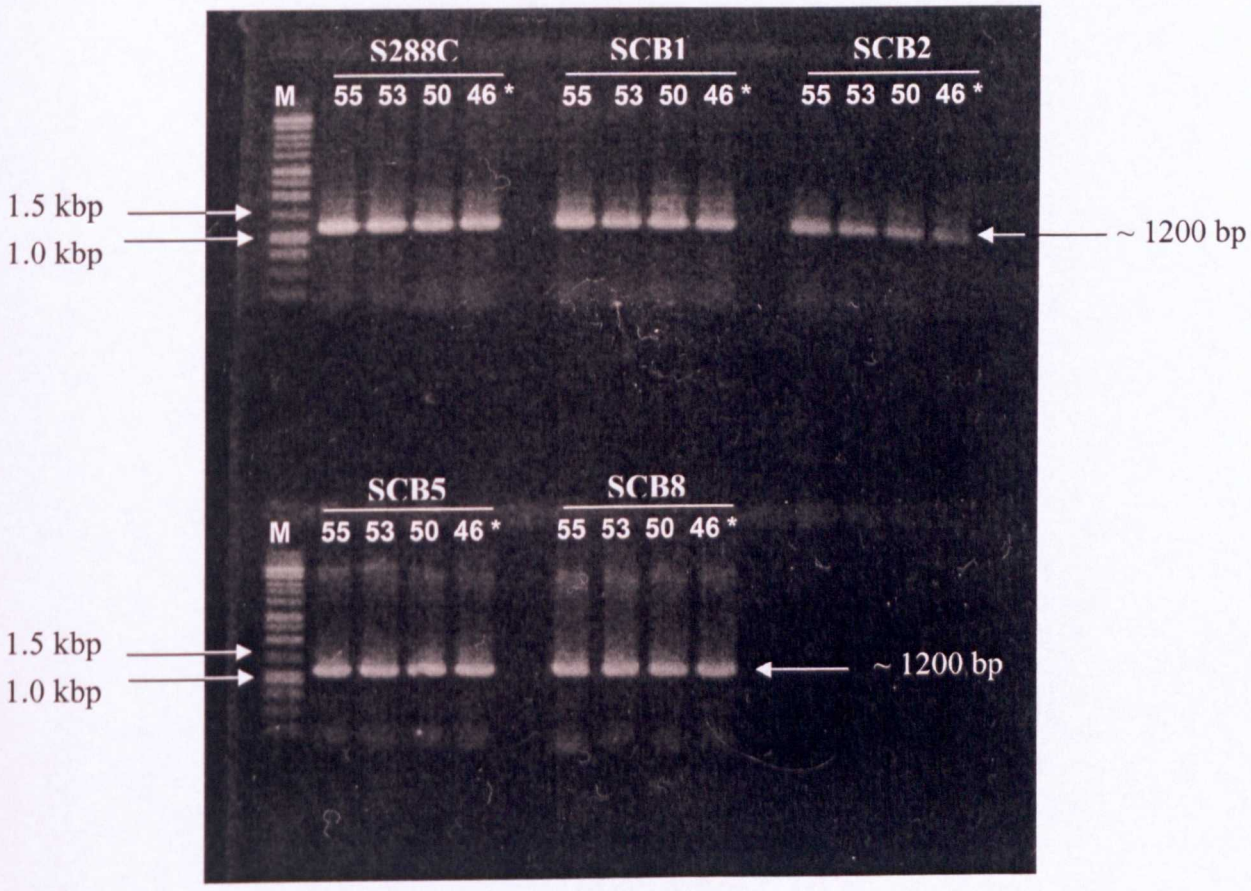


Figure 6.1. 0.7% agarose/TAE gel electrophoresis of PCR products formed using primers specific for *GPD1* (1176 bp) in strains S288C, SCB1, SCB2, SCB5 and SCB8. * denotes °C annealing temperature gradient. DNA was visualised with 0.5 μg/ml ethidium bromide using a standard UV transilluminator.

6.2.2 Cloning of the *GPD1* gene

GPD1 is considered to be the rate-limiting step in glycerol production in *S. cerevisiae* (Albertyn *et al.*, 1994) and is fundamental in the production of glycerol during osmotic stress. However, the organisation of this gene and its transcriptional/translational activity during hyperosmotic stress in brewing yeast strains is currently unknown. It has been demonstrated (in chapter5) that glycerol is produced in an aberrant manner, and consequently it is suggested that this may be mediated in some way by *GPD1*. It is possible that *GPD1* may be modified in brewing yeast strains, which may result in an altered structure and therefore function. In order to determine the nucleotide sequence of *GPD1* from brewing yeast strains it was first necessary to clone this gene in order that further manipulations could be made.

Gene cloning is the process by which a fragment of DNA is inserted into a circular vector molecule to produce a recombinant DNA molecule, identical to the sequence that was originally inserted in the vector. The vector carrying the target gene is transformed into a host cell (usually a bacterium), which undergoes replication, with a resultant amplification of the vector plus inserted gene (Brown, 2001). The purpose of gene cloning in many cases is the further manipulation, analysis and characterisation of a specific gene.

In order to facilitate further analysis of the genes identified by PCR, and their similarity to the published sequences for the laboratory haploid S288C strain, cloning of the genes was undertaken. In order to remove mismatching errors introduced with the use of *Taq* polymerase, PCR reactions were undertaken using *Pfu* turbo™ obtained from Stratagene Inc. U.K. Figure 6.2 demonstrates the amplification of *GPD1* using PCR with *Pfu* turbo™. Using the conditions and reaction protocols previously utilised with *Taq* polymerase, the *Pfu* turbo™ permitted the detection of the *GPD1* gene in ale strain SCB8 and in the lager strain SCB2 (figure 6.2). Amplification of *GPD1* yielded sufficient DNA for cloning purposes. In each case, the forward oligonucleotide primer used to amplify the gene *GPD1* contained a *Bam*HI restriction endonuclease site, and the reverse primer, a *Not*I restriction site (table 6.2).

The PCR products were purified to remove any excess oligonucleotides, deoxynucleotides, enzyme and buffer using a commercially available QIAquick™ PCR purification spin columns (section 2.14). The PCR products were digested with *Not*I

and *BamH*I restriction enzymes. Similarly, the phagemid vector pBlueScript® II SK+ was treated with the same enzymes to produce cohesive ends. The samples were gel-purifed using a QIAquick gel extraction kit following separation on a 0.7% agarose gel to remove impurities as shown in figure 6.3.

Ligations were performed using appropriate molar ratios of insert-to-vector DNA, according to the instructions of the manufacturer (section 2.16.9), in the presence of T4 DNA ligase and a commercially supplied buffer at 16°C overnight (section 2.16.9). Ligations were transformed into XL10-Gold® ultracompetent *E. coli* cells, including the appropriate controls (section 2.16.4). Cells were grown on ampicillin-containing L-broth agar medium and 20 isolated colonies were removed and grown as mini-preps. Plasmid DNA was subsequently extracted from mini-preparations, and screened for the insertion of the *GPD1* gene using PCR (figures 6.4/6.5) and double restriction digest analyses (figures 6.6). *GPD1*-positive transformants derived from SCB2 and SCB8 were subsequently selected for large scale preparation and sequencing.

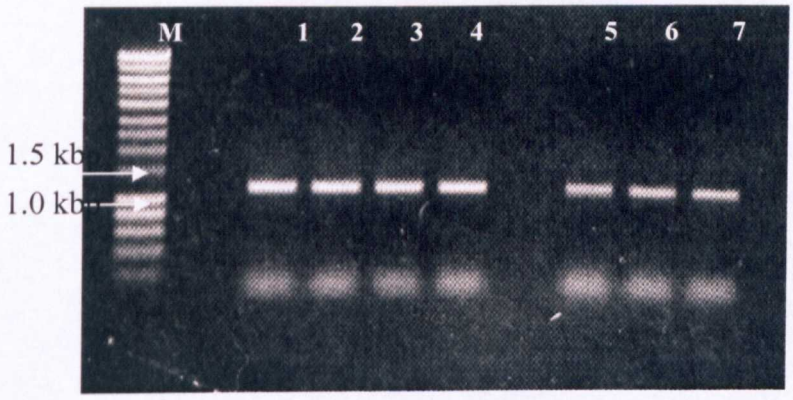


Figure 6.2 0.7% agarose/TAE gel electrophoresis of PCR products formed with primers specific for *GPD1* (1176 bp) using *Pfu* Turbo ™ DNA polymerase in strains SCB2 (1-4) and SCB8 (5-7). DNA was visualised with 0.5 μg/ml ethidium bromide using standard UV transilluminator.

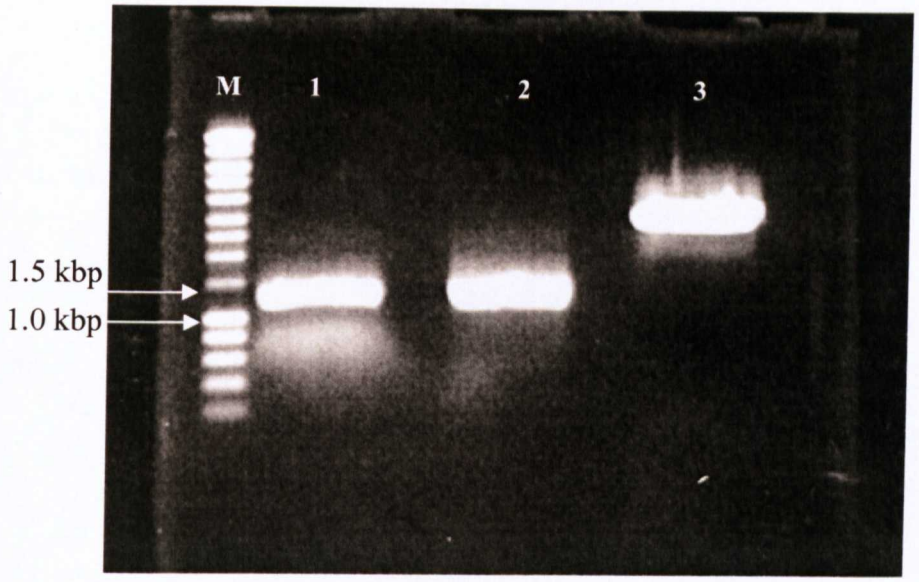


Figure 6.3 0.7% agarose/TAE gel electrophoresis of PCR products formed using primers specific for *GPD1* (1176 bp) using *Pfu* Turbo ™ following *BamH*I/*Not*I digests in strains SCB2(1) and SCB8(3) for cloning purposes 53°C annealing temperature. Lane 3 contains *BamH*I/*Not*I digested pBlueScript® II SK+. DNA was visualised with 0.5 μg/ml ethidium bromide on a standard UV transilluminator.

Figure 6.4 Positive clone screening for SCB2 transformants (1-10). Positive clone DNA isolated using small-scale preparations (mini-preps) and subsequently used in a PCR reaction with specific primers for *GPD1*. Lower band represents *GPD1* PCR product, upper bands represent plasmid carry-over (supercoiled and relaxed). Control (C) PCR performed using S288C genomic DNA. DNA was visualised with 0.5 μg/ml ethidium bromide on a standard UV transilluminator.

Figure 6.5 Positive clone screening for SCB2 transformants (1-10). Positive clone DNA isolated using small-scale preparations (mini-preps) and subsequently used in a PCR reaction with specific primers for *GPD1*. Lower band represents *GPD1* PCR product, upper bands represent plasmid background (supercoiled and relaxed). Control PCR performed using S288C genomic DNA. DNA was visualised with 0.5 μg/ml ethidium bromide on a standard UV transilluminator.

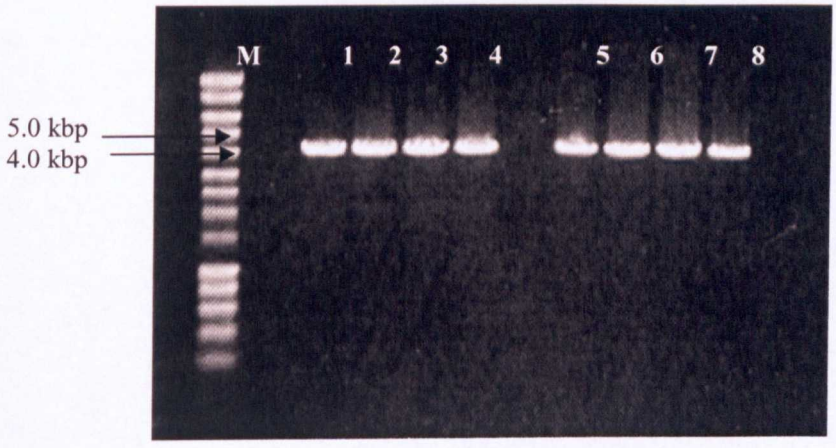


Figure 6.6 Positive clone screening for SCB2 (1-4) and SCB8 (5-8) transformants digested using *Bam*HI. DNA was visualised by staining with 0.5 μg/ml ethidium bromide on a standard UV transilluminator.

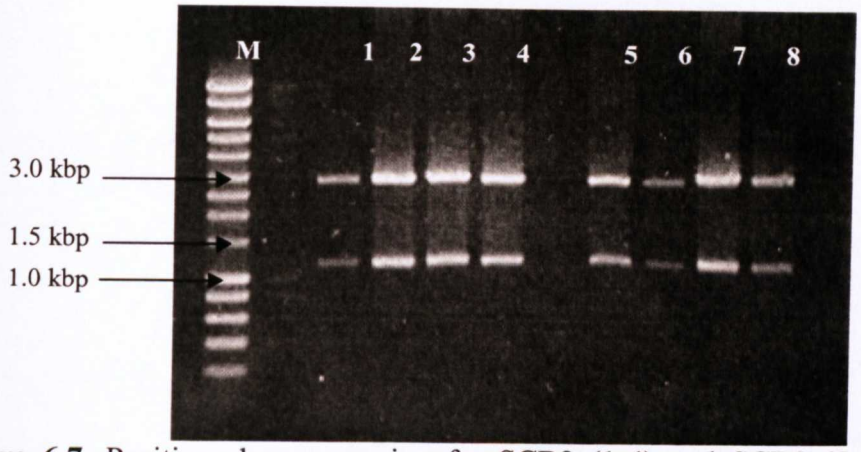


Figure 6.7. Positive clone screening for SCB2 (1-4) and SCB8 (5-8) transformants digested using *Bam*HI and *Not*I. DNA was visualised with 0.5 μg/ml ethidium bromide on a standard UV transilluminator.

The results presented here show that the *GPD1* gene isolated by high-fidelity PCR using *Pfu* Turbo ™ was directionally cloned into the polylinker region of pBlueScript® II SK+. PCR detection of the putatitive positive clones isolated from ampicillin plates revealed that there was good evidence for the incorporation of the *GPD1* gene, however high plasmid DNA carryover served to complicate the analysis of the results (figures 6.4 and 6.5). Subsequent single restriction enzyme digest treatment (*Bam*HI) of purified plasmid DNA revealed a band at *circa* 4Kb which corresponds to the combined length of the vector (3.0 kbp) and the *GPD1* insert (1.17 kbp). A further restriction enzyme (*Not*I) treatment of the *Bam*HI digested plasmid separated the two fragments as demonstrated in figure 6.7.

6.2.3 Sequencing and sequence alignment of *GPD1*

A thorough search of the literature suggested that no reports included the sequence of the *GPD1* gene from polyploid brewing ale and lager yeast strains. Furthermore, the predicted structure of Gpd1p from its nucleotide sequence may suggest an altered function, which may explain the atypical intracellular glycerol profiles detailed in chapter 5.

The vector pBlueScript® II SK+, containing *GPD1* genes from SCB2 and SCB8 was sequenced across the multiple cloning site (containing the insert) using oligonucleotide primers for the T7 and T3 promoters and the ABI Big Dye version 3.0.

The sequencing reactions and capillary sequencing run was performed commercially at the sequencing unit of the Department of Biochemstry, University of Oxford.

The results obtained suggested that there were three point mutations in the sequence of *GPD1* from SCB2 at bases 722 (guanine to cytosine) 867 (adenine to thymine) and 921 (thymine to adenine). These three point mutations were demonstrated to be genuine, as the same result was returned by subsequent rounds of sequencing from two further independently cloned isolates. In terms of the primary structure of the Gpd1p protein, only two out of three base changes were sufficient to alter the encoded amino acids. At base 722 the TGT codon standing for cysteine was replaced with TCT encoding a serine, and at position 869 the GAA codon for glutamic acid was replaced with aspartic acid (GAT) (figure 6.6). Neither the point mutations of the nucleotide sequence or the resultant change in protein primary structure is sufficient to effect either a change in predicted cellular localization or function according to the web-based protein identification and characterization programs. In contrast, no mutations were evident in the sequence of SCB8 *GPD1*.

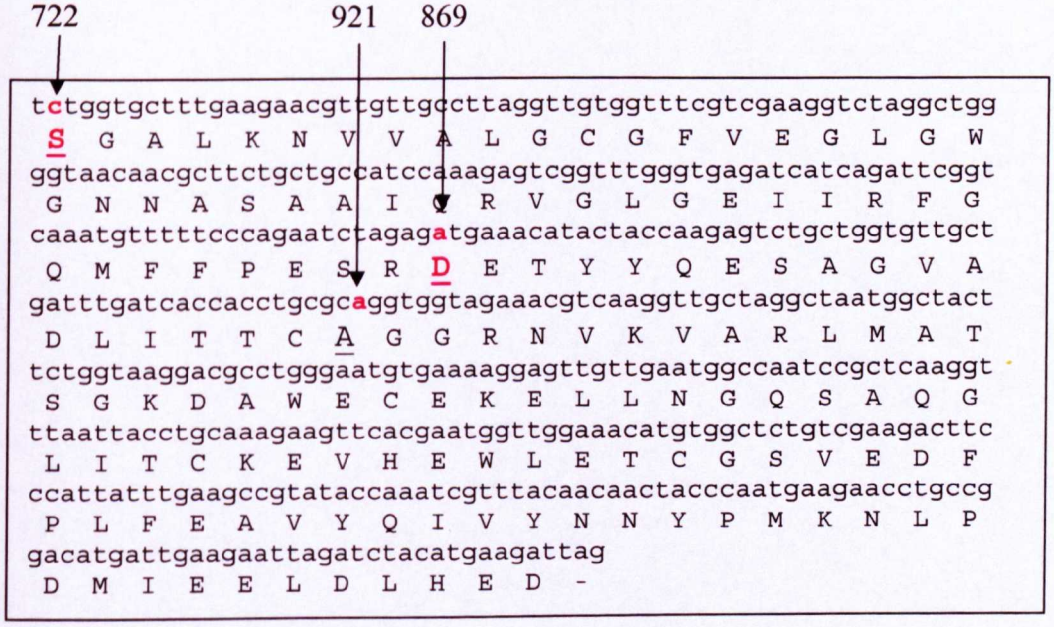


Figure 6.6. Sequence of SCB2 GPD1 translated into amino acid sequence showing point mutations at bases 722, 869 and 921.

