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The functional significance of Hsp12p and trehalose in desiccation and oxidative stress in the budding yeast *Saccharomyces cerevisiae*

by

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Foreword

The dissertation presented was performed under the supervision of Associate Professor George G. Lindsey at the Department of Molecular and Cell Biology, University of Cape Town, South Africa. I hereby declare that this dissertation, submitted for the degree of Master of Science in Molecular and Cell Biology at the University of Cape Town, is the result of my own investigations, apart from the referenced work of others.

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Abstract

The preservation of yeast viability and vitality during storage in the desiccated state is fundamental as several industrial processes utilise this technology. The significance of the stress response protein and putative hydrophilin, Hsp12p, was therefore examined *in vivo* under desiccation conditions. It was observed that the $\Delta hsp12$ strain was more desiccation tolerant than the wild type strain. The $\Delta hsp12$ strain was found to contain higher intracellular trehalose concentrations than the wild type strain. Furthermore, the results obtained suggest that trehalose acts as a ROS scavenger in yeast cells deficient in one or more stress response pathways. The results suggest that Hsp12p maintains the integrity of the plasma membrane *in vivo*. The significance of Hsp12p and trehalose in applied oxidative stress was consequently examined. We conclude that Hsp12p stabilises the yeast plasma membranes against membrane damaging stresses including desiccation and oxidative stress. Furthermore, we conclude that trehalose confers oxidative stress tolerance in *Saccharomyces cerevisiae* by acting as a ROS scavenger.

Abbreviations

α	alpha
β	beta
β -NADP	oxidised β -nicotinamide adenine dinucleotide
β -NADPH	reduced β -nicotinamide adenine dinucleotide
$^{\circ}\text{C}$	degree Celsius
Δ	delta, signifies gene deletion
$\Delta hsp12$	<i>HSP12</i> deletion strain
$\Delta\text{OD}_{340\text{nm}}$	change in optical density at 340 nm
μg	microgram (10^{-6} g)
μl	microliter (10^{-3} ml)
μm	micrometer
μmol	micromole
nmol	nanomole
ng	nanogram (10^{-9} g)
nm	nanometer
2-D	two dimensional
3-D	three dimensional
ARE	AP1 response element
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair(s)
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CDRE	calcineurin-dependant response elements
CFU	colony forming unit
CRE	Ca^{2+} /cAMP response-like element

CuRE	copper response element
Da	dalton (1 Da = 1.66×10^{-24} g)
DBD	DNA binding domain
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
FeRE	iron-responsive element
FRE	filamentous and invasion-responsive element
FS	forward scatter
G6P	glucose-6-phosphate
g	gram
GFP	green fluorescent protein
GSH	reduced glutathione
GSR	general stress response
GSSG	oxidised glutathione
h	hour
H	histone
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
HCl	hydrogen chloride
HOG	high osmolarity glycerol
Hsp	heat shock protein
Hsp12p	heat shock protein 12
HSE	heat shock element
HSR	heat shock response
IPTG	isopropyl- β -D-thiogalactopyranoside
K	kinase
kDa	kilodalton (10^3 Da)

l	litre
LEA	late embryogenesis-abundant
M	molar (mol.l^{-1})
MAP	mitogen activated protein
MDA	malondialdehyde
MgCl_2	magnesium chloride
mg	milligram (10^{-3} g)
min	minute
ml	millilitre (10^{-3} l)
mA	milliamperes (10^{-3} A)
mM	millimolar (10^{-3} M)
mW	milliwatts (10^{-3} W)
mU	milliunit of enzyme activity
N	normal
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium
NES	nuclear export signal
OH	hydroxyl group(s)
$\text{OD}_{600 \text{ nm}}$	optical density at 600 nm
ODU	optical density unit equivalent to approximately 3×10^7 cells ml^{-1}
PBS	phosphate buffered saline
PDSE	post-diauxic shift element
pI	isoelectric point
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
rpm	revolutions per minute

ROS	reactive oxygen species
RT	room temperature
s	seconds
SAB	sample application buffer
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
STRE	stress response element
T6P	trehalose-6-phosphate
TBARS	thiobarbituric acid reactive substances
TBP	TATA-box binding protein
TCA	trichloro-acetic acid
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	4-octylphenol polyethoxylate
Tween 80	Polyoxyethylenesorbitan monooleate
U	unit of enzyme activity
UDPG	Uridinediphosphate-glucose
V	volt
v/v	volume/volume
w/v	weight/volume
wt	wild type
YPD	Yeast extract peptone dextrose (glucose) media
ZRE	zymolyase response element

Chapter 1: General introduction

- 1.1 Yeast stress
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1.1 Yeast stress

One commonly used model eukaryotic microorganism used in genetic, physiological and applied studies is the budding yeast *Saccharomyces cerevisiae*. The response of yeast cells to stress reveals an immense amount of information that is pertinent to mammalian cells concerning ageing, cancer, apoptosis and even immunological responses. Industrial processes utilising yeast cells also benefit from yeast stress response studies. The response and adaptation of yeast cells to stress is complex, multifaceted and depends on the physiological state of the cells. Essentially, complex networks of sensing and signal transduction events occur in the cells in response to stress. The stress signals generated are transferred to the nucleus, activate transcriptional regulators and ultimately influence gene expression. This response allows adaptation to the imposed stress, which often involves the accumulation of both trehalose and stress proteins.