An alignment of the *GPD1* genes from both the strains examined in this study and related species of yeast yielded an interesting phylogeny. Figure 6.7 demonstrates the hierarchical organisation of six species of yeast, for which the sequence of the *GPD1*

gene is known, along with the data for the two polyploid brewing strains examined in this study. Unsurprisingly, *S. cerevisiae* and the ale strain SCB8 were grouped together as the sequence of their *GPD1* genes were identical.

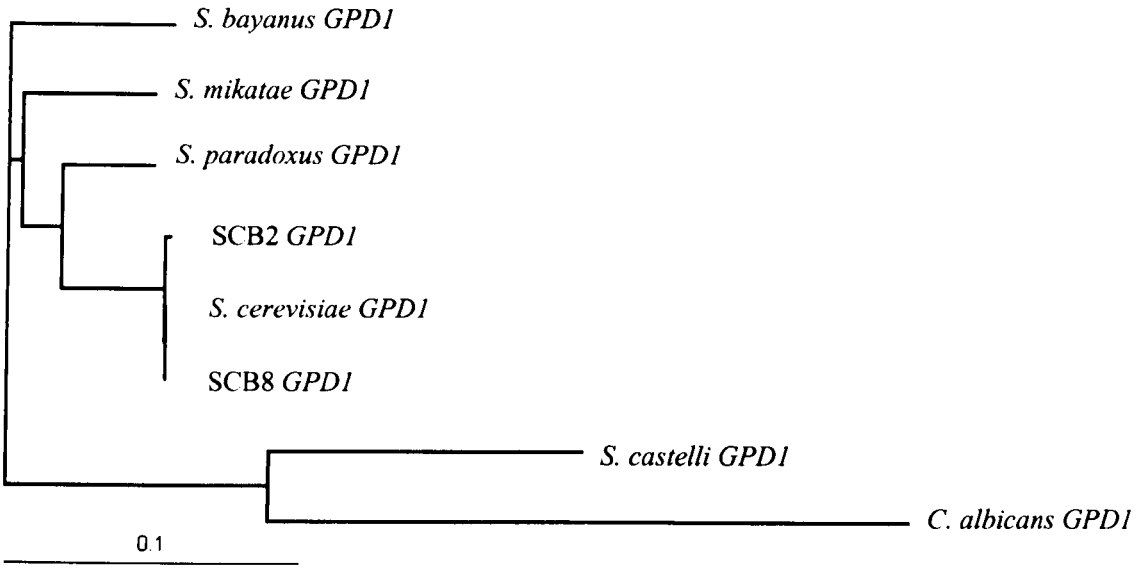


Figure 6.7. A phylogenetic tree demonstrating the relationships between the *GPD1* gene from 6 yeast species, one ale (SCB8) and one lager (SCB2) brewing yeast strain.

6.2.4 Quantitative RT-PCR analysis of *GPD1* expression.

The analysis of the *GPD1* gene derived from strains SCB2 and SCB8 demonstrated that there were no abnormalities in the predicted amino acid sequences, indicating no obvious reasons for a difference in function/localisation of the resulting protein. The aberrant glycerol profiles exhibited by the strains examined could therefore assumed to have occurred as a consequence of an alteration in the activity or abundance of glycerol-3-phosphate dehydrogenase. A viable marker for the production of Gpd1p is the level of mRNA transcripts coding for that protein, which should correspond to glycerol-3-phospate dehydrogenase production. Total RNA was extracted from osmotically-stressed cells, using the RNeasy kit (Qiagen) as described in section 2.20, and examined for purity by formaldehyde gel electrophoresis (section 2.20.5) (figure 6.8).

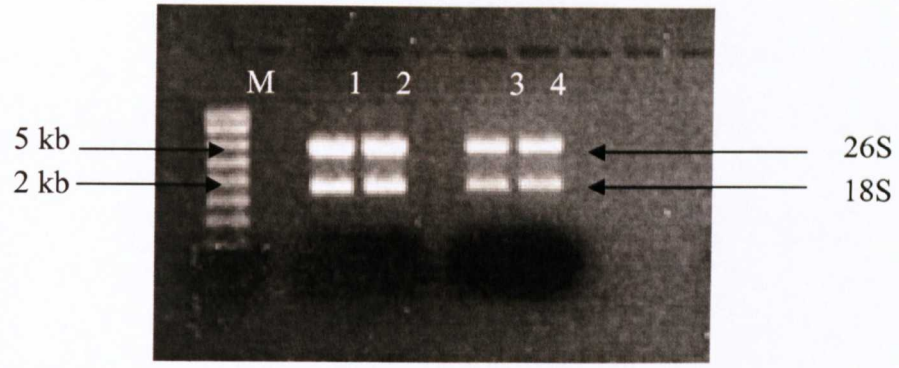


Figure 6.8. Denaturing (formaldehyde) gel electrophoresis of RNA extracted from brewing yeast cells. RNA was extracted from stationary phase SCB2 (lager) (1) and SCB8 (ale) (3), and exponential phase SCB2 (2) and SCB8 (4) yeast. The two ribosomal RNA subunits (26S (3.8 kb) and 18S (2kb)) were clearly visible as tight bands indicating that the RNA had not been degraded during extraction.

Complementary DNA (cDNA) to the extracted RNA was generated using reverse transcriptase with oligo (dT) primers (section 2.20.4), which were used to amplify only the polyadenylated mRNA strands. Using specific Taqman® FRET probes (section 2.13.4.1), the amount of cDNA present in the sample could be quantified against a carefully calibrated standard curve (R^2 of no less than 0.95). The amount of cDNA present, therefore, represents a correlative measure of the amount of specific mRNA transcripts of the *GPD1* gene and, hence, its transcriptional activity. It was observed that mRNA levels in osmotically stressed SCB2 (lager) cells demonstrated a similar pattern of abundance compared to the intracellular glycerol levels exhibited in chapter 5 (figure 5.3). A clear peak of *GPD1* mRNA was observed at 18% (w/v) sorbitol-induced stress, with a subsequent decline to levels associated with 0, 6, and 12% (w/v) sorbitol stress, when 24% (w/v) sorbitol challenge was applied. A slight elevation in *GPD1* transcriptional activity was demonstrated at 30% (w/v) sorbitol, but the expression levels were approximately half of that observed at 18% (w/v) sorbitol (figure 6.9).

The *GPD1* expression profile for osmotically stressed SCB8 (ale) brewing yeast (figure 6.10) yielded similar data to that observed for SCB2 (lager) cells (figure 6.9). However, SCB8 cell populations demonstrated a clear peak in osmotically-induced *GPD1* expression at a somewhat lower percentage of sorbitol (12% w/v). Furthermore, subsequent levels of *GPD1* mRNA were almost half those observed before this peak (figure 6.10).

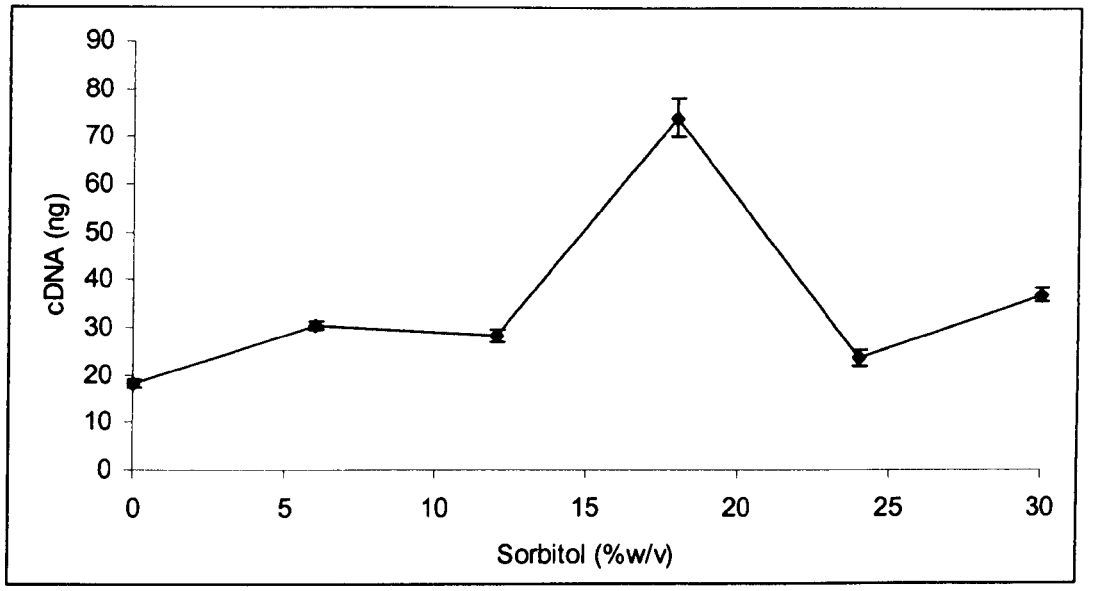


Figure 6.9. *GPD1* expression in SCB2 cells as determined by quantitative RT-PCR (qRT-PCR). Exponential phase cells were exposed to sorbitol concentrations for 48 hours at 25°C and the total RNA extracted. cDNA was generated from mRNA transcripts and *GPD1* levels assayed using quantitative PCR.

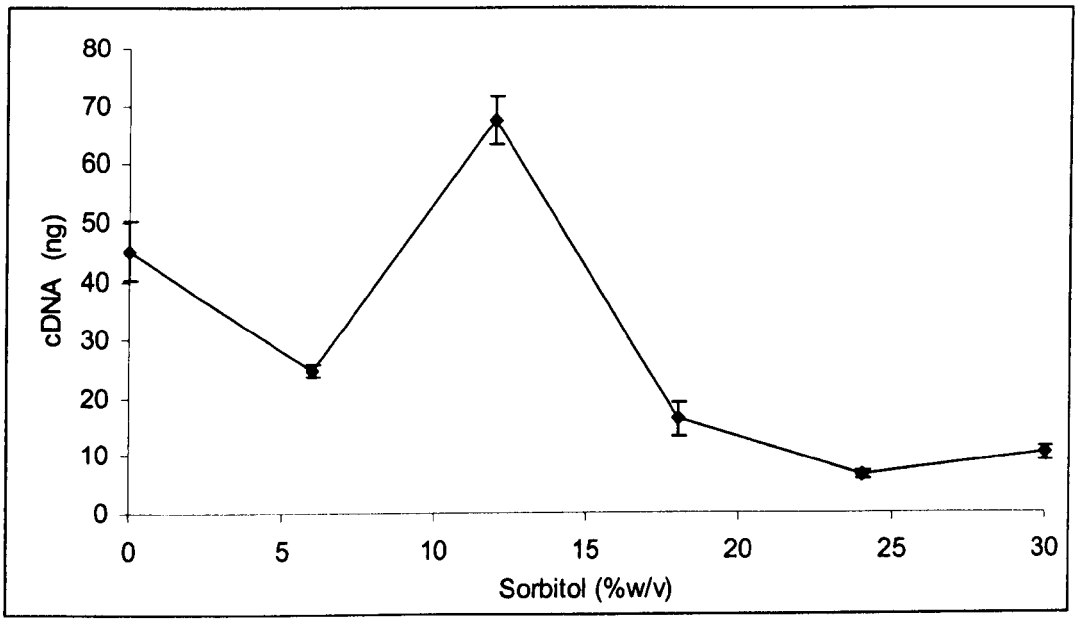


Figure 6.10 *GPD1* expression in SCB8 cells as determined by quantitative RT-PCR (qRT-PCR). Exponential phase cells were exposed to sorbitol concentrations for 48 hours at 25°C and the total RNA extracted. cDNA was generated from mRNA transcripts and *GPD1* levels assayed using quantitative PCR.

6.2.5 Western blot analysis of Gpd1p expression

The response of brewing ale (SCB8) and lager (SCB2) strains to sorbitol induced osmotic stress appeared to involve the expression of *GPD1* in a solute concentration-dependent manner (section 6.2.4). However, it has been previously established that transcription does not necessarily ensure translation of the protein, as previously described for ribosomal proteins in *Xenopus laevis* embryos (Pierandrei-Amaldi, 1985), ribulose-1,5-bisphosphate carboxylase in soybean (*Glycine max*) (Shirley and Meagher, 1990) and a wide array of proteins in yeast (Ghaemmaghami *et al.*, 2003).

The final consideration involved the existence of feedback loops, which may result in protein degradation introducing disparities between the level of transcription and the amount of protein observed. In order to establish the relationship between sorbitol-induced osmotic stress and production of Gpd1p, a study of protein expression was conducted in an attempt to correlate gene expression with protein production.

Initially, protein extraction methods were optimized in order that sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) could be performed (section 2.21.2). Protein extraction was performed using the protocol of Horvath and Riezmann (1994) with the exception that glass beads and sonication were used to disrupt the cell wall/membrane to liberate protein (section 2.21). The resultant extracts were resolved on a polyacrylamide gel and stained with Coomassie brilliant blue dye (section 2.21). Figure 6.11 demonstrates the consistency of this extraction method on independent duplicate samples of brewing lager (SCB2), ale (SCB8) and laboratory haploid (S288C) yeast cells.

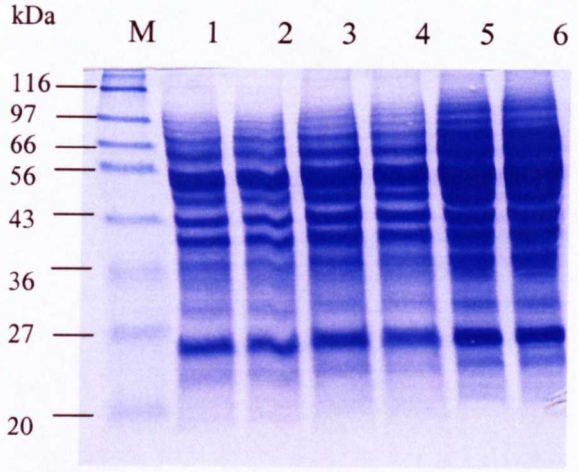
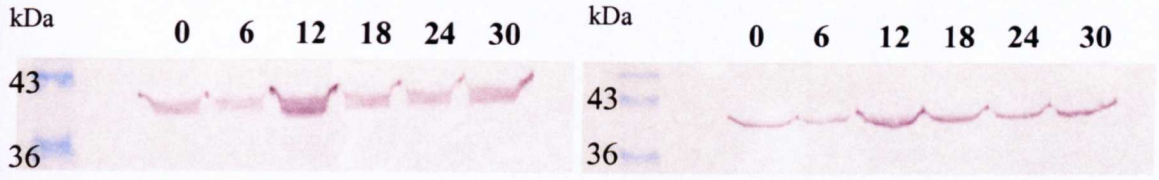


Figure 6.11. SDS-PAGE of yeast proteins extracted from stationary phase lager (SCB2) (1,2) ale (SCB8) (3,4) and laboratory haploid (S288C) (5,6) cells. Protein was extracted from 2×10^7 cells, and approximately 20μg of total protein per lane was analysed on 12% acrylamide gels. The proteins were stained using Coomassie brilliant blue.

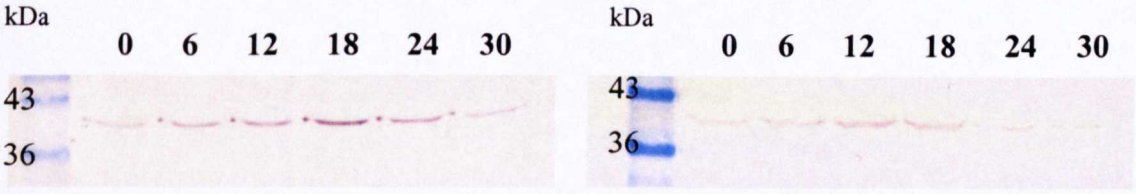
The optimisation of protein extraction from brewing yeast strains facilitated the further analysis of protein expression by Western blot analysis. Exponential phase brewing yeast cells were subjected to varying concentrations of sorbitol for 48 hours and total protein was extracted, as described above. In order to avoid artefacts associated with inconsistent loading of different protein concentrations, all extracts were assayed for protein concentration using the Bradford assay (section 2.21.1), and diluted accordingly to standardise the loading of protein. The SDS-PAGE separated proteins were transferred onto a nitrocellulose membrane (section 2.21.4) and subsequently probed for Gpd1p proteins using an anti-Gpdp1p antibody (section 2.21.5). Figures 6.12 and 6.13 demonstrate the profile of Gpd1p protein (42.8 kDa) production for the strains SCB2 (lager) and SCB8 (ale). In both strains examined, a variation in the amount of Gpd1p detected was observed and the levels did not correlate with the degree of osmotic stress incurred. Interestingly, the pattern of protein expression observed concurred with the mRNA expression profile previously demonstrated (section 6.2.4). Indeed, the lager strain SCB2 demonstrated a clear upregulation of protein production at 12% and 30% (w/v) sorbitol concentrations with an increasing gradient of expression of protein from 0% (distilled water) to a peak at12% sorbitol (figure 6.12). A decrease in Gpd1p levels was observed between 12% and 18% (w/v) followed by an increase in between 18% and 30% (w/v) sorbitol.