1.2 Yeast stress responses

The yeast stress response pathways control the induction, repression and de-repression of genes in response to various stresses. Most of the stress response pathways are mitogen activated protein (MAP) kinase pathways. These convey stress signals via phosphorelay events from the cell surface to transcription factors in the cytoplasm. Generally, a MAP kinase kinase kinase (MAPKKK) phosphorylates a MAP kinase kinase (MAPKK), which consequentially transfers a phosphate group to a MAP kinase (MAPK). The activated MAPK then phosphorylates transcription factors that appropriately alter gene expression. Non-MAP kinase pathways do exist e.g. target of rapamycin (Tor) pathway, as well as direct sensing mechanisms, which directly sense conformational changes in the cell structure [1, 2]. Extensive cross talk does occur between the response pathways. Thus it is not uncommon to find genes that are controlled by more than one pathway in response to a single stress. The four main MAP kinase pathways in *S. cerevisiae* are the general stress response (GSR), high osmolarity glycerol (HOG), heat shock response (HSR) and cell wall integrity pathways:

The GSR pathway is triggered by several stresses including, cold shock [3], heat shock, hyper-osmotic stress [2], alcohol, nutrient starvation [4] and oxidative stress [5]. There are approximately 60-190 general stress response genes [6]. The regulation of these genes is

controlled by the transcription factors Msn2/4p, which are redundant, interchangeable Cys₂His₂ zinc-finger proteins [7]. Under favorable conditions the GSR genes are negatively controlled by the Ras-cAMP-dependent protein kinase A (PKA) pathway [8]. The high PKA activity represses Msn2/4p, which are consequently located in the cytoplasm. During stress, the levels of cAMP diminish in the cells due to decreased adenylate cyclase activity, which results in low PKA activity. This consequentially alleviates Msn2/4p repression and allows Msn2/4p activation via phosphorylation. The activated Msn2/4p translocate to and accumulate in the nucleus, where they bind to the stress response elements (STREs). These elements are characterized by the consensus sequence AGGGG or CCCCT in the promoters of the stress response genes. They facilitate the transcription of the genes in response to numerous stresses [2, 9-11]. The Msn5p exportin contributes to the nuclear retention of Msn2/4p by recognising their phosphorylation states [12]. The GSR genes are involved in diverse cellular functions including carbohydrate metabolism and protein folding, transport and degradation.

The HOG pathway controls the expression of genes involved in adaptation to osmotic stress, which primarily involves glycerol accumulation. The two main proteins that control this pathway are Sln1p and Sho1p. Sln1p is an osmosensor and negative regulator of the HOG pathway. It senses stress via changes in the turgor of the cell. Sho1p is not an osmosensor and the mechanism by which Sho1p senses stress still requires elucidation [13]. The HOG pathway is activated when either Sln1p or Sho1p sense the stress and relay it to the MAP kinase effector Hog1p. Activated Hog1p translocates to the nucleus and binds to transcription factors including Msn2/4p, Hot1p and Sko1p. These transcription factors then induce the transcription of genes involved in downstream biochemical functions in response to osmotic stress [14, 15].

The HSR is induced by sublethal heat shock, heavy metal stress and anoxia [16]. It is primarily governed by the heat shock transcription factor Hsf1p. In unstressed cells, Hsf1p is present as inactive monomers. Each monomer possesses N- and C-terminal transcription activation domains, a helix-turn-helix DNA binding domain (DBD), and a leucine zipper trimerisation domain [12]. Hsf1p activation involves trimerisation and the acquisition of site-specific DNA-binding activity. Activation is mediated by a ribonucleoprotein complex containing translation elongation factor eEF1A and untranslated RNA, HSR1 (heat shock RNA-1). The non-coding HSR1 is essential for the HSR and is thought to be the

thermosensor that triggers the HSR [17]. Heat stress activates transcription of the heat shock genes by controlling the activity of both Hsf1p and Msn2/4p. Hsf1p binds to heat shock elements (HSEs) in the promoters of heat shock responsive genes. HSEs are highly conserved among many organisms and typical HSEs are composed of numerous, tandem inverted repeats of the 5 bp sequence nGAAn [18-20]. Atypical HSEs differ from typical HSEs by having 5 bp inserts between the inverted repeats [21]. A minimum of three repeats is required for Hsf1p to bind, due to it binding as a trimer. The architecture of the HSE is proposed to determine gene and stress specific responses [19]. The majority of heat shock genes contain at least one STRE or HSE, while some have been found to contain both. Genes containing both elements are independently controlled by Hsf1p and Msn2/4p [22-25].