(a) (b)

Figure 6.12. Western-blot analysis of Gpd1p production in sorbitol-stressed SCB2 (lager) yeast. Exponential phase cells were exposed to sorbitol (indicated as 0,6,12,18,24,30% w/v) concentrations for 48 hours at 25°C, samples (a) and (b) represent independent duplicate analyses.

For the ale strain SCB8 a fluctuation in Gpd1p levels also occurred (figure 6.13) with a gradual increase in the Gpd1p with sorbitol concentration until 18% (w/v). At higher concentrations the amount of Gpd1p detected decreased.



(a) (b)

Figure 6.13. Western-blot analysis of Gpd1p production in SCB8 (ale) yeast. Exponential phase cells were exposed to sorbitol (indicated as 0,6,12,18,24,30% w/v) concentrations for 48 hours at 25°C, samples (a) and (b) represent independent duplicate analyses.

6.2.6 Glycerol export

The data presented in this chapter thus far demonstrates that unique intracellular glycerol profiles previously observed (section 5.2.2) may be due to the expression of *GPD1* (section 6.2.4) and synthesis of glycerol-3-phosphate dehydrogenase (section 6.2.5).

Glycerol loss to the environment has been demonstrated to be efficiently regulated in *S. cerevisiae* via the transmembrane glycerol export channel (glyceroaquaporin) *FPS1* (Luyten *et al.*, 1994; Luyten *et al.*, 1995; Sutherland *et al.*, 1997; Tamàs *et al.*, 1999). It has been shown that Fps1p is able to mediate the accumulation of intracellular glycerol during osmotic stress by restricting glycerol export (Tamàs *et al.*, 1999; Oliveira *et al.*, 2003), a surprising finding as the yeast

membrane and cell wall barrier had previously been thought to be relatively permeable to glycerol (Biondi *et al.*, 1991; Andre, 1995).

In order to determine whether glycerol efflux contributes to the unusual glycerol accumulation profiles demonstrated by brewing yeast populations, cohorts of cells of SCB2 and SCB8 utilised in the analysis of *GPD1* and Gpd1p expression were concurrently assayed for intracellular and extracellular glycerol levels (section 2.7).

At higher concentrations of sorbitol (above 20% w/v), glycerol appeared to be lost from SCB2 cells and could be readily detected in the surrounding medium (figure 6.14) At lower concentrations of exogenous sorbitol (below 20% w/v), glycerol was also detected in the extracellular medium (figure 6.14). Furthermore, the extracellular levels exceeded intracellular levels.

In the ale strain SCB8 extracellular glycerol was detected at similar levels to those observed for intracellular levels with increasing sorbitol concentrations up to 18% (w/v) (figure 6.15). The concentration of extracellular glycerol continued to increase up to 30% (w/v), however, intracellular levels were observed to reduce and then plateau (figure 6.15).

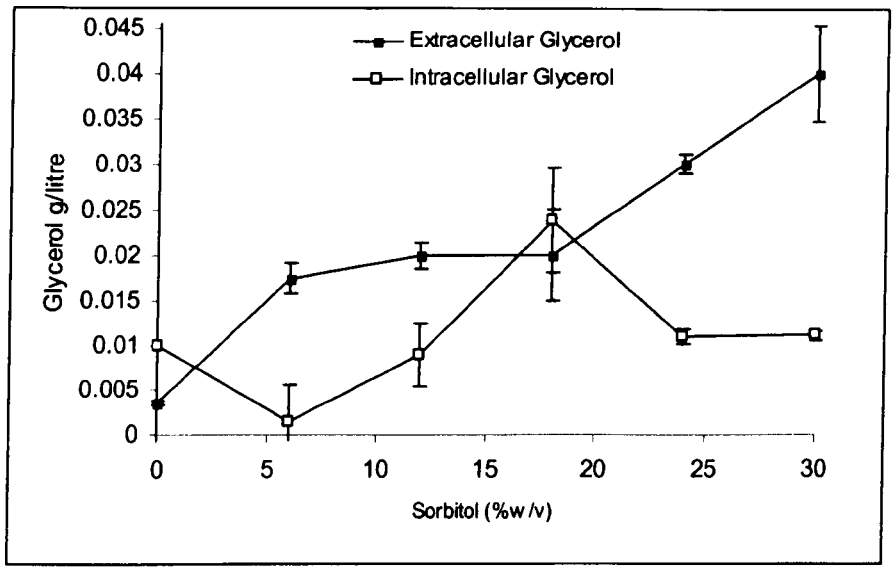


Figure 6.14. Intracellular and extracellular glycerol profiles for osmotically-stressed exponentially grown lager (SCB2) cell populations. Populations were exposed to sorbitol or distilled water (0%) for 48 hours at 25°C. Triplicate samples were assayed for intracellular glycerol content; error bars represent the standard deviation of a normal distribution.

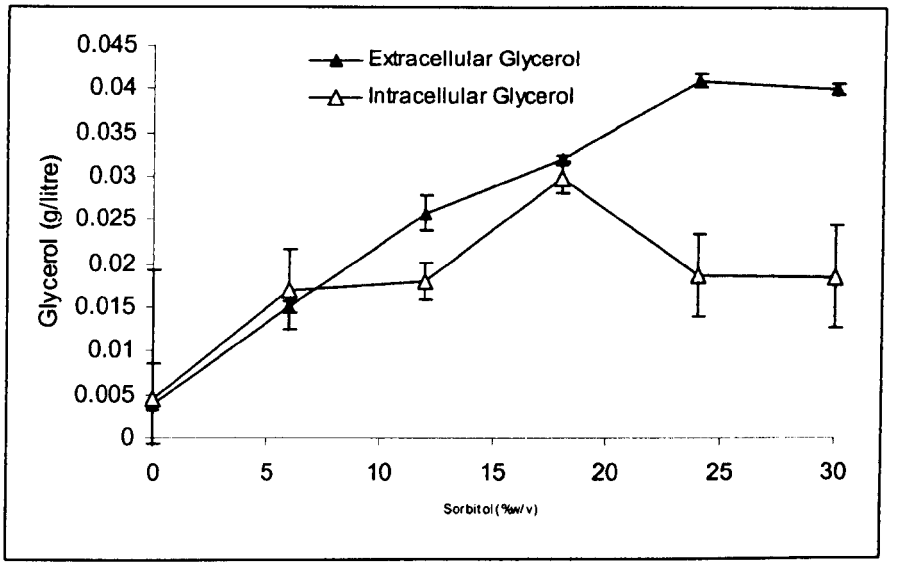


Figure 6.15. Intracellular and extracellular glycerol profiles for osmotically stressed exponentially grown ale (SCB8) cell populations. Populations were exposed to sorbitol or distilled water (0%) for 48 hours at 25°C. Triplicate samples were assayed for intracellular glycerol content; error bars represent the standard deviation of a normal distribution.

6.3 Discussion

The production and catabolism of glycerol via the glycolytic intermediate dihydroxyacetone phosphate is well documented in laboratory haploid strains (Pavlik *et al.*, 1993; Ronnow and Kielland-Brandt, 1993; Albertyn *et al.*, 1994; Norbeck and Blomberg, 1997; Pahlman *et al.*, 2001; Remize *et al.*, 2001). Despite this, no evidence concerning the conservation of this mechanism in production brewing yeast has been reported.

The genes involved in glycerol biosynthesis and degradation have not been previously identified in brewing yeast strains. Furthermore, the homology of these gene sequences in diploid, polyploid and aneuploid strains of *S. cerevisiae* has not been previously reported.

In this chapter, the genes involved in the production of key enzymes in the yeast glycerol biosynthesis pathway were screened for in brewing ale and lager yeast strains using the polymerase chain reaction (PCR). Oligonucleotide primers were designed from the known sequences of the genes *GPD1* and *GPD2* (encoding isoforms of glycerol-3-phosphate dehydrogenase), *GPP1* and *GPP2* (encoding isoforms of glycerol-3-phosphate phosphatase), *GUT1* (encoding glycerol kinase) *GUT2* (encoding glycerol-3-phosphate dehydrogenase) and *DAK1* (encoding dihydroxyacetone kinase) present in

the haploid *S. cerevisiae* strain S288C. This approach for detection had an associated risk in that it was assumed that a high degree of similarity existed at the nucleotide level between S288C; indeed there was an associated risk that genes would not be amplified due to polymorphic changes or gene truncations.

It was demonstrated that all of the genes screened for in brewing yeast strains were indeed present, indicating that the observation that glycerol is accumulated intracellularly in an aberrant manner in brewing yeast (chapter 5) is not due to the lack of a key gene. Furthermore, initial observations of PCR generated products for all of the genes examined in this study approximated in length to those seen in the haploid type strain.

The best studied gene in the osmotic stress response of *S. cerevisiae* is *GPD1*, which has been shown to be highly upregulated during osmotic stress (Larsson *et al.*, 1993; Albertyn *et al.*, 1994; Ansell *et al.*, 1997; Rep *et al.*, 1999). Furthermore, glycerol production is dependent upon correct function of *GPD1*, which has been described as the limiting step in glycerol biosynthesis. Cells lacking this enzyme produce little glycerol and are extremely osmosensitive (Albertyn *et al.*, 1994). The function of the glycerol-3-phosphate dehydrogenase isoenzyme encoded by *GPD2* is somewhat different, and is only highly expressed under anaerobic conditions (Ansell *et al.*, 1997), suggesting an important role in redox balance. *GPP1* and *GPP2* expression is also highly upregulated during osmotic stress, although the single gpp1Δ and gpp2Δ mutant strains do not demonstrate a reduction in viability or glycerol production during osmotic stress (Pahlman *et al.*, 2001). In addition, the double gpp1Δ gpp2Δ mutant is extremely reduced in its ability to produce glycerol demonstrating the redundancy (= functionally active paralogues) of this pair of genes (Pahlman *et al.*, 2001).

In contrast, the abolition of *GPD1* function is responsible for a loss in glycerol production, as it is the only point of glycerol biosynthesis that cannot be circumvented by an alternative route or isoenzyme (Larsson *et al.*, 1993).

In order to determine the functional homologies of *GPD1* and Gpd1p derived from S288C and brewing strains, the nucleotide sequence and expression profiles of this gene and corresponding protein were examined in SCB2 (lager) and SCB8 (ale) brewing strains.

The nucleotide sequence of the cloned *GPD1* derived from SCB8 (ale) cells revealed no deviations from that of the type strain S288C, a somewhat unsurprising

result given that ale strains have been identified as the most closely related of the industrial strains to *S. cerevisiae* (section 1.3.1). The lager strain, SCB2, revealed 3 base pair changes in the sequence of its *GPD1* gene when compared to the S288C haploid strain. These point changes were consistently exhibited by 3 independently cloned *GPD1* sequences derived from SCB2. This modification in sequence was not unexpected given that ale (*S. cerevisiae* (syn *S. pastorianus*)) strains have been suggested to derive from a hybrid of *S. cerevisiae* and *S. bayanus*. Unfortunately, the similarity of SCB2 *GPD1* to that of a *S. pastorianus* strain could not be examined.

The base changes at 722 base pairs in SCB2 were sufficient to affect an amino acid transposition (with cysteine being replaced by serine). Similarly, the base change at 869 bp altered the codon from glutamic acid to aspartic acid. Neither of these changes in the primary sequence of SCB2 Gpd1p was sufficient to alter either the predicted location (cytoplasm) or function, as determined using online protein analysis algorithms (http://www.expasy.org).

The fact that the two strains examined in this study were observed to possess full length and potentially functionally active *GPD1* genes did not explain the aberrant glycerol accumulation profiles observed; leading to the supposition that glycerol accumulation during osmotic stress was due to either the expression of these genes, activity of the enzyme produced or the export of the glycerol synthesised. To test the supposition that glycerol production is responsible for the aberrant intracellular glycerol levels demonstrated in chapter 5, expressional analyses of *GPD1* were conducted on the strains SCB2 and SCB8.

In haploid *S. cerevisiae* cells, *GPD1* expression is controlled by the osmotically induced activation of the HOG pathway. This signal transduction pathway is thought to be activated via one of two partially redundant transmembrane sensing proteins (Sln1p and Sho1p) (Maeda *et al.*, 1994; Maeda *et al.*, 1995) (section 1.5.6), which appear to effect a phosphorylation of the first MAP kinase protein, which is mediated by a change in cell turgor (Tamas *et al.*, 2000). The activation of the HOG-MAP kinase cascade has several outcomes but the most important in this context is the activation of the transcription factors Hot1p and Msn1p that are translocated to the nucleus and subsequently direct the expression of the *GPD1* (and *GPP1*) gene (Rep *et al.*, 1999).

In this study, it was observed that there were indeed different expression patterns of *GPD1*. The abundance of mRNA transcripts, as determined by quantitative RT-PCR,

altered according to extracellular sorbitol concentrations in a manner not dissimilar to the intracellular glycerol accumulation profiles previously observed. In addition, a direct (logarithmic) relationship between *GPD1* expression and extracellular osmolarity could not be identified. This novel mRNA abundance profile suggests a different regulation not governed by solute concentration, however the mechanism by which this functions is not known. It is suggested that the majority of the *GPD1* expression profile could be attributable to HOG activation (Maeda *et al.*, 1994; Maeda *et al.*, 1995; Posas and Saito, 1997; Gustin *et al.*, 1998; O'Rourke and Herskowitz, 1998; Posas *et al.*, 1998) (section 1.5.6), however, there may also be feedback control, which accounts for a decrease in expression at higher intracellular concentrations when the required intracellular osmolarity is reached (Hohmann, 2002).

Although *GPD1* expression profiling indicates the level of enzyme production, there are contributing factors which can affect the amounts of final functional protein produced. In any study of gene expression it is advisable to determine if the abundance of RNA corresponds to the levels of final protein production. Furthermore, disparities in the RNA/protein profiles may be due to post-transcriptional degradation of RNA (Pierandrei-Amaldi, 1985; Shirley and Meagher, 1990; Maquat; 2002) post-translational modification problems with protein (misfolding) and protein recycling. In this chapter the production of Gpd1p was examined by western blot analysis and revealed that Gpd1p levels approximately correlated to the transcriptional profiles obtained. The correlation of the protein and RNA profiles demonstrated that there were no fundamental degradative processes involved in *GPD1* expression, and that the parameters of RNA/protein and intracellular glycerol were related.

It has been demonstrated that although a major part of intracellular glycerol accumulation is mediated by an increase in production of glycerol catalysed by Gpd1p, an alternative mechanism of glycerol retention also occurs. Although the yeast cell membrane/cell wall barrier allows passive diffusion of glycerol out of the cell, this has been demonstrated to be remarkably low (Tamàs *et al.*, 1999; Oliveira *et al.*, 2003). Furthermore, glycerol export has been demonstrated to be mediated by the glyceroaquaporin channel, Fps1p. During hypoosmotic conditions, Fps1p is essentially 'open' and permits high rates of glycerol efflux which serves to balance water potentials (Luyten *et al.*, 1995; Tamas *et al.*, 1999). In addition hyperosmotic stress results in the gated closure of the Fps1p channel ensuring that glycerol is retained and this together

with an increase in *GPD1* expression should result in an overall increase in intracellular glycerol levels.

Glycerol export in osmotically stressed SCB2 and SCB8 cells was observed to be somewhat higher than one would expect, and it appears that when osmotically stressed these cells lose glycerol to the extracellular medium; a phenomenon not previously reported for any yeast strains. It is suggested that the aberrant profiles of intracellular glycerol obtained for osmotically stressed ale and lager brewing yeast cells is due partially to glycerol efflux, which subsequently mediates a HOG dependent expression of *GPD1* in order to counteract this glycerol loss.

Chapter 7

Conclusions

Chapter 7 Conclusions

7.1 Conclusions

In this study the impact of osmotic stress on brewing yeast viability, vacuolar morphology and compatible solute accumulation has been investigated in polyploid/aneuploid strains of *Saccharomyces cerevisiae* (ale) and *Saccharomyces cerevisiae* (syn. S. *pastorianus*) (lager) yeast. Osmotolerance, which may be defined as the capacity of a yeast cell population to retain viability during osmotic stress, was strain dependent with no statistically significant differences between ale and lager populations evident. Thus the mechanism of osmotolerance appears to be conserved for *Saccharomyces* species but subject to slight differences in genetic regulation dependent on strain genotype. Osmotolerance was also solute dependent reflecting independent mechanisms of osmotic defence for sorbitol and NaCl induced osmotic stress. Finally, osmotolerance was growth phase dependent with stationary phase populations exhibiting higher tolerance to stress than corresponding exponential phase cells (Werner-Washburne *et al*, 1993; 1998; Gasch and Werner-Washburne, 2002). It is postulated that this was probably due to the up-regulation of global stress defences and the more general changes that occur in cellular metabolism and physiology exhibited following the completion of the transition from post-diauxic shift to stationary phase (Werner-Washburne *et al*, 1993; 1998; Gasch and Werner-Washburne, 2002).

Exposure to osmotic stress appears to induce genetic, physiological and morphological changes in *Saccharomyces* species including modifications in cell wall and plasma membrane ultrastructure and functionality, modifications in vacuolar size and appearance, changes in cell shape and size and the accumulation of compatible solutes. Some of these biomarkers including changes to wall and plasma membrane functionality occur during exposure to other stresses typically encountered during the brewing process and may therefore represent global biomarkers for stress. Others such as changes in cell size and shape may occur as a result of shear stress and hydrostatic pressure to which yeast cells are exposed during handling or fermentation. In addition, cell size and shape may inherently change in response to individual replicative cell age (Barker and Smart, 1996) and as a consequence may not be indicative of exposure to stress at all.

The potential of the yeast vacuole to serve as a biomarker of osmotic stress was investigated in this study on the premise that changes in the morphology of this organelle would only represent an osmotic stress biomarker if no other physiological states and stresses yielded the same response. Confocal microscopical analysis of a large number of samples revealed that the vacuole in *S. cerevisiae* and *S.cerevisiae* (syn *S. pastorianus*) brewing yeast strains was consistently fragmented with very few entire vacuoles recorded.

Fragmentation was observed irrespective of growth phase and exposure to relative levels of osmotic stress. In agreement with the observations of Condradt *et al.*, (1992) vacuolar fragmentation was observed during mitotic growth providing a mechanism for vacuolar inheritance from mother to daughter cells. It is concluded that environments promoting replication would also promote vacuolar fragmentation irrespective of the relative osmolarity of the surrounding medium. As a consequence, vacuolar fragmentation in brewing yeast strains may occur as a result of replication and/or osmotic stress at the beginning of fermentation and therefore would not represent a sufficiently specific biomarker for hyperosmotic exposure.

Another candidate biomarker for this stress was postulated to involve the accumulation of compatible solutes. These solutes are a group of molecules or ions which can be accumulated intracellularly without deleterious effects upon cellular physiology and metabolism. In a number of organisms, including plants, the imino acid proline is readily accumulated during osmotic stress, and serves to increase intracellular osmotic pressure, and thus cytosolic water retention (Gilbert *et al.*, 1998). The supposition that proline could be accumulated intracellularly in osmotically stressed *S. cerevisiae* and *S. cerevisiae* (syn *S. pastorianus*) brewing strains has not been previously examined. The results presented in this report provide strong evidence to support the hypothesis that proline is not accumulated during osmotic stress, as determined by TLC and also using a ninhydrin-based quantitative assay. Examination of the amino acid pool of osmotically stressed, and non-stressed cells revealed that there were discreet alterations in the composition of the cytosolic pool of amino acids, however it was not established whether one amino acid above all others was accumulated during osmotic stress. In order to address this an analysis of total intracellular amino acid levels would be required. However even if certain amino acids were accrued following exposure to osmotic stress, a critical analysis of those amino acids required in the construction of a

number of global and specific 'stress proteins' would be required (Mager and De Kruijff, 1995).

In *S. cerevisiae* cells, glycerol has been demonstrated as a compatible solute (Brown, 1978; Reed *et al*, 1987; Bellinger and Larher, 1987; Meikle *et al*, 1991; Blomberg and Adler, 1992; Albertyn *et al*, 1994; Hohmann, 1997, Remize *et al*, 2001; Pahlman *et al*, 2001). It has been observed that haploid *S. cerevisiae* cells preferentially accumulate this polyhydric sugar alcohol during osmotic stress; however the data in this report could only partly corroborate these findings in polyploid and aneuploid *S. cerevisiae* and *S. cerevisiae* (syn *S. pastorianus*) brewing strains. Glycerol accumulation during osmotic stress was observed to be a strain dependent phenomenon, however statistical analysis did not clearly demonstrate a difference between ale *S. cerevisiae* and lager *S. cerevisiae* (syn *S. pastorianus*) strains.

Furthermore intracellular glycerol levels were observed to be partially dependent upon growth phase, when higher concentrations of sorbitol were used to elicit an osmotic stress response. Stationary phase populations of cells exhibited twice the intracellular glycerol levels as comparative exponential phase cells when exposed to 30% (w/v) sorbitol. For lower concentrations of sorbitol, statistical analyses were unable to differentiate between intracellular glycerol levels in exponential and stationary phase populations.

In contrast intracellular glycerol levels during NaCl-induced osmotic stress were observed to be significantly lower ($P<0.05$) than those observed when sorbitol was used to elicit the osmotic stress response. Compatible solute accumulation is dependent on the specific solute that generates the stress. In the case of NaCl a different signal transduction pathway (the calcineurin pathway (Gaxiola *et al.*, 1992) (section 1.5.7)) is activated, which affects a change in physiology and metabolism in order to detoxify the cell from the effects of Na^+ ions. The re-routing of cellular defences in order to remove toxic monovalent cations has the result of diverting cellular energy away from glycerol overproduction.

This study did not include a comparison of the osmotolerances other than in relation to weight/volume (w/v) percentages. Initial studies using an osmometer proved unsuitable due to the high concentrations used in this study. Furthermore it is suggested that for future experimental work a better comparison of results using sorbitol and NaCl

would result from the use of molar solutions, or better attempts to quantify either water activities or osmolarities of solutions.

Glycerol accumulation in brewing yeast strains in response to sorbitol and NaCl solute challenge did not appear to be related to the solute concentration applied, a phenomenon not observed in laboratory haploid strains. In an attempt to understand the pattern of glycerol accumulation in osmotically stressed *S. cerevisiae* and *S cerevisiae* (syn *S. pastorianus*) brewing cells a molecular approach was undertaken. PCR screening for the genes involved in glycerol biosynthesis and dissimilation was undertaken. The results from this investigation demonstrated that brewing strains of *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae* (syn *pastorianus*) did possess all of the genes encoding enzymes involved in both pathways.

The nucleotide sequence GPD1 derived from SCB8 (ale) cells revealed no deviations from that of the strain S288C. The lager strain SCB2, however, revealed 3 base pair changes in the sequence of its *GPD1* gene as compared to the S288C haploid. The base changes at 722 base pairs in SCB2 was sufficient to affect an amino acid transpositions (cysteine is replaced by serine), similarly the base change at 869bp altered the codon from glutamic acid to aspartic acid. Neither of these changes in the primary sequence of SCB2 Gpd1p was sufficient to alter either the predicted location (cytoplasm) or function of protein. It could therefore be concluded that the differences in glycerol accumulation profiles observed for brewing and laboratory haploid strains did not derive from a genomic deletion or truncation of the *GPD1* gene.

Furthermore the expression profile of *GPD1* in both brewing in terms of mRNA transcripts and Gpd1p expression mirrored the intracellular accumulation profiles. It is suggested that the regulation of expression of this gene may therefore be responsible for the glycerol accumulation profiles observed.

It has been demonstrated that expressional control of *GPD1* and therefore glycerol production is mediated by the high osmolarity glycerol (HOG) pathway (section 1.5.6) (Maeda *et al.*, 1994; Maeda *et al.*, 1995; Varela and Mager, 1996; Hohmann, 2002). The osmotically induced expression of the HOG pathway results in the translocation of phosphorylated transcritption factors to the nucleus with a resultant upregulation of gene expression. It is suggested that the same mechanism is in operation in brewing yeast strains, preliminary data indicated that hog1p is indeed phosphorylated in brewing yeast during conditions that induce *GPD1* expression). In

addition it is also suggested that the same feedback mechanisms may be active during osmotic stress in brewing yeast strains that is evident in the laboratory haploid type strain S288C (Hohmann, 2002).

Facilitated glycerol export in *S. cerevisiae* has been demonstrated to be mediated by the glyceroaquaporin Fps1p. This major intrinsic protein is gated and therefore able to effect control of the retention of glycerol (Luyten *et al.*, 1994; Tamas *et al.*, 1995). During osmotic stress the Fps1p channel is effectively closed to glycerol and therefore allows for the intracellular accumulation of this molecule. It is suggested that this mechanism may be defective in brewing yeast strains given the high levels of extracellular glycerol observed in osmotically stressed cells. It may, however be the case that the cell membrane/cell wall barrier in brewing yeast strains is more permeable to glycerol than haploid cells which accounts for the increased levels of efflux observed.

7.2 Future Work

This study detailed the intrinsic dynamicity of the yeast vacuolar compartment, and its variable morphology during the replication cycle. To provide further evidence for the supposition that vacuolar fragmentation is a function of cell cycle progression it is proposed that real time (time lapse) confocal microscopy be used to follow the cellular replication cycle in budding laboratory haploid and brewing yeast cells. The utilisation of vacuolar dyes in this thesis may not prove sufficient due the inability to continuously supply dye to cells with a correlative consistency in staining. For long term time lapse microscopy, however the localisation of the yeast vacuole may be achieved using green fluorescent (GFP) protein fusions to currently known tonoplast or vacuolar lumen proteins. Potential candidates for GFP fusions to localise the tonoplast include *AMS1* (vacuolar alpha-mannosidase), *LSB6* (1-phosphatidylinositol 4-kinase) (Han *et al.*, 2002) and *VAM3* (syntaxin-related protein; required for vacuolar assembly) (Pelham *et al.*, 1999). It is further proposed that an red fluorescent protein fusion (RFP) could be constructed to localise the vacuolar lumen, possibly to a protein such as Sna3p (an integral lumen protein) (Reggiori *et al.*, 2001). The use of tagging methodologies for vacuolar inheritance studies can be problematic as confusion arises whilst the tagged protein is being targeted to the vacuole via the endoplasmic reticulum and golgi.

Preliminary results using an antibody that recognises phosphorylated hog1p in yeast has indicated that hog1p phosphorylation occurs during osmotic stress in brewing yeast. It is proposed that the level of *GPD1* (and other HOG dependent genes) could be investigated for correlation to the levels of phosphorylated Hog1p in brewing yeast strains. Furthermore quantification of the level of Hog1p phosphorylation could be made using a radiolabelled or chemiluminescent reporter assay in western blot analysis.

The relationship between intracellular glycerol levels and glycerol export in brewing yeast cells subject to osmotic stress remain to be elucidated. It is not known whether glycerol export is facilitated via the Fps1p channel or if passive diffusion mediates the majority of efflux. Experimental determination of glycerol efflux patterns coupled with expressional analysis of the *FPS1* and Fps1p would demonstrate whether the expression of this protein is upregulated during osmotic stress. Cells grown on solute augmented YPD plates would be monitored for their capacity to acclimatise to the stress, and subsequent expression of *FPS1* and Fps1p monitored by qRT-PCR and western blot analysis. If a differential pattern of Fps1p expression was observed, cells with up/downregulated Fps1p levels could be subjected to osmotic stress and the levels of intra/extracellular glycerol studied.

The contribution that other solutes might make to intracellular osmolarity during osmotic stress in brewing yeast strains is currently not known, it is suggested that a detailed gas chromatographic (GC-MS) or near-infrared spectroscopic (NIR) examination of intracellular extracts could be made in order to screen for the involvement of 'alternative' compatible solutes. Special attention is to be paid the groups of traditionally accumulated compatible solutes, including amino acids (using HPLC analysis), quaternary amines and other sugar alcohols (mannitol, arabitol).

8.1 References

Agre, P., Bonhivers, M. and Borgnia, M.J. (1998). The aquaporins, blueprints for cellular plumbing systems. *J. Biol. Chem.* **273(24)** 14659-62.

Aigle, M., Erbs, D. and Moll, M. (1984). Some molecular structures in the genome of lager brewing yeast. *J. Am. Soc. Brew. Chem.*, **42** 1

Akhtar, N., Blomberg, A. and Adler, L. (1997) Osmoregulation and protein expression in a pbs2delta mutant of *Saccharomyces cerevisiae* during adaptation to hypersaline stress. *FEBS Lett.* **403(2)**,173-80.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002). in Molecular biology of the cell 4th Ed. Garland Science publishing New York.

Albertyn, J., Hohmann, S., Thevelein, J.M. and Prior BA. (1994). *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol. Cell. Biol.* **(6),** 4135-44.

Algeri, A., Tassi, F., Ferrero, I., Puglisi, P.P. (1978) Different phenotypes for the lactose utilization system in *Kluyveromyces* and *Saccharomyces* species. *Antonie Van Leeuwenhoek.* **44(2)**,177-82.

Anderson, R.G., Falck, J.R., Goldstein, J.L. and Brown, M.S. (1984). Visualization of acidic organelles in intact cells by electron microscopy. *Proc. Natl. Acad. Sci. USA* **81**(15), 4838-42.

Ando, S., Mikata, K., Tahara, Y. and Yamada, Y. (1996) Phylogenetic relationships of species of the genus *Saccharomyces* Meyen ex Reess deduced from partial base sequences of 18S and 26S ribosomal RNAs (saccharomycetaceae). *Biosci. Biotechnol. Biochem.* **60(7)**,1070-5.

André, B. (1995). An overview of membrane transport proteins in *Saccharomyces cerevisiae. Yeast*, **11,** 1575-1611.

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.* **16(9),** 2179-87.

Attfield, P.V., Kletsas, S., Veal, D.A., van Rooijen, R., and Bell, P.J. (2000) Use of flow cytometry to monitor cell damage and predict fermentation activity of dried yeasts. *J. Appl. Microbiol.* **89(2)**,207-14.

Attfield, P.V., Choi, H.Y., Veal, D.A., and Bell, P.J. (2001) Heterogeneity of stress gene expression and stress resistance among individual cells of *Saccharomyces cerevisiae*. *Mol. Microbiol.* **40(4)**,1000-8.

Banta, L. M., Robinson, J. S., Klionsky, D. and Emr, S. D. (1988). Organelle assembly in yeast: characterisation of yeast mutants defective in vacuolar biogenesis and protein sorting. *J. Cell Biol.* **107**, 1369-1383.

Barberio, C., Fani, R., Raso, A., Carli, A. and Polsinelli, M. (1994) DNA fingerprinting of yeast strains by restriction enzyme analysis. *Res. Microbiol.* **145(9)**, 659-66.

Barker, M.G. and Smart, K. A. (1996). Morphological changes associated with the cellular ageing of a brewing yeast strain. *J. Am. Soc. Brew. Chem.* ,**54**, 121-126.

Barnett, J.A., (1968). In. The Fungi. Volume 3. Eds. G.C. Ainsworth, F.K. Sparroe, and A.S. Sussman. Academic Press, New York and London. 557-595.

Bartoletti, D.C., Harrison, G.I., and Weaver, J.C. (1989) The number of molecules taken up by electroporated cells: quantitative determination. *FEBS Lett.* **256(1-2)**,4-10.

Bellinger, Y. and Lahrer, F. (1987). A ^{13}C comparative nuclear magnetic resonance study of organic solute production excretion by the yeasts *Hansenula anomala* and *Saccharomyces cerevisiae* in saline media. *Can. J. Microbiol.*, **34**, 605-612.

Bendiak, D. S. (2000). Review of metabolic activity tests and their ability to predict fermentation performance. In Brewing Yeast Fermentation Performance. pp 34-35. Ed K.A. Smart. Blackwell Science, Oxford.

Beney, L., Martinez de Maranon, I., Marechal, P.A., and Gervais P. (2000) Influence of thermal and osmotic stresses on the viability of the yeast *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **55(1-3)**,275-9

Beney, L., Marechal, P.A., and Gervais P. (2001) Coupling effects of osmotic pressure and temperature on the viability of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **56(3-4)**, 513-6.

Bertl, A., Bihler, H., Kettner, C. and Slayman, C.L. (1998) Electrophysiology in the eukaryotic model cell *Saccharomyces cerevisiae*. *Pflugers Arch.* **436(6)**,999-1013.

Bienz, M. and Pelham, H.R. (1986) Heat shock regulatory elements function as an inducible enhancer in the *Xenopus* hsp70 gene and when linked to a heterologous promoter. *Cell.* **45(5)**,753-60.

Bihler, H., Slayman, C.L.and Bertl, A. (1998) NSC1: a novel high-current inward rectifier for cations in the plasma membrane of *Saccharomyces cerevisiae. FEBS Lett.* **432(1-2)**,59-64.

Biondi, A.C., Feliz, M.R. and Disalvo, E.A. (1991) Surface changes induced by osmotic stress and its influence on the glycerol permeability in lipid bilayers. *Biochim. Biophys. Acta.* **1069(1)**, 5-13.

Blomberg, A. (1997). Osmotic hypersensitivity of the yeast *Saccharomyces cerevisiae* is strain and growth media dependent: quantitative aspects of the phenomenon. *Yeast* **13**(6), 529-39.

Blomberg, A. and Adler, L. (1989). Roles of glycerol and glycerol 3-phosphate dehydrogenase (NAD^+) in acquired osmotolerance of *Saccharomyces cerevisiae. J. Bacteriol.* **171**, 1087-1092.

Blomberg, A., and Adler, L. (1992). Physiology of osmotolerance in fungi. *Adv. Microbial Phys.*, **33**,145-212.

Boller, T. H., Dürr, M. and Wiemken, A. (1976). Asymmetric distribution of concanavalin A binding sites on yeast plasmalemma and vacuolar membrane. *Arch. Microbiol.*, **109** (1-2), 119.

Bonangelino, C.J., Chavez, E.M. and Bonifacino, J.S. (2002a) Genomic Screen for Vacuolar Protein Sorting Genes in *Saccharomyces cerevisiae. Mol. Biol. Cell.* 13(7), 2486-501.

Bonangelino, C. J., Nau, J. J., Duex, J. E., Brinkman, M., Wurmser, A. E., Gary, J. D., Emr, S. D. and Weisman, L. S. (2002b) Osmotic stress-induced increase of phosphatidylinositol 3,5-bisphosphate requires Vac14p, an activator of the lipid kinase Fab1p. *J. Cell. Biol.* **156**(6), 1015-28

Bonangelino, C. J., Catlett, N.L. and Weisman, L.S.(1997). Vac7p, a novel vacuolar protein, is required for normal vacuole inheritance and morphology. *Mol. Cell. Biol.* **17**(12), 6847-58.

Bone, N., Millar, J.B., Toda, T. and Armstrong J. (1998) Regulated vacuole fusion and fission in *Schizosaccharomyces pombe*: an osmotic response dependent on MAP kinases. *Curr Biol.***8(3)**,135-44.

Bonhivers, M., Carbrey, J.M., Gould, S. J. and Agre, P. (1998). Aquaporins in *Saccharomyces*. Genetic and functional distinctions between laboratory and wild-type strains. *J. Biol. Chem.* **273**(42) 27565-72.

Borginia. M., Nielsen, S., Engel, A. and Agre P. (1999). Cellular and molecular biology of the aquaporin water channels. *Ann. Rev. Biochem.* **68**, 425-458.

Boucherie, H. (1985). Protein synthesis during transition and stationary phases under glucose limitation in *Saccharomyces cerevisiae*. *J. Bacteriol.* 161(1), 385-92.

Boulton, C. A. (1991a). Yeast management and the control of brewery fermentations. *Brewers Guardian*, **120**(3), 25-29.

Boulton, C. A. (1991b). Developments in brewery fermentation. *Biotech. Genet. Eng. Rev.* **19**, 127-181.

Boulton, C. A. and Quain, D. E. (2001) In Brewing yeast and fermentation. Blackwell Science Ltd, Oxford

Boy-Marcotte, E., Perrot, M., Bussereau, F., Boucherie, H. and Jacquet, M. (1998) Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. *J. Bacteriol.* **180(5)**, 1044-52.