The cell wall integrity or protein kinase C (PKC) pathway responds to stresses that directly affect the assembly or integrity of the cell wall. These stresses comprise increased temperature [26], mechanical injury, hypo-osmotic conditions [27, 28] and the presence of cell wall disrupting agents such as Congo Red [15], Zymolyase and Calcofluor White [29]. These stresses cause the cell wall localised sensors Mtl1p, Wsc1/2/3p and Mid2p to activate protein kinase C (Pkc1p) via the Rho1p and Rom2p GTPases [30]. Pkc1p consequently activates gene expression through the transcription factors Swi4/6p and Rlm1p. The former regulates the cell cycle and cell wall biosynthesis genes, while the latter regulates the cell wall synthesis and assembly genes [15]. The occurrence of plasma membrane stretch, as seen in hypo-osmotically stressed cells, has been suggested to activate the PKC pathway [31]. During ionic stress certain ion transporters, for instance Mid1p, are stretch-activated and control the intracellular ion levels [32]. Ionic stress results in the increase in cytosolic Ca^{2+} , predominantly due to Ca^{2+} influx through the single uptake system Cch1p-Mid1p. The increase in cytosolic Ca^{2+} triggers calcineurin-dependent signalling via activation of calmodulin, which in turn activates calcineurin (calmodulin-specific serine-threonine protein phosphatase). Calcineurin dephosphorylates the transcriptional activator Crz1p, resulting in it translocating to the nucleus and binding to the calcineurin-dependant response elements (CDREs) of the target genes [13, 32-34]. Exquisite cross-talk occurs between the PKC and calcineurin pathways when they synergistically induce genes e.g. *FKS2*, which encodes a subunit of the cell wall biosynthetic enzyme β -1,3-glucan synthase, in response to cell wall damage [35].

1.3 Trehalose

Trehalose (α -D-glucopyranosyl-1,1- α -D-glucopyranoside) is a non-reducing disaccharide ubiquitously found in fungi, bacteria and in some animals [36]. Trehalose was originally thought to be a storage carbohydrate, as it accumulates to high levels in stressed and stationary phase cells [37]. However, it has since been implicated in the protective responses to hyper-osmotic, heat, cold, ethanol, oxidative, desiccation and nutrient limitation stresses. [3, 38-42].

1.3.1 Trehalose synthesis

Trehalose accumulation occurs through endogenous synthesis or via the uptake of exogenous trehalose [43]. Although the specific factor that stimulates trehalose synthesis still requires elucidation, plasma membrane alterations are thought to be the likely trigger [44]. Under typical growth conditions when no stresses are present, trehalose accumulation commences during the diauxic shift and peaks at the onset of stationary phase. Endogenous trehalose is synthesized from glucose-6-phosphate (G6P) and UDP-glucose (UDPG) in two successive enzymatic reactions catalysed by trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase [45]. Essentially, a hexosyl group transfer occurs between G6P and UDPG resulting in the formation of trehalose-6-phosphate (T6P). T6P then undergoes phosphoric ester hydrolysis and is converted into trehalose [7]:



The 56 kDa trehalose-6-phosphate synthase (*TPS1*), 102 kDa trehalose-6-phosphate phosphatase (*TPS2*), 115 kDa Tps3p (*TPS3*) and 123 kDa Tsl1p (*TSL1*) make up the four subunits of the trehalose synthase complex [9, 27, 46, 47]. Tps3p and Tsl1p function to maintain the integrity of the complex and are involved in its regulation [7, 46]. The *TPS1*, *TSP2* and *TSL1* promoters all have numerous STRE present in them [3, 9, 44]. The expression of the genes affecting trehalose synthesis does not always correlate with the internal trehalose levels in the cells [48]. In reality, the intracellular trehalose concentration is a result of the balance between the synthetic activity of the synthase complex and the degradative activity of the trehalases [44].

1.3.2 Trehalose degradation

During stationary phase, trehalose is slowly degraded due to its possible utilisation as a reserve energy source [31, 49]. The disappearance of trehalose is usually accompanied by

the loss of viability [27]. Additionally, unstressed cells maintain low internal trehalose levels by activating trehalases and deactivating the trehalose synthase complex [7]. Trehalose degradation by trehalases is achieved via O-glycosyl bond hydrolysis. There are two functional trehalases in *S. cerevisiae* namely the neutral trehalase (*NTH1*) and the vacuolar acid trehalase (*ATH1*) [50]. The trehalose level in the cells is mainly controlled by the neutral trehalase enzyme, which is located in the cytosol and is responsible for the hydrolysis of cytosolic trehalose. This trehalase is activated by cAMP-dependent phosphorylation [41, 42, 50, 51]. The acid trehalase is localized in the vacuole and functions primarily during growth on trehalose as a carbon source. It appears to be involved in the uptake and utilisation of exogenous trehalose [51]. The regulation of the vacuolar acid trehalase is unknown but catabolite repression and compartmentation have been suggested as probable regulatory mechanisms [50].