Braun, E.L., Fuge, E.K., Padilla, P.A. and Werner-Washburne, M. (1996). A stationary-phase gene in *Saccharomyces cerevisiae* is a member of a novel, highly conserved gene family. *J. Bacteriol.* **178**(23), 6865-72.

Breeuwer, P., Drocourt, J.L., Rombouts, F.M. and Abee, T. (1994) Energy-dependent, carrier-mediated extrusion of carboxyfluorescein from *Saccharomyces cerevisiae* allows rapid assessment of cell viability by flow cytometry. *Appl. Environ. Microbiol.* **60(5)**, 1467-72.

Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. and Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. *Science*, **259** 1760-1763.

Brown, A. D. (1978). Compatible solute and extreme water stress in eukaryotic microorganisms. *Adv. Microbial Phys.*, 17, 181-242.

Brown, A. D., Mackenzie, K. F. and Singh, K. K. (1986). Selected aspects of microbial osmoregulation. *FEMS Microbiol. Rev.* **39**, 31-36.

Brown, T. A. (2001). In Gene cloning: an introduction. 4th Edition. Blackwell Science (UK).

Buehrer, B.M. and Errede, B. (1997). Coordination of the mating and cell integrity mitogen-activated protein kinase pathways in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **17**(11), 6517-25.

Bullock, W.O., Fernandez, J. M., Short, J. M. (1987). XL1-BLUE: A high efficiency plasmid transforming RECA *Escherichia coli* strain witch Beta- galactosidase selection. *Biotechniques.*, **15** (4), 376-379.

Cahill, G., Murray, D. M., Walsh, P. K. and Donnelly, D. (2000) Effect of the Concentration of Propagation Wort on Yeast Cell Volume and Fermentation Performance. *J. Am. Soc. Brew. Chem.* **58(1)**,14-20.

Carbrey, J. M., Bonhivers, M., Boeke, J. D. and Agre, P. (2001). Aquaporins in *Saccharomyces*: Characterization of a second functional water channel protein. *Proc. Natl. Acad. Sci. USA* , **98** (3), 1000–1005.

Casey, G. P (1996). Practical applications of pulsed field electrophoresis and yeast chromosome fingerprinting in brewing QA and R&D. *MBAA Tech. Quart.* **33**, 1-10.

Castrillo, J.I., Hayes, A., Mohammed, S., Gaskell, S.J. and Oliver SG. (2003) An optimized protocol for metabolome analysis in yeast using direct infusion electrospray mass spectrometry. *Phytochemistry.* **62(6)**,929-37.

Chatterjee, M.T., Khalawan, S.A. and Curran, B.P. (2000). Cellular lipid composition influences stress activation of the yeast general stress response element (STRE). *Microbiology* **146**(4), 877-84.

Cheng, S., Fockler, C., Barnes, W.M. and Higuchi, R.. (1994). Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci.* USA **91**(12), 5695-9.

Cherry, J.M., Adler, C., Ball, C., Chervitz, S.A., Dwight, S.S., Hester, E.T., Jia, Y., Juvik, G., Roe, T., Schroeder, M., Weng, S., and Botstein, D. (1998) SGD: *Saccharomyces* Genome Database. *Nucl. Acids. Res.* **26**, 73-79.

Chiang, H. L. (1995). Protein targeting and degradation in the yeast vacuole. *Can. J. Bot.*, **73(Suppl. 1)**, S347-S351.

Chvatchko, Y., Howald, I. and Riezman, H. (1986) Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell.* **46(3)**,355-64.

Cline, J., Braman, J.C. and Hogrefe, H. H. (1996). PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res.* **24**(18), 3546-51.

Cohen, P. (1992). Signal integration at the level of protein kinases, protein phosphatases and their substrates. *Trends Biochem. Sci.* **(10)** 408-13.

Cole, L., Orlovich, D. A. and Ashford, A. E. (1998). Structure, Function, and Motility of Vacuoles in Filamentous Fungi. *Fungal Genet. Biol.* **(1/2),** 86-100.

Colson, I., Delneri, D. and Oliver, S. G. (2004) Effects of reciprocal chromosomal translocations on the fitness of *Saccharomyces cerevisiae. EMBO Rep.* **5(4)**,392-8.

Conradt, B., Shaw, J., Vida, T., Emr, S. and Wickner, W. (1992) *In vitro* reactions of vacuole inheritance in *Saccharomyces cerevisiae. J Cell Biol.* **119(6)**,1469-79.

Cooke, F.T., Dove, S.K., McEwen, R.K., Painter, G., Holmes, A.B., Hall, M.N., Michell, R.H. and Parker, P.J. (1998) The stress-activated phosphatidylinositol 3-phosphate 5-kinase Fab1p is essential for vacuole function in *S. cerevisiae. Curr Biol.* **8(22)**,1219-22.

Costenoble, R., Valadi, H., Gustafsson, L., Niklasson, C. and Franzen, C.J. (2000). Microaerobic glycerol formation in *Saccharomyces cerevisiae. Yeast.* **16(16)**, 1483-95.

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* **947(2)**,367-84.

Csonka, L.N. and Hanson, A.D. (1991). Prokaryotic osmoregulation: genetics and physiology. *Annu. Rev. Microbiol.* **45**, 569-606.

D'Amore, T. (1992). Improving yeast fermentation performance. *J. Inst. Brew.* **98**, 375-382.

Darzynkiewicz, Z., Traganos, F., Staiano-Coico, L., Kapuscinski, J. and Melamed, M.R. (1982) Interaction of rhodamine 123 with living cells studied by flow cytometry. *Cancer Res.* **42(3)**, 799-806.

Davenport, K.R., Sohaskey, M., Kamada, Y., Levin, D.E. and Gustin, M.C. (1995). A second osmosensing signal transduction pathway in yeast. Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway. *J. Biol. Chem.* **270** (50), 30157-61.

De Deuve, C. (1973). The lysosome in retrospect. In. Lysosomes in biology and pathology. Vol I. 2nd Ed. Eds. J. T. Dingle and H. D. Fell. North Holland, Amsterdam. pp 3-40.

Deere, D., Shen, J., Vesey, G., Bell, P., Bissinger, P. and Veal, D. (1998). Flow cytometry and cell sorting for yeast viability assessment and cell selection. *Yeast* **14**(2), 147-60.

DeRisi, J. L., Iyer, V.R. and Brown, P.O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278**(5338), 680-6.

Diaper, J.P, Tither, K. and Edwards, C. (1992) Rapid assessment of bacterial viability by flow cytometry. *Appl. Microbiol. Biotechnol.* **38(2)**, 268-72.

Dickinson, J.R. (1999). Carbon metabolism. In The metabolism and molecular physiology of *Saccharomyces cerevisiae*. Eds. Dickinson, J.R. and Schweizer, M. Taylor and Francis Ltd. pp 23-46.

Dihazi, H., Kessler, R. and Eschrich, K. (2001) Phosphorylation and inactivation of yeast 6-phosphofructo-2-kinase contribute to the regulation of glycolysis under hypotonic stress. *Biochemistry.* **40(48)**,14669-78.

Dove, S.K., Cooke, F.T., Douglas, M.R., Sayers, L.G., Parker, P.J. and Michell, R. H. (1997). Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature* **390**(6656), 187-92.

Dove, S.K., McEwen, R.K., Mayes, A., Hughes, D.C., Beggs, J.D. and Michell, R.H. (2002) Vac14 controls PtdIns(3,5)P(2) synthesis and Fab1-dependent protein trafficking to the multivesicular body. *Curr Biol.* **12(11)**,885-93.

Dujon, B. (1998) European Functional Analysis Network (EUROFAN) and the functional analysis of the Saccharomyces cerevisiae genome. *Electrophoresis.* **19(4)**,617-24.

Eggers, D.K. and Valentine, J. S. (2001). Crowding and hydration effects on protein conformation: a study with sol-gel encapsulated proteins . *J. Mol. Biol.* **314**(4) 911-22.

Eitzen, G., Wang. L., Thorngren, N. and Wickner, W. (2002) Remodeling of organelle-bound actin is required for yeast vacuole fusion. *J. Cell Biol.* **158(4)**,669-79.

Elion, E.A. (2000). Pheromone response, mating and cell biology. *Cur. Opin. Microbiol.* **(6)**, 573-81.

Eriksson, P., Andre, L., Ansell, R., Blomberg, A. and Adler, L. (1995) Cloning and characterization of *GPD2*, a second gene encoding sn-glycerol 3-phosphate dehydrogenase (NAD^+) in *Saccharomyces cerevisiae*, and its comparison with *GPD1*. *Mol. Microbiol.* **17(1)**,95-107.

Fernandez, S. S., Gonzalez, M. G. and Sierra, J. A. (1993) Evaluation of the effect of acid washing fermentative and respiratory behaviour of yeasts by the acidification power test. *Tech. Quart. MBAA.* **30**, 1-8.

Finnerty, W. R. (1989) Microbial lipid metabolism. In Microbial lipids Vol 2. Ratledge, C. and Wilkinson, S. G. Eds) Academic press, London.

Fiol, J.B. (1976) Systematic study of *Saccharomyces*: oxidases and vitamin requirements *Mycopathologia.* **58(1)**, 49-58.

Fischer, G., James, S.A., Roberts, I. N., Oliver, S.G. and Louis, E.J. (2000) Chromosomal evolution in *Saccharomyces. Nature* **405(6785)**,451-4.

Flaman, J.M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C., Ishioka, C., Friend, S.H., and Iggo, R. (1994). A rapid PCR fidelity assay . *Nucleic. Acids. Res.* **22**(15), 3259-60.

Francois, J., Eraso, P. and Gancedo, C. (1987). Changes in the concentration of cAMP, fructose 2,6-bisphosphate and related metabolites and enzymes in *Saccharomyces cerevisiae* during growth on glucose. *Eur. J. Biochem.* **164**(2), 369-73.

Fuge, E.K., Braun, E.L. and Werner-Washburne, M. (1994). Protein synthesis in long-term stationary-phase cultures of *Saccharomyces cerevisiae. J. Bacteriol.* **176**(18), 5802-13.

Galinski, E. A. and Trüper, H. G. (1994). Microbial behaviour in salt-stressed ecosystems. *FEMS Microbiol. Rev.*, **15**, 95-108.

Galinski, E.A., Stein, M., Amendt, B. and Kinder, M. (1997). The kosmotropic (structure-forming) effect of compensatory solutes. *Comp. Biochem. and Phys.* **117A**(3), 357-365.

Ganster, R. W., McCartney, R. R. and Schmidt, M. C. (1998). Identification of a Calcineurin-Independent Pathway Required for Sodium Ion Stress Response in *Saccharomyces cerevisiae. Genetics* **150:** 31–42.

Gary, J.D., Wurmser, A.E., Bonangelino, C.J., Weisman, L.S. and Emr, S.D (1998). Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. *J. Cell. Biol.***143**(1), 65-79.

Gasch, A.P. and Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. *Funct. Integr. Genomics* **2**(4-5),181-92.

Gaxiola, R., de Larrinoa, I. F., Villalba, J. M. and Serrano, R. (1992). A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J.* **11**, 3157- 3164.

Ghaemmaghami, S., Huh, W.K., Bower, K., Howson ,R.W., Belle, A., Dephoure, N., O'Shea, E. K. and Weissman, J.S. (2003) Global analysis of protein expression in yeast. *Nature*. 425(6959),737-41.

Gilbert, G. A., Gadush, M.V., Wilson, C. and Madore, M.A (1998). Amino acid accumulation in sink and source tissues of *Coleus blumei* Benth. During salinity stress *J. Exp. Bot.* **49** (318): 107-114.

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H. and Oliver, S.G. (1996) Life with 6000 genes *Science* 274(5287), 563-7.

Goffeau, A., *et al.*,(1997). The Yeast Genome Directory. *Nature* **387**(Suppl.), 5–105.

Gomes de Mesquita, D.S., Shaw, J., Grimbergen, J.A., Buys, M.A., Dewi, L. and Woldringh, C.L. (1997). Vacuole segregation in the *Saccharomyces cerevisiae vac2-1* mutant: structural and biochemical quantification of the segregation defect and formation of new vacuoles. *Yeast* **13**, 999-1008.

Gomez, M.J., Luyten, K. and Ramos, J. (1996). The capacity to transport potassium influences sodium tolerance in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **135**(2-3),157-60.

Götte, M. and Lazar, T. (1999). The ins and outs of yeast vacuole trafficking. *Protoplasma*. **209**, 9-18.

Govindan, B., Bowser, R. and Novick, P. (1995) The role of Myo2, a yeast class V myosin, in vesicular transport. *J. Cell Biol* **128**, 1055-1068.

Graham, R.K. and Caiger, P. (1969). Fluorescence staining for the determination of cell viability. *Appl. Microbiol.* **17**(3), 489-90.

Grauslund, M., Lopes, J.M. and Ronnow, B. (1999). Expression of *GUT1*, which encodes glycerol kinase in *Saccharomyces cerevisiae*, is controlled by the positive regulators Adr1p, Ino2p and Ino4p and the negative regulator Opi1p in a carbon source-dependent fashion. *Nucleic Acids Res.* **27**(22), 4391-8.

Green, L.J., Marder, P., Mann, L.L., Chio, L.C. and Current, W.L. (1999). LY303366 exhibits rapid and potent fungicidal activity in flow cytometric assays of yeast viability. *Antimicrob. Agents Chemother.* **43**(4), 830-5.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I. and Kornfeld, S. *(1988)* The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* **52**, 329-341.

Gustin, M. C., Albertyn, J., Alexander, M. and Davenport, K. (1998). MAP Kinase Pathways in the Yeast *Saccharomyces cerevisiae Micro. Mol. Biol. Rev.*, **62** (4),1264–1300.

Guthrie, B. A. and Wickner, W. (1988). Yeast vacuole fragment when microtubules are disrupted. *J. Cell Biol.i,* **107**, 115-120.

Haas, A., Conradt, B. and Wickner, W. (1994). G-protein ligands inhibit in vitro reactions of vacuole inheritance. *J Cell Biol* **126(1),**87-97.

Haas, A. and Wickner, W. (1996). Homotypic vacuole fusion requires Sec17p (yeast alpha-SNAP) and Sec18p (yeast NSF). *EMBO J.* **15**(13), 3296-305.

Haase, S.B., and Lew, D.J. (1997) Flow cytometric analysis of DNA content in budding yeast. *Methods Enzymol.* **283**,322-32.

Haase, S.B., and Reed, S.I. (2002) Improved flow cytometric analysis of the budding yeast cell cycle. *Cell Cycle.* **1(2)**,132-6.

Hammond, J. R. M. (1993). Brewer's Yeasts. In. The yeasts. 2nd Ed. Vol. II. Yeast technology. Eds. A. H. Rose and J. S. Harrsion. Academic Press Ltd.

Hammond, J., Davis, D., Lee, M. and Storey, K. Does osmotic pressure affect yeast performance in high gravity fermentation? *Proceedings of the European Brewing Convention, Budapest 2001.*

Han, G.S., Audhya, A., Markley ,D.J., Emr, S.D. and Carman, G.M.(2002)The *Saccharomyces cerevisiae* LSB6 gene encodes phosphatidylinositol 4-kinase activity. *J. Biol. Chem.* **277(49)**, 47709-18.

Hanson, A. D., Rathinasabapathi, B., Rivoal, J., Burnet, M., Dillon, M.O. and Gage, D.A. (1994). Osmoprotective compounds in the *Plumbaginaceae*: a natural experiment in metabolic engineering of stress tolerance. *Proc. Natl. Acad. Sci. USA* **91(1),**306-10.

Hazen, K.C. and Hazen, B.W. (1992). Hydrophobic surface protein masking by the opportunistic fungal pathogen *Candida albicans*. *Infection and Immunity*, **60**, 1499-1508.

Heidelberg, J.F., Shahamat, M., Levin, M., Rahman, I., Stelma, G., Grim, C., and Colwell, RR. (1997) Effect of aerosolization on culturability and viability of gram-negative bacteria. *Appl. Environ. Microbiol.* **63(9)**,3585-8.

Hernandez-Saavedra, N. Y., Ochoa, J. L. and Vazquez-Dulhalt, R. (1995). Osmotic adjustment in marine yeast. *J. Plankton. Res.* **17**(1), 59-69.

Hewitt, C. J., and Nebe-Von-Caron, G. (2001) An industrial application of multiparameter flow cytometry: assessment of cell physiological state and its application to the study of Microbial Fermentations. *Cytometry* 44,179–187.

Hill, K.L., Catlett, N.L.and Weisman, L.S. (1996). Actin and myosin function in directed vacuole movement during cell division in *Saccharomyces cerevisiae. J. Cell Biol* **135**,1535-1549.

Ho, K.H. and Miller, J. J. (1978). Free proline content and sensitivity to desiccation and heat during yeast sporulation and spore germination. *Can. J. Microbiol.* **24**(3), 312-20.

Hohmann, S. (1997). Shaping up: The response of yeast to osmotic stress. In. yeast stress responses. Eds. S. Hohmann and W. H. Mager. R.G. Landes Company.

Hohmann, S. (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev.* **66(2)**,300-72.

Hommes, F.A. (1966) Mechanism of the Crabtree effect in yeast grown with different glucose concentrations. *Arch. Biochem. Biophys.* **113(2)**,324-30.

Horak, J. (1986) Amino acid transport in eucaryotic microorganisms. *Biochim Biophys Acta.* **864(3-4)**,223-56.

Horvath, A. and Riezman, H. (1994) Rapid protein extraction from *Saccharomyces cerevisiae. Yeast* **10(10)**, 1305-10.

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.* **219(1-2)**,187-93.

Hough, J.S., Briggs, D.E., Stevens, R. and Young, T.W., (1995). Malting and Brewing Science. Volume 2: Hopped Wort and Beer. Chapman and Hall.

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*. **144**, 671-680.

Hulse, G., Bihl, G., Morakile, G. and Axcell, B. (2000). Optimisation of storage and propagation for consistent lager fermentations. In. Brewing Yeast Fermentation Performance. Ed. K. A. Smart. Blackwell Science, Oxford. 161-169.