1.3.3 Trehalose function

Trehalose accumulation increases the resistance of yeast to heat shock, freezing, desiccation and ethanol exposure. Previous studies have suggested that trehalose stabilises the intracellular water structure of the cells and preserves the structural and functional integrity of the plasma membrane [40, 52-54]. The observation that the rotational motion of water molecules around trehalose is restricted, provides evidence that trehalose does indeed stabilise water structures. During desiccation, trehalose replaces the bound water content of the cells [55]. It intercalates between adjacent phospholipid head groups in the plasma membrane and hydrogen bonds subsequently form between the hydroxyl groups on trehalose and the polar head groups of the phospholipids. This decreases the melting temperature (T_m) of plasma membranes, preventing phase transitions and membrane fusion during desiccation. Additionally, it has been shown *in vitro* that one trehalose molecule can specifically interact with one *cis*-double bond of an unsaturated fatty acid through hydrogen bonding involving the OH-6 and OH-3 or OH-2 hydroxyl groups of trehalose [39, 49, 56, 57]. *In vitro* studies using liposomes have shown that trehalose must be present on both sides of the lipid bilayer to stabilise the membrane during desiccation [58]. During heat stress, trehalose has been demonstrated to protect native proteins from heat inactivation and prevent aggregation of denatured proteins. One hypothesis is that trehalose maintains these proteins in a partially folded state, facilitating the chaperone action of heat shock proteins [27, 54, 59-61].

1.4 Stress proteins

Two types of stress proteins are synthesised by *S. cerevisiae* during adaptation to adverse conditions: the general stress proteins and the classical heat shock proteins (Hsps). The former are regarded as the proteins that contain only STREs in their gene promoters, while the latter contain only HSEs or both [51]. Two major classes of Hsp are synthesized in *S. cerevisiae*, namely the 70 – 100 kDa and the 17 – 30 kDa class, in response to different stress conditions. The former class is highly conserved between species, while limited sequence conservation exists between species within the latter class [62]. Most Hsps act as molecular chaperones. They prevent protein aggregation, refold heat-damaged proteins and stabilise cellular proteins [40]. Other Hsps form part of the proteolytic machinery of cells and facilitate the degradation of irreversibly damaged proteins [59]. Some Hsps, for instance Hsp48p, Hsp34p and Hsp96p, have been found to be involved in carbohydrate metabolism [38].

1.5 Heat shock protein 12 (Hsp12p)

Heat shock protein 12 (Hsp12p) is one of the small heat shock proteins synthesized by *S. cerevisiae*. It was primarily discovered due to its induction in response to glucose limitation and increased temperatures [62, 63]. It was therefore initially named *GLPI* (glucose and lipid regulated protein) but was subsequently renamed *HSP12*, when its expression in response to stress was quantified. Some authors have suggested that Hsp12p be classified as a late embryogenesis abundant (LEA)-like protein due to its lack of structure and up-regulation during desiccation [64]. Garay-Arroyo *et al.* 2000 identified twelve proteins in *S. cerevisiae* that could function as hydrophilins. These proteins have a high glycine content (> 6%), have predicted random coil configurations and high hydrophilicity indexes (> 1.0). Hsp12p was one of the identified proteins, as it contains 12 mol % glycine and has a 61 % predicted random coil configuration [65, 66]. Goyal *et al.* 2005 suggested that hydrophilins, due to their unordered and flexible structures, could stabilise cellular structures by forming physical barriers between neighbouring molecules. Hydrophilins may also assist in maintaining the minimum cellular water content needed for survival [67]. Although Hsp12p is defined as a heat shock protein, it is regarded more as a general stress response protein due to its induction in response to a diverse range of stresses.

1.5.1 *HSP12* gene information

HSP12 (YFL014W) is a single copy gene, located on chromosome VI (coordinates: 107250-107579) [62, 63]. The extended 5'-flanking region of *HSP12* comprises the *MDJI* gene on the opposite Crick strand. Both *HSP12* and *MDJI* are located between the *SUF9* and *CDC4* genes [9].

1.5.2 *HSP12* promoter sequence

The *HSP12* promoter is complex and contains multiple positive and negative *cis*-acting elements (Fig. IA and IB). These include two HSEs, five STREs, one Rap1-binding site, one Rlm1p binding domain (MADS-box), one zymolyase response element (ZRE) encompassing a Ca²⁺/cAMP response-like element (CRE), one filamentous and invasion-responsive element (FRE) and one Post-diauxic Shift Element (PDSE) [9, 15, 62]. The *HSP12* promoter is also reported to contain a weak iron-responsive element (FeRE) involved in iron uptake and a weak copper response element (CuRE) used in copper uptake [68-70]. Furthermore, the *HSP12* promoter contains numerous features typical of most yeast promoters including two TATA boxes, a pyrimidine-rich region spanning nucleotides -98 to -80 and an A-rich 5' untranslated leader sequence. The sequence structure ahead of the initiation codon, A at position -3 and T at position -6, is characteristic of highly expressed yeast genes. The CA sequence at position -38 is reported to be the transcription initiation site [62]. Mig1p has been implicated in the down-regulation of *HSP12* under glucose repression, as STRE2 and the 5' flanking sequence (tttccAGGGG) bears resemblance to the mig1 consensus GC-box sequence [9]. The *HSP12* promoter also seems to contain an autonomously replicating sequence element, which is bound and repressed by the global transcriptional regulator Abf1p under favourable conditions. Moreover, *HSP12* is repressed by the global transcriptional regulator Mot1p, which binds to the TATA box binding protein (TBP) and causes it to dissociate from the DNA [71].