Humphreys, M.J., Allman, R. and Lloyd, D. (1994) Determination of the viability of *Trichomonas vaginalis* using flow cytometry. *Cytometry* **15(4)**, 343-8.

Hyde, G.J., Davies, D.S., Cole, L. and Ashford, A.E. (2003) Retention of fluorescent probes during aldehyde-free anhydrous freeze-substitution *J. Microscopy.* **210 (2),**125-30.

Indge, K. J. (1968). The isolation and properties of the yeast cell vacuole. *J. Gen. Microbiol.* **51**(3), 441-6.

Innis, M. A., Gelfand, D. H. and Sninsky, J. J. (1999). In PCR Applications: Protocols for Functional Genomics. Academic press.

Ishikawa, K., Catlett, N.L., Novak, J.L., Tang, F., Nau, J.J. and Weisman, L.S. (2003) Identification of an organelle-specific myosin V receptor. *J Cell Biol.***160(6)**, 887-97.

James, S. A., Cai, J., Roberts, I. N. and Collins, M. D. (1997). A phylogenetic analysis of the genus *Saccharomyces* based on 18S rRNA gene sequences: description of *Saccharomyces kunashirensis* sp. nov. and *Saccharomyces martiniae* sp. nov. *Int. J. Syst. Bacteriol.* **47,** 453–460.

Jamieson, D. J. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae. Yeast.* **14,** 1511-1572.

Jayapal, V., Sharmila, K.M., Selvibai, G., Thyagarajan, S.P., Shanmugasundaram, N., and Subramanian, S. (1991). Fluorescein diacetate and ethidium bromide staining to determine the viability of *Mycobacterium smegmatis* and *Escherichia coli. Lept. Rev.* **62**,310– 314.

Jenkins, M.B., Anguish, L.J., Bowman, D.D., Walker, M.J. and Ghiorse, W.C. (1997) Assessment of a dye permeability assay for determination of inactivation rates of *Cryptosporidium parvum* oocysts. *Appl Environ. Microbiol.* **63(10)**, 3844-50.

Jerpseth, B., Callahan, M., Greener, A. (1997). New Competent Cells for Highest Transformation Efficiencies. *Strategies.*, **10**, 37-39.

Jespersen, L., van der Aa Kuhle, A., and Petersen, K.M. (2000). Phenotypic and genetic diversity of *Saccharomyces* contaminants isolated from lager breweries and their phylogenetic relationship with brewing yeasts. *Int. J. Food Micro.* **60**(1), 43-53.

Johnston, G.C., Prendergast, J.A. and Singer, R.A. (1991) The *Saccharomyces cerevisiae MYO2* gene encodes an essential myosin for vectorial transport of vesicles. *J. Cell Biol* **113**, 539-551.

Josepa, S., Guillamon, J. M., Cano, J. (2000). PCR differentiation of *Saccharomyces cerevisiae* from *Saccharomyces bayanus/Saccharomyces pastorianus* using specific primers. *FEMS Microbiology Letters*, **193**(2), 255-259.

Kaneshiro, E.S, Wu, Y.P. and Cushion, M.T. (1991) Assays for testing *Pneumocystis carinii* viability. *J. Protozool.* **38(6)**, 85S-87S.

Kang, M. S., Elango, N., Mattia, E., Au-Young, J., Robbins, P. W. and Cabib, E. (1984). Isolation of chitin synthetases from *Saccharomyces cerevisiae.* Purification of an enzyme by entrapment in the reaction product. *J. Biol. Chem.* **259**, 14966-14972.

Kempf, B. and Bremer, E. (1995) OpuA, an Osmotically Regulated Binding Protein-dependent Transport System for the Osmoprotectant Glycine Betaine in Bacillus subtilis. *J. Biol. Chem.* **270**, 16701-16713

Kempf, B. and Bremer, E. (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* **170**(5), 319-30

Kennedy, A. I. (2000) Yeast handling in the brewery. In. Brewing Yeast Fermentation Performance. Ed. K. A. Smart. Blackwell Science, Oxford. 129-134.

Kennedy, A. I., Taidi, B., Dola, J. L. and Hodgson. J. A. (1997) Optimisation of a fully defined medium for yeast fermentation studies. *Food Technol. Biotechnol.* **35**, 261-265

Keogh, R., Seoighe, C. and Wolfe K. H. (1998) Evolution of Gene Order and Chromosome Number in Saccharomyces, Kluyveromyces and Related Fungi. *Yeast* **14**, 443–457

Ketela, T., Brown, J.L., Stewart, R.C. and Bussey, H. (1998) Yeast Skn7p activity is modulated by the Sln1p-Ypd1p osmosensor and contributes to regulation of the HOG pathway. *Mol Gen Genet.* 259(4):372-8.

Kets, E.P., Galinski, E.A., de Wit, M., de Bont, J.A. and Heipieper, H.J. (1996) Mannitol, a novel bacterial compatible solute in Pseudomonas putida S12. *J Bacteriol.* **178(23)**, 6665-70.

Kielland-Brandt, M.C., (1994). Industrial *Saccharomyces* yeasts. In. Molecular Genetics of Yeast, A Practical Approach. Eds. D. Rickwood and B.D. Hames, Oxford University Press. 245-259.

King, L. M., Schisler, D. O., and Ruocco, J. J. (1981) Epifluorescent method for detection of non-viable yeast. *J. Am. Soc. Brew. Chem.* **39**,52-55.

Kirsop, B. E. (1991) Maintenance of yeasts. In maintenance of microorganisms and cultures cells pp161-182. Eds. B. E. Kirsop and A. Doyle. Academic Press.

Klionsky, D. J. (1998). Nonclassical protein sorting to the yeast vacuole. *J. Biol. Chem.* **273**(18), 10807-10810

Knop, M., Schiffer, H.H., Rupp, S, and Wolf, D.H. (1993). Vacuolar/lysosomal proteolysis: proteases, substrates, mechanisms. *Curr. Opin. Cell Biol.* **5**(6), 990-6.

Knudsen, F. B. (1985). Fermentation variables and their control. *J. Am. Soc. Brew. Chem.* **43**, 91-95.

Kobayashi, N and McEntee, K. (1993) Identification of cis and trans components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae. Mol Cell Biol.* **13(1)**, 248-56.

Kollár, R., Petrakova, E., Ashwell, G., Robbins, P. W. and Cabib, E. (1995). Architecture of the yeast cell wall. The linkage between chitin and beta(1→ 3)-glucan. *J. Biol. Chem.* **270**(3), 1170-8.

Kollár, R., Reinhold, B. B., Petrakova, E., Yeh, H. J., Ashwell, G., Drgonova, J., Kramer, R., Kopp, F., Niedermeyer, W. and Fuhrmann, G. F. (1997) Architecture of the yeast cell wall. Beta(1→ 6)-glucan interconnects mannoprotein, beta(1→ 3) glucan, and chitin. *J. Biol. Chem.* **272**(28):17762-75.

Kreger van Rij, N.J.W and Veenhuis M., (1975). Electron microscopy of ascus formation in the yeast *debaryomyces hansenii. J. Gen. Microbiol.* **89**(2), 256-64.

Kreger van Rij, N.J.W., (1987). Classification of yeasts. In. The Yeasts, Volume 1. Biology of the yeasts. Eds. A.H. Rose and J.S. Harrison. Academic press. 5-61.

Kucharczyk, R. and Rytka, J. (2001). *Saccharomyces cerevisiae* a model organism for the studies on vacuolar transport. *Acta Biochim. Pol.* **48**(4), 1025-42.

Kurtzman, C.P., (1984). In. The Yeasts, a taxonomic study. Ed. N.J.W. Kreger van Rij. Elsevier Biomedical Press, Amsterdam. 164-223.

Kurtzman, C.P. (1994) Molecular taxonomy of the yeasts. *Yeast.* **10(13),** 1727-40.

Laizé, V., Tacnet, F., Ripoche, P., and Hohmann, S. (2000). Polymorphism of Saccharomyces cerevisiae aquaporins. *Yeast,* **(16)** 897-903.

Larsson, K., Ansell, R., Eriksson, P. and Adler L (1993). A gene encoding sn-glycerol 3-phosphate dehydrogenase (NAD+) complements an osmosensitive mutant of *Saccharomyces cerevisiae. Mol. Microbiol.* **10**(5), 1101-11.

Laize, V., Tacnet, F., Ripoche, P. and Hohmann S (2000) Polymorphism of *Saccharomyces cerevisiae* aquaporins. *Yeast.* 16(10),897-903.

Latterich, M. and Watson, M. D. (1991). Isolation and characterization of osmosensitive vacuolar mutants of *Saccharomyces cerevisiae. Mol. Microbiol.* **5**(10), 2417-26.

Latterich, M. and Watson, M.D. (1993). Evidence for a dual osmoregulatory mechanism in the yeast Saccharomyces cerevisiae. *Biochem. Biophys. Res. Commun.* **191**(3), 1111-7.

Lentini, A., Jones, R. D. and Wheatcroft, R. (1990) Metal ion uptake by yeast. *Proc. Inst. Guild Brew. Conv.* Melbourne, 198-9.

Lentini, A. (1993) A review of the various methods available for monitoring the physiological status of yeast: yeast viability and vitality. *Ferment* **6**, 321-327.

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.* **1192(1),** 7-13.

Leslie, S.B., Israeli, E., Lighthart, B., Crowe, J.H. and Crowe, L.M (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Appl Environ Microbiol.* **61(10)**:3592-7.

Levy, M.S., Lotfian, P., O'Kennedy, R., Lo-Yim, M.Y. and Shamlou, P.A.(2000). Quantitation of supercoiled circular content in plasmid DNA solutions using a fluorescence-based method. *Nucleic. Acids. Res.* **28**(12)57.

Lewis, C. W., Johnston, J. R. and Martin, P. A. (1976). The genetics of yeast flocculation *J. Inst. Brew.*, **82** 158.

Lewis, J.G., Learmonth, R.P. and Watson, K.(1993). Role of growth phase and ethanol in freeze-thaw stress resistance of Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **59**(4):1065-71.

Lewis, M. J. and Young, T. W. (1995) in Brewing. Chapman and Hall, London

Li, H., Lee, S. and Jap, B. K. (1997). Molecular design of aquaporin-1 water channel as revealed by electro crystallography. *Nature Struc. Biol.* 4 263-265.

Liden, G., Walfridsson, M., Ansell, R., Anderlund, M., Adler, L. and Hahn-Hagerdal, B. (1996) A glycerol-3-phosphate dehydrogenase-deficient mutant of *Saccharomyces cerevisiae* expressing the heterologous *XYL1* gene. *Appl. Environ. Microbiol.* **62(10),** 3894-6.

Lie, S., and Jacobsen, T. (1983) The absorption of zinc by brewer's yeast. *Proc. Eur. Brew. Conv.* London. 145-51.

Lieckfeldt, E., Meyer, W. and Borner, T. (1993) Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. *J Basic Microbiol.* **33(6)**,413-25.

Lillie, S. H. and Pringle, J. R. (1980). Reserve carbohydrate metabolism in *Saccharomyces cerevisiae* : responses to nutrient limitation. *J. Bacteriol.* **143**, 1384-1394.

Lindquist S. (1986) The heat-shock response. *Annu. Rev. Biochem.*55, 1151-91.

Lloyd, D., and Hayes, A. J. (1995) Vigour, vitality and viability of microorganisms. *FEMS Micro. Lett.* **133**,1-7.

Lockhart, L., Oliver, S.G. and Delneri, D. (2002). Tools for the study of genome rearrangements in laboratory and industrial yeast strains. *Yeast,* **19(5)**:441-8.

Lodder, J., (1970). In. The Yeasts. 2nd Ed. North Holland Publishing Company. Amsterdam and London.

Lorenz, R.T., and Parks, L.W. (1991) Involvement of heme components in sterol metabolism of *Saccharomyces cerevisiae. Lipids.* **26(8)**,598-603.

Loveridge, D., Ruddlesden, J. D., Noble, C. S. and Quain, D. E., (1999) Improvements in brewery fermentation performance by 'early' yeast cropping and reduced storage time. *Proc. 7th Inst. Brew. Conv.* Nairobi 95-9.

Lucau-Danila, A., Wysocki, R., Roganti, T. and Foury, F. (2000) Systematic disruption of 456 ORFs in the yeast *Saccharomyces cerevisiae. Yeast.***16(6)**,547-52.

Lucht, J. M. and Bremer, E. (1994). Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system proU. *FEMS Microbiol. Rev.*, **14(1)**, 3-20.

Lum, P.Y. and Wright, R. (1995) Degradation of HMG-CoA reductase-induced membranes in the fission yeast, *Schizosaccharomyces pombe. J Cell Biol.* **131(1)**,81-94

Lundberg, K.S., Shoemaker, D.D., Adams, M.W., Short, J.M., Sorge, J.A. and Mathur, E.J. (1991). High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus. Gene* **108**(1),1-6.

Luyten, K., Albertyn, J., Skibbe, F., Prior, B.A., Ramos, J., Thevelein, J.M., and Hohmann, S. (1994). The FPS1 gene product functions as a glycerol facilitator in the yeast *Saccharomyces cerevisiae. Folia Microbiol (Praha)* **39(6)**, 534-6.

Luyten, K., Albertyn, J., Skibbe, F., Prior, B.A., Ramos, J., Thevelein, J.M., and Hohmann, S. (1995). Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *EMBO J* **14(7)**,1360-71.

Lyons, T. P. and Hough, J. S. (1970). The role of yeast cell walls in brewing. *Brew. Digest.* **Aug**. 52-60.

Mackenzie, K. F., Blomberg, A. and Brown, A. D. (1986). Water stress plating hypersensitivity of yeast: protective role of trehalose in *Saccharomyces cerevisiae. J Gen Microbiol* **12**, 2053-2056.

Madhani, H.D. and Fink, G.R. (1998). The riddle of MAP kinase signalling specificity. *Trends Genet.* **14**(4) 151-5.

Maeda, T., Takekawa, M. and Saito, H. (1995). Activation of yeast PBS2 MAPKK by MAPKKK or by binding of an SH3-containing osmosensor. *Science*, **269**, 554-558.

Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature,* **369**, 242-245.

Mager, W.H. and De Kruijff, A. J. (1995). Stress-induced transcriptional activation. *Microbiol. Rev.* **59**(3), 506-31.

Mager, W. H. and Varela, J. C. S. (1993). Osmostress response of the yeast *Saccharomyces. Mol Microbiol* **10**, 253-258

Magné, C. and Larher, F. (1992). High sugar content of extracts interferes with colorimetric determination of amino acids and free proline *Anal. Biochem.* **200**, 115-118.

Maquat, L.E.(2002) Molecular biology. Skiing toward nonstop mRNA decay. *Science.* **295(5563)**,2221-2.

Marchler, G., Schuller, C., Adam, G. and Ruis, H. A. (1993) *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J.* **12(5)**, 1997-2003.

Marechal, P.A., and Gervais, P. (1994) Yeast viability related to water potential variation: influence of the transient phase. *Appl. Microbiol. Biotech.* **42**, 617-622.

Marquez, J.A., Pascual-Ahuir, A., Proft, M. and Serrano, R. (1998) The Ssn6-Tup1 repressor complex of *Saccharomyces cerevisiae* is involved in the osmotic induction of HOG-dependent and -independent genes. *EMBO J.* **17(9)**,2543-53.

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.* **15(9)**, 2227-35

Martini, A.V. and Martini, A. (1987) Three newly delimited species of *Saccharomyces sensu stricto. Antonie Van Leeuwenhoek.* **53(2)**,77-84.

Masneuf, I., Aigle, M., and Dubourdieu, D. (1996). Development of a polymerase chain reaction/restriction fragment length polymorphism method for *Saccharomyces cerevisiae* and *Saccharomyces bayanus* identification in enology. *FEMS Microbiology Letts*, **138** (2), 239-244.

Matile, P. H., and Wiemken, A. (1967). The vacuole as the lysosome of the yeast cell. *Ann. Rev. Plant Phys.*, **29**,193.

Matile, P. (1978). Biochemistry and function of vacuoles. *Annu. Rev. Plant Physiol.* **29,** 193-213.

Matsumoto, T. K., Ellsmore, A. J., Cessna, S. G., Low, P. S., Pardo, J. M., Bressan, R. A. and Hasegawa, P. M. (2002). An osmotically induced cytosolic Ca^{2+} transient activates calcineurin signalling to mediate ion homeostasis and salt tolerance of *Saccharomyces cerevisiae J. Biol. Chem.* **277(36)**, 33075-80.

McCaig, R. (1990) Evaluation of the fluorescent dye 1-anilino-8-naphthalene sulphonic acid for yeast viability determination. *J. Am. Soc. Brew. Chem.* **48**, 22-25

McIntosh, A.L., Gallegos, A.M., Atshaves, B.P., Storey, S.M., Kannoju, D. and Schroeder, F. (2003) Fluorescence and multiphoton imaging resolve unique structural forms of sterol in membranes of living cells. *J Biol. Chem.* **278(8)**,6384-403.

McLaurin, J., Franklin, T., Fraser, P.E. and Chakrabartty, A.(1998) Structural transitions associated with the interaction of Alzheimer beta-amyloid peptides with gangliosides. *J. Biol. Chem.* **273**, 4506-4515.

Meilgaard, M. (1975) Flavour and threshold of beer volatiles. *Tech, Quart. MBAA*, **11**, 87-9.

Mendoza, I., Quintero, F. J., Bressan, R. A., Hasegawa, P. M., and Pardo, J. M. (1996). Activated calcineurin confers high tolerance to ion stress and alters the budding pattern and cell morphology of yeast cells. *J. Biol. Chem.* **271**, 23061-23067.

Mendoza, I., Rubio, F., Rodriguez-Navarro, A., and Pardo, J. M. (1994). The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **269**, 8792-8796.

Mewes, H.W., Albermann, K., Bahr, M., Frishman, D., Gleissner. A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, S.G. and Pfeiffer F, Zollner (1997). An Overview of the yeast genome. *Nature* **387**(6632 Suppl) 7-65.