1.5.3 *HSP12* coding sequence

The *HSP12* coding sequence (Fig. IIA) is comprised of 109 codons. It has an exceptionally high codon bias index of 0.82, which is characteristic of highly expressed yeast genes [62].

1.5.4 *HSP12* expression

A variety of stresses induce *HSP12* expression via either one or more of the aforementioned yeast stress response pathways. Numerous transcription factors (Table 1) alter *HSP12*

expression according to the perceived stress. The broad and high stress inducibility of *HSP12* at the transcriptional level has been attributed to its upstream promoter sequence, especially the multiple copies of STREs present [9]. Moreover, the high codon bias allows efficient transcription and translation of *HSP12* [62]. Studies on *HSP12* expression predominantly examine mRNA transcript levels using both micro-array and Northern blot analyses. These studies are limited in that no information regarding the translation or post-translational control is revealed and it is assumed that subsequent protein synthesis does occur [48]. However, this is not always the case. It has been observed that yeast grown at elevated temperatures have decreased Hsp12p levels despite *HSP12* transcription being upregulated in response to heat shock [64]. This indicates some degree of post-transcriptional regulation of Hsp12p, either via mRNA stability or post-translational control.

1.5.5 *HSP12* inducing stresses

HSP12 is induced by heat shock [62], water deficit stress or desiccation [65, 72] acetaldehyde stress [73], salt stress [9], osmotic stress, entry into stationary phase, glucose limitation, nutrient depletion [62], agents affecting the cell wall integrity [74], barometric pressure [75], as well as the presence of oleate [63], mannitol [30] or ethanol in the medium [76, 77]. Polyene antibiotics (amphotericin B and nystatin), DNA damaging agents (methylmethanesulfonate) and herbicides (2,4-dichlorophenoxyacetic acid) [78-80] have also been observed to induce *HSP12* expression. Furthermore, *HSP12* induction has been observed in response to cold (18 °C) and near freezing (0 °C) temperatures [3, 8]. Several studies have revealed that oxidative stress resulting from exposure to oxidising conditions or agents, such as iodine [81], excess copper [70], cadmium [82], zinc depletion and the presence of H₂O₂ [83] results in *HSP12* up-regulation. Although there is little data regarding the function of stress proteins in eukaryotic micro-organisms in response to oxidative stress [84], these findings suggest that Hsp12p may be involved in oxidative stress tolerance in *S. cerevisiae*.

1.5.6 Hsp12p polypeptide

The Hsp12p polypeptide is 109 amino acids long (Fig. IIA), has a molecular weight of 11 693 Da and an isoelectric point (pI) of 5.1. Hsp12p has a high relative concentration of glycine, alanine and charged amino acid residues (20% negatively and 18% positively

A)

```

ATGTCGTGACGCGCAGGTAGAAAAGGATTCCGGTGAANAAGCTTCTGAAAGCTTTGGTAGCCAGAC
M S D A G R K G F G E K A S E A L K P D

TCTCAAAAGTCAATACGCTGAAACAAGGTAAGGAATACATCACTGACAAGGCCGACAAAGSTC
S Q K S Y A E Q G K E Y I T D K A D K V

GCTGTAAGGTTCACACCAGTAGACAACTAGGGTGTCTTCCAAAGGTCTCCACCACCTCTCCC
A G K V Q P E D N K G V F Q G V H D S A

CBAABACCCAAAGATAACGGTGAAGSTCAAGGTCAATCTTCCGACAGCCAAAGCTAGAGAT
E K G K D N A E G Q G E S L A D Q A R D

TACATGGGAGCCCGCAAGTCCAAGTTGAACGATGCCGTGGAATATGTTCCGGTGGTGTG
Y M G A A K S K L N D A V E Y V S G R V

CACCGTGAAGAGACCCCAACCTACTACTAA
H G E E D P T K K

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B)

	Amino acid	Num	mol/mol%	w/w%
A	Ala	13	11.93	8.49
C	Cys	0	0.00	0.00
D	Asp	11	10.09	10.74
E	Glu	11	10.09	11.87
F	Phe	2	1.83	2.42
G	Gly	13	11.93	7.16
H	His	2	1.83	2.28
I	Ile	1	0.92	0.96
K	Lys	15	13.76	16.07
L	Leu	3	2.75	2.88
M	MET	2	1.83	2.19
N	Asn	3	2.75	2.91
P	Pro	3	2.75	2.53
Q	Gln	6	5.50	6.43
R	Arg	3	2.75	3.83
S	Ser	8	7.34	6.17
T	Thr	2	1.83	1.73
V	Val	7	6.42	6.01
W	Trp	0	0.00	0.00
Y	Tyr	4	3.67	5.31

Figure II: A) Hsp12p coding sequence (top) and amino acid sequence (bottom, bold blue). B) Amino acid composition of Hsp12p, as determined using DNAMAN 4.13 software (Lynnsoft Biosoft, Quebec, Canada).

Table 1: The promoter elements found in the *HSP12* promoter, their consensus sequences and position, their respective transcription factors and the regulatory functions they control.

Promoter element	Consensus sequence	Position (relative to ATG)	Colour code ^a	Transcription factor	Regulatory function ^b
CuRE (Copper response element)	TTTGC(T/G)C(A/G)	-579 to -583	Green	Mac1p	Copper Homeostasis ⁵⁹
PDSE (Post-diauxic shift element)	T(T/A)AGGGAT	-576 to -572	Grey	Gis1p	Nutrient limitation ^{60, 61}
Promoter specific	ACCCANCA	-568 to -556	Brown	Rap1p	Activation or repression of transcription ⁶²
FeRE (iron-responsive element)	(C/T)(A/G)CACCC(A/G)	-555 to -559	Olive	Atf1p	Iron utilisation and homeostasis ⁶³
FRE (filamentous and invasion-responsive element)	GATTC(C/T)(C/T)	-542 to -536	Dark red	Tec1p	Filamentous and invasive growth ^{15, 67}
MASS-box	CTA(T/A) ₄ TAG	-520 to -505	Dark green	Rlm1p	Cell wall integrity ^{26, 65}
HSE (Heat shock element)	NGAAN	459 to 450	Blue	Hsf1p	Heat shock response ⁶⁶
		-177 to -170			
STRE (Stress response element)	AGGGG or CCCCT	-435 to -431	Red	Msn2/4p	General stress response ⁶⁸
		-414 to -410			
		-377 to -373			
		-232 to -228			
		-180 to -186			
GC-box	(A/T) ₄ (C/G)(C/T)GGGG	-232 to -223	Light red	Mig1p	Glucose repression ⁹
ZRE (Zymolyase response element)	ATGACGT	-332 to -327	Dark blue	Unknown	Cell wall integrity ⁹
CRE-like (Ca ²⁺ /cAMP response element)	ACGTCA	-327 to -340	Dark blue	Ska1p	Osmotic responses ^{9, 62}
ARS (autonomously replicating sequence) element	(A/G)TC(A/G)(C/T)N ₃ ACG or TNNCG1NNNNNTGAT	-265 to -253	Red	Abf1p	Global transcriptional regulator ⁶⁵
TATA-box	TATA(A/T)A(A/T)N	-145 to -139	Yellow	Tbp1p	Directs transcription ⁶⁶
		-77 to -72			
TATA-box	Forms a complex with TBP at the TATA-box	-145 to -139	Yellow	Mot1p	Global transcriptional regulator ^{61, 65}
		-77 to -72			

^a Colour code same as in Figure 1.

^b Respective references indicated.

charged) (Fig. IIB). Consequently, Hsp12p has a negative hydrophobicity plot (Fig. III), which is characteristic of small Hsps [62]. It is predicted that Hsp12p has a random coil configuration with no transmembrane domains or signal peptide(s). Both the hydrophilic amino acid content and lack of tertiary structure renders Hsp12p soluble at 80 °C. [64]. Hsp12p, unlike most other small Hsps, does not share any sequence homology with α -crystallin proteins. In fact, no protein shares domains/motifs in common with those found in Hsp12p [9, 62].

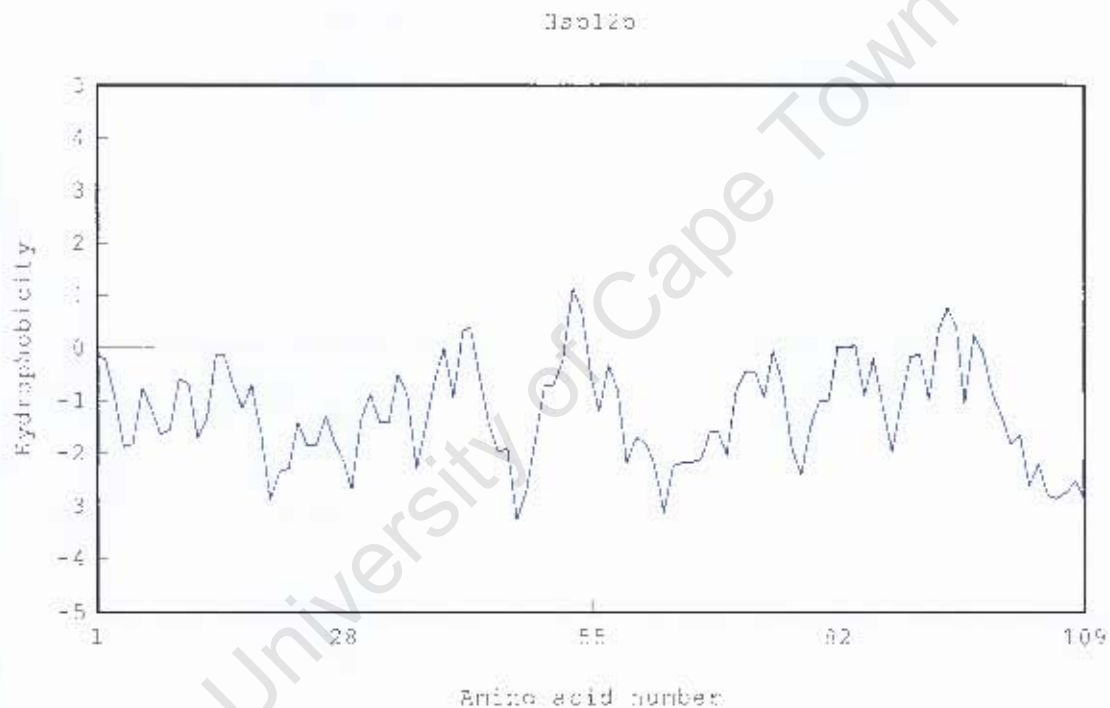


Figure III: Hydrophobicity plot of Hsp12p, as determined using DNAMAN 4.13 software (Lynnon Biosoft, Quebec, Canada).

1.5.7 Hsp12p cellular localisation

Immunocytochemical studies have revealed that Hsp12p is located in the peripheral regions of the yeast cell, notably close to the plasma membrane [58] and in the cell wall [30].

1.5.8 Hsp12p function

Functionally conserved “classical” Hsps mediate protein-protein interactions and assist in the transportation of proteins through membranes [7]. Additionally, some Hsps prevent protein aggregation, facilitate protein degradation and promote protein refolding during stress [40]. It is unlikely that Hsp12p fulfils any of these functions. Although the functional significance of Hsp12p is considered to be largely undefined (<http://www.yeastgenome.org/>), there is extensive evidence that suggest various functions for Hsp12p in *S. cerevisiae*.