Meyer, S. A. and Phaff, H. J. (1969) Deoxyribonucleic Acid Base Composition in Yeasts. *J. Bacteriol.* **97(1)**, 52–56.

Meyrial, V., Laize, V., Gobin, R., Ripoche, P., Hohmann, S. and Tacnet, F. (2001). Existence of a tightly regulated water channel in *Saccharomyces cerevisiae Eur. J. Biochem*, **268**, 334-343.

Middaugh, C.R., Evans, R.K., Montgomery, D.L. and Casimiro, D. R. (1998). Analysis of plasmid DNA from a pharmaceutical perspective. *J. Pharm. Sci.* **87**(2),130-46.

Miekle, A. J., Chudeck, J. A., Reed, R. H. and Gadd, G. M. (1991). Natural abundance ^{13}C nuclear magnetic resonance spectroscopic analysis of acyclic polyol and trehalose accumulation by several yeast species in response to salt stress. *FEMS Microbiol. Letts.*, **82**, 163.

Millard, P.J., Roth, B.L., Thi, H.P., Yue. S.T. and Haugland, R.P. (1997). Development of the FUN-1 family of fluorescent probes for vacuole labelling and viability testing of yeasts. *Appl. Environ. Microbiol.* **63**(7),2897-905.

Miller, S. M. and Magasanik, B. (1990). Role of NAD-linked glutamate dehydrogenase in nitrogen metabolism in *Saccharomyces cerevisiae*. *J. Bacteriol.* **172**(9), 4927-35.

Molzahn, S.W., (1993). Developments in yeast science and technology. *Proc. Euro. Brew. Conv. Cong.*, **24**, 203-219.

Mondal, A.K., Parui, S. and Mandal, S. (1998). Analysis of the free amino acid content in pollen of nine Asteraceae species of known allergenic activity. *Ann. Agric. Environ. Med.* 5(1), 17-20.

Morimoto, R.I., Kroeger, P.E. and Cotto, J.J. (1996) The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions. In: Stress inducible cellular responses. pp 139-163 Feige, U, Morimoto, R. I., Yahara, I, Polla, B.S.(Eds) Birkhauser Verlag, Boston.

Montrocher, R., Verner, M.C., Briolay, J., Gautier, C. and Marmeisse, R. (1998) Phylogenetic analysis of the *Saccharomyces cerevisiae* group based on polymorphisms of rDNA spacer sequences.*Int. J. Syst. Bacteriol.* **48(1)**,295-303.

Moon, C., Preston, G.M., Griffin, C.A., Jabs, E.W. and Agre, P. (1993). The human aquaporin-CHIP gene. Structure, organization, and chromosomal localization. *J. Biol. Chem.* **268**(21),15772-8.

Montijn, R. C., Vink, E., Müller, W. H., Verkleij, A. J., Van Den Ende, H., Henrissat, B. and Klis, F. M. (1999). Localization of Synthesis of β1,6-Glucan in *Saccharomyces cerevisiae. J. Bacteriol.* **181**, 7414-7420.

Moon, C., Williams, J.B., Preston, G. M., Copeland, N.G., Gilbert, D.J., Nathans, D., Jenkins, N. A. and Agre, P. (1995). The mouse aquaporin-1 gene. *Genomics,* **30**(2), 354-7.

Morris, G. J., Winters, L., Coulson, G. E and Clarke, K. J. (1986). Effect of osmotic stress on the ultrastructure and viability of the yeast *Saccharomyces cerevisiae. J. Gen. Microbiol.*, **132**, 2023-2034.

Moye, W.S., Amuro, N., Rao, J.K. and Zalkin, H. (1985). Nucleotide sequence of yeast *GDH1* encoding nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase. *J. Biol. Chem.* **260** (14), 8502-8.

Murguia, J. R., Belles, J. M. and Serrano, R. (1995). A salt-sensitive 3'(2'),5'-bisphosphate nucleotidase involved in sulfate activation. *Science* **267,** 232–234.

Murguia, J. R., Belles, J. M. and Serrano, R. (1996). The yeast *HAL2* nucleotidase is an in vivo target of salt toxicity. *J. Biol. Chem.* **271,** 29029–29033.

Murray, C . R., Barich, T. and Taylor, D. (1984). *Tech. Quart. Master Brewers Assoc. Americas*, **21**, 189.

Murty, V.N. and Stevens, C.F. (1998) Vesicles retain their identity through the endocytic cycle. *Nature* 392, 497-501.

Mycielska, M.E., Schafer, M., Fraser, S.P., Djamgoz, M.B. and Bashford, C.L. (2000) A study of membrane activity in rat prostate cancer cells: an evaluation of the FM1-43 dye technique. *Biosci. Rep.* **20**, 31-40.

Nass, R. and Rao, R. (1999). The yeast endosomal Na+/H+ exchanger, Nhx1, confers osmotolerance following acute hypertonic shock. *Microbiology* **145**(11), 3221-8.

Naumov, G. I. (1996).Genetic identification of biological species in *Saccharomyces sensu stricto* complex. *Can. J. Microbiol.* **42**, 335-339.

Nedergaard, M., Desai, S. and Pulsinelli, W. (1990). Dicarboxy-dichlorofluorescein: a new fluorescent probe for measuring acidic intracellular pH. *Anal. Biochem.* **187**(1):109-14.

Nelson, H., Mandiyan, S.and Nelson, N. (1995) A bovine cDNA and a yeast gene (VMA8) encoding the subunit D of the vacuolar H(+)-ATPase. *Proc. Natl. Acad. Sci.USA.* **92(2)**, 497-501.

Nelson, B., Parsons, A.B., Evangelista, M., Schaefer, K., Kennedy, K., Ritchie, S., Petryshen, T.L. and Boone, C. (2004) Fus1p interacts with components of the HOG1p mitogen-activated protein kinase and Cdc42p morphogenesis signalling pathways to control cell fusion during yeast mating. *Genetics.* **166(1)**,67-77.

Nevitt, T., Pereira, J., Azevedo, D., Guerreiro, P. and Rodrigues-Pousada, C. (2004) Expression of YAP4 in *Saccharomyces cerevisiae* under osmotic stress. *Biochem J.* **379(2)**, 367-74.

Nevoigt, E., and Stahl, U. (1997). Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae. Microbiol. Rev.*, **21**, 231-241.

Nishikawa, S., Umemoto, N., Oshumi, Y., Nakano, A. and Anraku, Y. (1990). Biogenesis of vacuolar glycoproteins of yeast *Saccharomyces cerevisiae. J. Biol. Chem.*, **265**(13), 7440-7448.

Nishino,T., Hata, S., Osumi, T. and Katsuki, H. (1980) Biosynthesis of ergosterol in cell-free system of yeast. *J. Biochem.* **88(1)**247-54.

Norbeck, J. and . Blomberg, A. (1996). Protein expression during exponential growth in 0.7M NaCl medium of *Saccharomyces cerevisiae. FEMS Microbiol. Letts.*, **137**,1-8.

Norbeck, J. and Blomberg, A. (1997). Metabolic and regulatory changes associated with growth of *Saccharomyces cerevisiae* in 1.4M NaCl: evidence for osmotic induction

of glycerol dissimilation via the dihydroxyacetone pathway. *J. Biol. Chem.*, **272**, 5544-5554.

Norbeck, J. and Blomberg. A. (1998). Amino acid uptake is strongly affected during exponential growth of *Saccharomyces cerevisiae* in 0.7M NaCl medium. *FEMS Microbiol. Letts.*, **158,** 121-126.

Norbeck, J., Pahlman, A.K., Akhtar, N., Blomberg, A, and Adler L. (1996). Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from *Saccharomyces cerevisiae*. Identification of the corresponding *GPP1* and *GPP2* genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway *J. Biol. Chem.* **271**(23), 13875-81.

O'Connor-Cox, E. (1997). Improving yeast handling in the brewery. Part 1: Yeast cropping. *The Brewers Guardian*, **126**, 26-34.

O'Connor-Cox, E. (1998). Improving yeast handling in the brewery. Part 2: Yeast collection. *The Brewers Guardian*, **122**(2), 22-34.

Odumeru, J. A., D'Amore, T., Russell, I. and Stewart, G. G. (1992). Effect of heat shock and ethanol stress on the viability of a *Saccharomyces uvarum* (*carlsbergensis*) brewing yeast strain during fermentation of high gravity wort. *J. Ind. Micro.* **10**, 111-116.

Ohsumi, Y. and Anraku, Y. (1983) Calcium transport driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae. J Biol Chem.* **258(9),** 5614-7.

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.* **1613(1-2),**57-71.

O'Rourke, S.M and Herskowitz, I. (1998). The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in *Saccharomyces cerevisiae. Genes Dev.* **12**(18), 2874-86.

Osumi, M. (1998). The ultrastructure of yeast: wall structure and formation. *Micron*, **29**, 207-223.

Padilla, P.A., Fuge, E.K., Crawford, M.E., Errett, A. and Werner-Washburne, M. (1998). The highly conserved, coregulated SNO and SNZ gene families in *Saccharomyces cerevisiae* respond to nutrient limitation. *J. Bacteriol.* **180**(21), 5718-26.

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.* **6**(5), 3555-63.

Panaretou, B. and Piper, P.W. (1992). The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and the entry to stationary phase. *Eur. J. Biochem.* **206**(3), 635-40.

Panchal, C. J., and Stewart, G. G. (1980). The effect of osmotic pressure on the production and excretion of ethanol and glycerol in brewing yeast strains *J. Inst. Brew.*, **86**: 207-210.

Parks, L.W. (1978) Metabolism of sterols in yeast. *Crit. Rev. Microbiol.***6(4)**, 301-41.

Parrou, J. L., Teste, M. A. and Francois, J. (1997). Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. *Microbiology* **143**(6), 1891-1900.

Parrou, J.L., Enjalbert, B., Plourde, L., Bauche, A., Gonzalez, B. and Francois, J. (1999a). Dynamic responses of reserve carbohydrate metabolism under carbon and nitrogen limitations in *Saccharomyces cerevisiae*. *Yeast* **15**(3),191-203.

Parrou, J.L., Enjalbert, B., and Francois, J. (1999b). STRE- and cAMP-independent transcriptional induction of *Saccharomyces cerevisiae GSY2* encoding glycogen synthase during diauxic growth on glucose. *Yeast* **15**(14):1471-84.

Pavlik, P., Simon, M., Schuster, T. and Ruis, H. (1993). The glycerol kinase (*GUT1*) gene of *Saccharomyces cerevisiae*: cloning and characterization. *Curr. Genet.* **(1-2)**,21-5.

Pelham, H.R. (1999) SNAREs and the secretory pathway-lessons from yeast. *Exp. Cell. Res.* **247(1)**,1-8.

Pierandrei-Amaldi, P., Beccari, E., Bozzoni, I. and Amaldi, F. (1985) Ribosomal protein production in normal and anucleolate Xenopus embryos: regulation at the posttranscriptional and translational levels. *Cell.* **42(1)**, 317-23.

Phaff, H.J., (1984). In. The Yeasts, a taxonomic study. Ed. N.J.W. Kreger van Rij. Elsevier Biomedical Press, Amsterdam. 125-129.

Philips, J. and Herskowitz, I. (1997). Osmotic balance regulates cell fusion during mating in *Saccharomyces cerevisiae*. *J. Cell Biol.* **138**(5), 961-74.

Pierce, J. (1970) Institute of Brewing Analysis Committee: Measurement of yeast viability, *J. Inst. Brew.* **34**, 306-312.

Planta, R.J., Brown, A.J., Cadahia, J.L., Cerdan, M.E., de Jonge, M., Gent, M.E., Hayes, A., Kolen, C.P., Lombardia, L.J., Sefton, M., Oliver, S.G., Thevelein, J., Tournu, H., van Delft, Y.J., Verbart, D.J. and Winderickx, J.(1999) Transcript analysis of 250 novel yeast genes from chromosome XIV. *Yeast.* **15(4)**,329-50.

Poolman, B. and Glaasker, E. (1998). Regulation of compatible solute accumulation in bacteria. *Mol. Microbiol.*, **29**(2), 397-407.

Posas, F. and Saito, H. (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science* **276**(5319) 1702-5.

Posas, F., Takekawa, M and Saito, H. (1998). Signal transduction by MAP kinase cascades in budding yeast. *Curr. Opin. Microbiol.* **1**(2) 175-82.

Posas, F., Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., Thai, T. C. and Saito, H. (1996). Yeast *HOG1* MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the *SLN1-YPD1-SSK1* "two-component" osmosensor. *Cell.*, **86**(6), 865-75.

Posas, F., Chambers, J.R., Heyman, J.A., Hoefflerm J.P., de Nadal, E. and Arino, J. (2000) The transcriptional response of yeast to saline stress. *J. Biol. Chem.* **275(23)**,17249-55

Postma, E., Verduyn, C., Scheffers, W. A. and Van Dijken ,J. P. (1989) Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **55 (2)**, 468–477.

Powell. C.D (chair) (2003) 2003 Subcommittee report: Determination of Yeast Viability by Fluorescent Staining. *J. Am. Soc. Brew. Chem.* **61(4)**, 231-232.

Pratt-Marshall, P., Stewart, G.G. and Bryce,J. (2002a) High gravity brewing effects on yeast vacuolar morphology. In, Proceedings of the 27th Convention of the Institute and Guild of Brewing Asia Pacific Section, Paper 10, pp.1-10.

Pratt-Marshall, P.L., Brey, S.E., de Costa, S.D., Bryce, J.H. and Stewart, G.G. (2002b) High gravity brewing - an inducer of yeast stress. *Brewers' Guardian*, **131**, 22-26.

Prista, C., Almagro, A., Loureiro-Dias, M.C. and Ramos, J. (1997). Physiological basis for the high salt tolerance of *Debaryomyces hansenii*. *Appl. Environ. Microbiol.* **63**(10) 4005-9.

Projan, S.J., Carleton, S. and Novick, R.P. (1983). Determination of plasmid copy number by fluorescence densitometry. *Plasmid* **9**(2),182-90.

Prudencio, C., Sansonetty, F., and Corte-Real, M. (1998) Flow cytometric assessment of cell structural and functional changes induced by acetic acid in yeasts *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*. *Cytometry* **31**,307–313.

Puig, S. and Perez-Ortin, J. E. (2000). Stress response and expression patterns in wine fermentations of yeast genes induced at the diauxic shift. *Yeast* **16**(2),139-48.

Quain, D. E. (1986). Differentiation of brewing yeast. *J. Inst. Brew.* **92**, 435-438.

Quain, D. E. (1995). Yeast supply – the challenge of zero defects. *Proc. Eur. Brew Con.* **25**, 309-318.

Quain, D. E., Thurston, P. A. and Tubb, R. S. (1981). The structural and storage carbohydrates of *Saccharomyces cerevisiae*: changes during fermentation of wort and a role for glycogen catabolism in lipid biosynthesis. *J. Inst. Brew.*, **87**, 108-111.

Rainina EI, Zubatov AS, Luzikov VN (1980). Glucose repression and autolysis of *Saccharomyces cerevisiae* cells: alterations in the cytochemical localization of acid phosphatase. *Histochem. J.* **12**(1), 57-69.

Rainina, E.I., Zubatov, A.S., Buchwalow, I.B., and Luzikov, V,N. (1979). A cytochemical study of the localization of acid phosphatase in *Saccharomyces cerevisiae* at different growth phases. *Histochem. J.* **11**(3),299-310.

Ramos J.P.; Valente P.; de Souza R.A.; Rosa C.A.; Leoncini O (2001). Heteroduplex mobility assay of the D1/D2 region of the 26S rDNA for differentiation of *Saccharomyces* species. *Lett Appl Microbiol.* **33**(3), 206-210.

Raymond, C.K., O'Hara, P.J., Eichinger, G., Rothman, J.H.and Stevens, T.H. (1990). Molecular analysis of the yeast *VPS* 3 gene and the role of its product in vacuolar protein sorting and vacuolar segregation during the cell cycle. *J. Cell Biol.* **111**, 877-92.

Raynal, L., Barnwell, P., and Gervais, P. (1994). The use of epi-fluorescence to determine the viability of Saccharomyces cerevisiae subjected to osmotic shifts. J. *Biotechnol.* **36**,121–127.

Reed, R.H., Chudeck, J. A., Foster, R. and Gadd, G. M.(1987).Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Appl. Env. Micro.*, **53** (9), 2119-2123.

Reggiori, F., and Pelham, H.R.(2001) Sorting of proteins into multivesicular bodies: ubiquitin-dependent and-independent targeting. *EMBO J.* **20(18)**,5176-86.

Reilly, D.I., O'Shea, D. G. and Walsh, P. K. (2003) Development of an image analysis technique to monitor vacuolar morphology during high-gravity brewing. *Proc. 29th EBC (Dublin).* P59.

Reiser, V., Raitt, D.C. and Saito, H. (2003) Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure. *J. Cell. Biol.* 161(6),1035-40.

Remize, F., Barnavon, L. and Dequin, S. (2001). Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in Saccharomyces cerevisiae. *Metab. Eng.* **(4)**, 301-12.

Rep, M., Reiser, V., Gartner, U., Thevelein, J.M., Hohmann, S., Ammerer, G. and Ruis, H. (1999) Osmotic stress-induced gene expression in Saccharomyces cerevisiae requires Msn1p and the novel nuclear factor Hot1p. *Mol Cell Biol.* **19(8)**, 5474-85.

Rep, M., Krantz, M., Thevelein, J.M. and Hohmann, S. (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *J Biol Chem.***275(12)**, 8290-300.

Reynolds, T.B., Hopkins, B.D., Lyons, M.R., Graham, T.R. (1998) The high osmolarity glycerol response (HOG) MAP kinase pathway controls localization of a yeast golgi glycosyltransferase. *J. Cell. Biol.* **143(4)**,935-46.

Rhymes. M. R. and Smart, K.A. (1996). Effect of starvation on the surface properties of brewing yeast. *Proc. 2nd Con. Ferm. Physiol: Inst. Chem. Eng.*, 34-36.