Hsp12p has been implicated in both plasma membrane protection and cell wall organization. It was thought that Hsp12p might stabilise membranes during desiccation by providing a source of hydrogen-bonding groups in the absence of water [64]. It was demonstrated that Hsp12p could protect liposomes against desiccation and subsequent rehydration in a manner analogous to trehalose. Furthermore, Hsp12p was able to protect liposomes from ethanol-induced lysis. Sales *et al.* [58] proposed a simple mechanism by which Hsp12p might afford this protection. They suggested that Hsp12p hydrogen bonds to membrane proteins and glycolipids resulting in the formation of a hydrophilic surface, which prevents membrane fusion during water deficit. Hsp12p would perform this by increasing the bound water content of the membrane, as some water molecules would be sufficiently bound to the charged residues on Hsp12p to such an extent that their removal would be prevented during water deficit. It is suggested these bound water molecules stabilise and protect the membrane [58].

Hsp12p is suggested to be involved in cell adhesion, as the ability of sherry-producing yeast strains to form biofilms is dependent on the presence of Hsp12p [97], which is consistent with its cell wall localisation. Moreover, the fact that *HSP12* is upregulated in response to cell wall perturbing agents suggests that Hsp12p may also function in cell wall remodeling and flexibility in response to stress. The initial suggestion that Hsp12p may function in cell wall flexibility came from the observation that the volume of the $\Delta hsp12$ cells was less

affected by changes in external osmolarity than wild type cells. Additionally, $\Delta hsp12$ cells were observed to be more sensitive to increased pressure and burst earlier than wild type cells [30]. The function of Hsp12p as a plasticiser in cell wall flexibility was further investigated in a study using polymerised agarose as a model for the yeast cell wall. Incorporation of Hsp12p into the agarose decreased the agarose gel strength making it more flexible [74]. Recent studies, performed by the same group using atomic force spectroscopy, showed that Hsp12p does indeed decrease the rigidity of the cell wall thus making it more flexible. Furthermore, this study demonstrated that Hsp12p also increases the cell wall permeability of *S. cerevisiae* [96]. A mechanism by which Hsp12p increases cell wall flexibility was proposed by Motshwene *et al.* 2004. They suggested that Hsp12p increases the flexibility of the cell wall by interrupting the hydrogen bonding and ionic interactions between the cell wall glucan chains (typically β -1,3-glucans), which would allow the chains to move more freely relative to each other [30].

1.5.9 Hsp12p applications

HSP12 has been used in several studies as a general stress status marker gene due to its broad and high stress inducibility [9, 98]. These studies range from using *HSP12* induction as an indicator in bioassay systems that screen for toxic compounds [82] to an indicator that allows the monitoring of fermentation processes [99]. The ability of numerous stresses to induce *HSP12* expression motivated the creation of an *HSP12-GFP2* construct, a fusion between Hsp12p and the green fluorescent indicator protein Gfp2p. This construct allowed the rapid determination of the stress status of yeast by monitoring Gfp2p fluorescence in the cells upon exposure to stress [100].

1.5.10 Effects of *HSP12* disruption

Disrupting *HSP12* has no effect on cell viability or growth at normal temperatures [62]. However, $\Delta hsp12$ cells are unable to grow in the presence of caffeine and Congo Red, both of which affect yeast cell wall integrity [30]. The $\Delta hsp12$ strain was also observed to be less able to grow in ethanol-supplemented media [58] and displayed increased sensitivity to copper [70]. Furthermore, $\Delta hsp12$ cells exhibit increased sensitivity in response to both elevated levels and rapid changes in barometric pressure [30].

1.6. Oxidative stress