Ribchester, R. R., Mao, F. and Betz, W. J. (1994) Optical measurements of activity-dependent membrane recycling in motor nerve terminals of mammalian skeletal muscle. *Proc. R. Soc. Lond. B. Biol. Sci.* **255**, 61-66.

Riezman, H. (1985) Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell.* **40(4)**, 1001-9.

Roberts, C.J., Raymond, C.K., Yamashiro, C.T. and Stevens, T.H. (1991)Methods for studying the yeast vacuole. *Methods Enzymol.* **194**, 644-661.

Ronnow, B. and Kielland-Brandt, M.C. (1993). *GUT2*, a gene for mitochondrial glycerol 3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. *Yeast* **(10),** 1121-30.

Rotmann, B. and Papermaster, B. W. (1966). Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc. Natl. Acad. Sci. USA*. **55**, 134-141.

Ruis, H. and Schüller, C. (1995). Stress signalling in yeast. *Bioessays* **17**, 959-965.

Ryu, S.L., Murooka, Y. and Kaneko, Y. (1996). Genomic reorganization between two sibling yeast species, *Saccharomyces bayanus* and *Saccharomyces cerevisiae*. Yeast.**12**(8),757-64.

Sajbidor, J. (1997). Effect of some environmental factors on the content and composition of microbial membrane lipids. *Crit. Rev. Biotechnol.* **17**(2), 87-103.

Sambrook, J., Maniatis, T., and Fritsch, E.F. (1989).In Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3.

Sano, A., Kurita, N., Iabuki, K., Coelho, R., Takeo, K., Nishimura, K., and Miyaji, M. (1993). A comparative study of four different staining methods for estimation of live yeast from cells of *Paracoccidioides brasiliensis*. *Mycopathologia* **124**,157– 161.

Santos E, Leal F, Sentandreu R (1982). The plasma membrane of Saccharomyces cerevisiae. Molecular structure and asymmetry. *Biochim. Biophys. Acta.* **685** (3), 329-39.

Schenberg-Frascino, A. and Moustacchi, E. (1972). Lethal and mutagenic effects of elevated temperature on haploid yeast. I. Variations in sensitivity during the cell cycle. *Mo.l Gen. Genet.* **115**, 243-257.

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. USA*. **93(12)**, 5777-82.

Schrick, K., Garvik, B. and Hartwell, L.H. (1997). Mating in *Saccharomyces cerevisiae*: the role of the pheromone signal transduction pathway in the chemotropic response to pheromone. *Genetics* **147**(1), 19-32.

Schwencke, J. (1977). Characteristics and integration of the yeast vacuole and cellular functions. *Phys. Vég.* **15**(3), 491-517.

Schwencke, J. (1991). Vacuoles, Internal membranous systems and vesicles. In. The Yeasts. Volume 4 2nd Ed. Academic Press Ltd.

Schwencke, J., Magana-Schwencke, N. and Laporte, J. (1977). Yeast protoplasts from stationary and starved cells: preparation, ultrastructure and vacuolar development. *Ann. Microbiol.* **128A**(1)3-18.

Seoighe, C. and Wolfe K. H. (1999). Yeast genome evolution in the post-genome era. *Curr. Op. Microbiol.* **2,** 548–554.

Serrano, R. (1996). Salt tolerance in plants and microorganisms: toxicity targets and defence responses. *Int. Rev. Cytol.*, **165**, 1-52.

Serrano, R., Márquez, J. A. and Ríos, G. (1997). Crucial factors in salt stress tolerance. In. yeast stress responses. Eds. S. Hohmann and W. H. Mager. R.G. Landes Company.

Sharma, S.C., Raj, D., Forouzandeh, M. and Bansal, M.P. (1996) Salt-induced changes in lipid composition and ethanol tolerance in *Saccharomyces cerevisiae. Appl Biochem Biotechnol.* **56(2)**,189-95.

Sheppard, C. J. R. and Shotton, D. M. (1997). In Confocal Scanning Microscopy. BIOS Scientific Publishers, Oxford UK.

Shirley, B.W. and Meagher, R.B. (1990) A potential role for RNA turnover in the light regulation of plant gene expression: ribulose-1,5-bisphosphate carboxylase small subunit in soybean. **18(11)**, 3377-85.

Simpson, W. J. and Hammond, J. R. M. (1989). The response of brewing yeast to acid washing. *J. Inst. Brew.*, **95**, 347-354.

Singer, M. A. and Lindquist, S. (1998). Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. *TIBTECH,* **16**, 460-468.

Smart, K. A. (1997). Brewing yeast fermentation performance. *Proc. 1st brewing yeast fermentation performance congress*, Oxford Brookes University Press.

Smart, K. A., (1999). Ageing in Brewing Yeast. *Brewers Guardian*, **132**(2), 19-24.

Smart, K. A., Boulton, C. A., Hinchcliffe, E., and Molzahn, S. (1995). Effect of physiological stress on the surface properties of brewing yeasts. *J. Am. Soc. Brew. Chem.*, **53**, 33-38.

Smart, K.A. (2000). The death of the yeast cell. In. Brewing Yeast Fermentation Performance. Ed. K. A. Smart. Blackwell Science, Oxford. 105-113.

Smart, K.A., Chambers, K. M., Lambert, I. and Jenkins, C. (1999). Use of methylene violet staining procedures to determine yeast viability and vitality. *J. Am. Soc. Brew. Chem.* **57**(1), 18-23.

Smart, K. A. (2001). The management of Brewing yeast stress. *Proc.28th Eur. Brew. Cong.* Budapest.

Sobczak, H. (1985) A simple disk-diffusion test for differentiation of yeast species *J. Med. Microbiol.* **20(3)**,307-16.

Spormann, D. O., Heim, J and Wolf, D. (1992). Biogenesis of the yeast vacuole (Lysosome). *J. Biol. Chem.* **267**(12), 8021-8029.

Srinivasan, S., Seaman, M,, Nemoto, Y., Daniell, L., Suchy, S.F., Emr, S., De Camilli, P. and Nussbaum, R. (1997). Disruption of three phosphatidylinositol-polyphosphate 5-phosphatase genes from *Saccharomyces cerevisiae* results in pleiotropic abnormalities of vacuole morphology, cell shape, and osmohomeostasis. *Eur. J. Cell. Biol.* **74**(4), 350-60.

Stark, M.J.R. (1999). Protein phosphorylation and dephosphorylation. In The metabolism and molecular physiology of *Saccharomyces cerevisiae.* Eds. Dickinson, J.R. and Schweizer, M. Taylor and Francis Ltd. pp 209-256.

Steels, E.L., Learmonth, R.P. and Watson, K. (1994). Stress tolerance and membrane lipid unsaturation in *Saccharomyces cerevisiae* grown aerobically or anaerobically. *Microbiology* **140** (3),569-76.

Stefan, C.J. and Blumer, K.J. (1999). A syntaxin homolog encoded by *VAM3* mediates down-regulation of a yeast G protein-coupled receptor. *J. Biol. Chem.* **274**(3), 1835-41.

Stewart, G. G. (1996). Yeast performance and management. *The Brewer*, 211-215

Stewart, G.G. and Russell, I. (1993). Fermentation, the black box of the brewing process. *MBAA Technical Quarterly,* **30**, 159-168.

Stewart, G.G., (1975). Yeast flocculation, practical implications and experimental findings. *Brew. Dig.*, **50**, 42-46.

Straver, M. H., Traas, V.M., Smit, G. and Kijne, J.W. (1994). Purification and partial characterisation of a flocculin from brewer's yeast. *Appl. Env. Micro.*, **60**, 2754-2758.

Sutherland, F. C. W., Lages, F., Lucas, C., Luyten, K., Albertyn, J., Hohmann, S., Prior, B. A., and Kilian, S. G. (1997). Characteristics of Fps1-Dependent and Independent Glycerol Transport in *Saccharomyces cerevisiae J. Bacteriol.*, **179**(24), 7790–7795.

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.* **47(4)**,405-11.

Tamai, Y., Momma, T., Yoshimoto. H. and Kaneko Y. (1998). Co-existence of two types of chromosome in the bottom fermenting yeast, *Saccharomyces pastorianus. Yeast,* **14**(10), 923-33.

Tamas, M. J., Luyten, K., Sutherland, F.C., Hernandez, A., Albertyn, J., Valadi, H., Li, H., Prior, B.A., Kilian, S.G., Ramos, J., Gustafsson, L,, Thevelein, J.M. and Hohmann, S. (1999). Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.*, **31**(4), 1087-104.

Tamas, M.J., Luyten, K., Sutherland, F.C., Hernandez A., Albertyn. J, Valadi, H., Li, H., Prior, B.A., Kilian, S.G., Ramos, J., Gustafsson, L., Thevelein, J.M and Hohmann, S. (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.* **31(4)**,1087-104.

Tamas, M. J. Rep. M., Thevelein, J.M. and Hohmann, S. (2000). Stimulation of the yeast high osmolarity glycerol (HOG) pathway: evidence for a signal generated by a change in turgor rather than by water stress. *FEBS Lett.* **472**(1):159-65.

Tamas, M. J. and Hohmann, S (2003) In yeast stress responses. Hohmann, S. and Mager W. H.(Eds). Springer-Verlag. Berlin. pp 121-200.

Tanghe, A., Van Dijck, P., Dumortier, F., Teunissen, A., Hohmann, S. and Thevelein, J.M. (2002) Aquaporin expression correlates with freeze tolerance in baker's yeast, and overexpression improves freeze tolerance in industrial strains. *Appl. Environ. Microbiol.* **68(12)**, 5981-9.

Toone, W. M. and Jones, N. (1998). Stress-activated signalling pathways in yeast. *Genes Cells,* **3**(8) 485-98.

Tornai-Lehoczki, J. and Dlauchy, D. (1996) An opportunity to distinguish species of *Saccharomyces sensu stricto* by electrophoretic separation of the larger chromosomes. *Lett. Appl. Microbiol.* **23(4)**,227-30.

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.* **243(1)**, 13-9.

Troll, W. and Lindsley, J. (1955). A photometric method for the determination of proline. *J. Biol. Chem.* **215**, 655-660.

Tsuji, T., Kawasaki, Y., Takeshima, S., Sekiya, T., and Tanaka, S. (1995). A new fluorescence staining assay for visualizing living microorganisms in soil. *Appl. Environ. Microbiol.* **61**,3415– 3421.

Tuller, G., Nemec, T., Hrastnik, C. and Daum, G. (1999) Lipid composition of subcellular membranes of an FY1679-derived haploid yeast wild-type strain grown on different carbon sources. *Yeast.***15(14)**, 1555-64.

Tunblad-Johansson, I. and Adler, L. (1987). Effect of sodium chloride concentration on phospholipid fatty acid composition of yeasts differing in osmotolerance. *FEMS Microbiol. Lett.* **43**, 275-278.

Uesono, Y. and Toh, E.A. (2002). Transient inhibition of translation initiation by osmotic stress. *J Biol Chem* **277**(16), 13848-55.

Ulber, R., Frerichs, J.G., and Beutel, S. (2003) Optical sensor systems for bioprocess monitoring. *Anal. Bioanal. Chem.* **376(3)**,342-8.

Valente, P., Gouveia, F.C., de Lemos, G.A., Pimentel. D., van Elsas, J.D., Mendonca-Hagler, L.C. and Hagler, A.N. (1996). PCR amplification of the rDNA internal transcribed spacer region for differentiation of *Saccharomyces* cultures. *FEMS Microbiology Letts.*, **137** (2), 253-256.

Van der Walt, J. P. (1970) Criteria and methods used in classification. In The yeasts – a taxonomic study. J. Lodder (Ed.) North-Holland Publishing Co., Amsterdam.

Van Dijken, J.P., Weusthuis, R.A. and Pronk, J.T. (1993) Kinetics of growth and sugar consumption in yeasts. *Antonie Van Leeuwenhoek* **63(3-4)**,343-52.

Van Drogen, F., Stucke, V.M., Jorritsma, G. and Peter, M. (2001). MAP kinase dynamics in response to pheromones in budding yeast. *Nature Cell Biol.* **(12),** 1051-9.

Van Zandycke, S.M., Simal, O., Gualdoni, S., and Smart, K.A. (2003) Determination of yeast viability using fluorophores. *J. Am. Soc. Brew. Chem.* **61(1)**, 15-22.

Varela, J.C. and Mager, W.H. (1996). Response of *Saccharomyces cerevisiae* to changes in external osmolarity. *Microbiology* **142** (4) 721-31.

Vaughan-Martini, A. and Martini, A. (1995). Facts, myths and legends on the prime industrial microorganism. *J. Ind. Microbiol.* **(6),** 514-22.

Vera-Estrella, R., Barkla, B.J., Bohnert, H.J., and Pantoja, O. (1999). Salt stress in *Mesembryanthemum crystallinum* L. cell suspensions activates adaptive mechanisms similar to those observed in the whole plant. *Planta* **207(3),** 426-35.

Vida, T.A. and Emr, S.D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J Cell Biol* **128**(5) 779-92.

Vidal-Leiria, M., Buckley, H. and van Unden, N., (1979). *Mycologia,* **71**, 497.

von Blohn, C., Kempf, B., Kappes, R.M. and Bremer, E. (1997) Osmostress response in Bacillus subtilis: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B. *Mol. Microbiol.* **1**, 175-87

Von Blohn C, Kempf B, Kappes RM, Bremer E. (1997) Osmostress response in *Bacillus subtilis:* characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B. *Mol. Microbiol.* 25(1),175-87.

Walker, G. M. (1998). In. Yeast Physiology and Biotechnology. John Wiley and Sons Ltd., Chichester, U.K.

Webster, J. (1993). In. Introduction To Fungi. 2nd Ed. Cambridge University Press. 267-278.

Wegmann, K. (1986). Osmoregulation in eukaryotic algae. *FEMS Microb. Rev.* **39**, 37-43.

Weisman, L.S., Emr, S.D. and Wickner, W.T. (1990). Mutants of *Saccharomyces cerevisiae* that block intervacuole vesicular traffic and vacuole division and segregation. *Proc. Natl. Acad. Sci.* USA **87**(3), 1076-80.

Weisman, L.S. (2003) Yeast vacuole inheritance and dynamics. *Annu Rev Genet.*;**37**, 435-60.

Welsh, D. T. and Herbert, R. A. (1999). Osmotically induced trehalose, but not glycine betaine accumulation promotes desiccation tolerance in *Escherichia coli. FEMS Microbiol. Letts.*, **174**, 57-63.

Werner-Washburne, M., Becker, J., Kosic-Smithers, J. and Craig, E.A. (1989). Yeast Hsp70 RNA levels vary in response to the physiological status of the cell. *J Bacteriol.* **171**(5), 2680-8.

Werner-Washburne, M., Braun, E., Johnston, G.C. and Singer, R.A. (1993). Stationary phase in the yeast *Saccharomyces cerevisiae. Microbiol. Rev.* **57**(2),383-401.

Werner-Washburne, M., Braun, E.L., Crawford, M.E. and Peck, V.M. (1996). Stationary phase in *Saccharomyces cerevisiae. Mol. Microbiol.* **19**(6),1159-66.

Whitmarsh, A.J. and Davis, R. J. (1998). Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. *Trends Biochem. Sci,* **(12)**, 481-5.

Wickerham, L.J., (1951). Taxonomy of the yeasts. Technical bulletin no.1029, United States Department of Agriculture. Washington D.C.

Winston, F., Dollard, C. and Ricupero-Hovasse, S.L. (1995) Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast.* **11(1)**,53-5.

Wiemken, A. (1990). Trehalose in yeast: stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek* **58**. 209-217.

Willer, M., Regnacq, M., Reid, P.J., Tyson, J.R., Cui, W., Wilkinson, B.M. and Stirling, C.J. (2000)Disruption and functional analysis of six ORFs on chromosome XII of *Saccharomyces cerevisiae*: YLR124w, YLR125w, YLR126c, YLR127c, YLR128w and YLR129w. *Yeast.* **16(15)**,1429-35.

Weisman, L.S., Bacallao, R. and Wickner, W. (1987) Multiple methods of visualizing the yeast vacuole permit evaluation of its morphology and inheritance during the cell cycle. *J. Cell Biol.* **105(4)**, 1539-47.

Wilcocks, K. L. and Smart, K. A. (1995). The importance of surface charge and hydrophobicity for the flocculation of chain-forming brewing yeast strains and resistance of these parameters to acid washing. *FEMS Microbiol. Letts.*, **134**, 293-297

Wilson, K. and McLeod, B.J. (1976) The influence of conditions of growth on the endogenous metabolism of *Saccharomyces cerevisiae*: effect on protein, carbohydrate, sterol and fatty acid content and on viability. *Antonie Van Leeuwenhoek.***42(4)**397-410.

Wolfe, K. H. and Shields, D. C. (1997). Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387,** 708–713.

Wood, J.M. (1999) Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol. Mol. Biol. Rev.* **63(1)**,230-62.

Wosnich, W., Zellnig. G., Kohlwein, S. D., Schaur, R . J., Bilinski, T., Tatzber, F. and Esterbauer, H. (2001). Ultrastructural analysis of HNE-treated *Saccharomyces cerevisiae* cells reveals fragmentation of the vacuole and an accumulation of lipids in the cytosol. *Cell Biochem. Funct.*, **19**, 51-58.

Yale, J. and Bohnert, H. J. (2001). Transcript Expression in *Saccharomyces cerevisiae* at High Salinity. *J. Biol. Chem.* **276** (19), 15996–16007.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. and Somero, G. N. (1982). Living with water stress: evolution of osmotic systems. *Science*, **217**, 1214-1222.

Yang, H.-C., Nemoto, Y., Homma, T., Matsuoka, H., Yamada, S., Sumita, O., Takatori, K., and Kurata, H. (1995). Rapid viability assessment of spores of several fungi by an ionic intensified fluorescein diacetate method. *Curr. Microbiol.* **30**,173–176.

Yurkow, E.J., and McKenzie, MA. (1993) Characterization of hypoxia-dependent peroxide production in cultures of *Saccharomyces cerevisiae* using flow cytometry: a model for ischemic tissue destruction. *Cytometry*.**14(3)**,287-93.