Oxidative stress arises when an imbalance between the generation and elimination of reactive oxygen species (ROS) overwhelms the oxidative defense mechanisms in the cells [57,101]. ROS are formed either as a result of exposure to oxidative agents or as a result of electron leakage from the electron transport chain in the mitochondria during normal cellular aerobic respiration [98]. ROS damage all cellular structures (lipids, proteins, carbohydrates and DNA) and disrupt the internal reducing environment of the yeast cytoplasm [27, 102]. Yeast cells relieve oxidative stress by synthesising trehalose and stress proteins and via activating oxidative defense mechanisms [103].

There are two oxidative defence mechanisms in *S. cerevisiae*: enzymatic and non-enzymatic. The former involves enzymes that repair and remove the products of oxidatively damaged components and includes the peroxidase, superoxide dismutase and catalase enzymes. The latter employs antioxidants such as glutathione, ascorbate and β -carotene, which either eradicate ROS or sequester metal ions [59, 60, 104, 105].

ROS stimulate gene expression either via direct activation of transcription factors e.g. Yap1p or by acting as secondary messengers in MAP kinase cascades [101]. Yap1p controls an oxidative stress regulon of at least 32 proteins [106]. During favourable conditions, Yap1p is localised in the cytoplasm due to interactions between the Yap1p nuclear export signal (NES) and the Crm1p nuclear exportin. The interactions between Yap1p and Crm1p are disrupted upon oxidative stress exposure, which allows Yap1p to translocate into the nucleus and facilitate gene expression. Yap1p responds directly to oxidative stress by acting as a redox state sensor, independently of cell surface localised sensors. It possesses a cysteine-rich domain that is oxidised upon ROS exposure. The resultant Yap1p with a intramolecular disulphide bond masks the NES and consequently allows Yap1p to translocate into the nucleus [12]. Yap1p binds as a homodimer to AP1-response elements (AREs), characterized by the consensus sequence TGACTCA, found in the promoters of many oxidative stress response genes [10, 107]. However, approximately half of the Yap1p target genes do not contain AREs [106]. It has been suggested that Yap1p induces these genes either via binding to STREs [50, 108] or by operating upstream of the STRE-binding factors via co-operative binding to *cis*-acting elements [9, 109].

An additional transcription factor, Skn7p, induces approximately half of the genes in the Yap1p regulon. These genes are involved in cell cycle control and cell wall biosynthesis. Skn7p is constitutively localised in the nucleus, however its consensus binding sequence remains to be elucidated [106]. Skn7p has been shown to bind to HSEs *in vitro* with similar specificity to Hsf1p [18]. Skn7p has a helix-turn-helix DNA binding domain similar to Hsf1p and a receiver domain similar to that found in two-component signal transduction systems of prokaryotes. Skn7p has been shown to be the response-regulator protein in the Sln1p-branch of the HOG pathway and plays a role in the osmotic stress response [106]. Skn7p is suggested to be the factor that integrates many different stress signals at the level of gene expression, as it interacts with numerous other transcription factors including Yap1p, Hsf1p and Crz1p [27]. Additional transcription factors, in particular Msn2/4p, have also been suggested to be involved in the oxidative stress response in yeast [107].

1.7 Research question

Several industrial processes require yeast cells to be preserved for storage in the desiccated state due to both logistical and financial reasons. It is fundamental that such yeast cells retain their viability and vitality after subsequent rehydration to ensure efficient functioning in downstream processes. Since Hsp12p has been defined as a putative hydrophilin [65] and has been shown to stabilise model membranes against desiccation *in vitro* [58], the role of Hsp12p in yeast desiccation tolerance was investigated *in vivo*. Elucidation of the role of Hsp12p in yeast desiccation tolerance could possibly lead to the engineering of an industrially important, desiccation tolerant yeast strain.

Chapter 2: Materials and methods

- 2.1 Yeast strains
- 2.2 Culture conditions
- 2.3 Rapid total yeast protein extraction
- 2.4 Protein concentration
- 2.5 Gel electrophoresis (SDS-PAGE)
- 2.6 Western blotting
 - 2.6.1 Transfer of proteins onto nitrocellulose
 - 2.6.2 Visualisation of transferred proteins
- 2.7 Desiccation conditions
- 2.8 Cell viability
- 2.9 Membrane integrity
- 2.10 Intracellular trehalose determination
 - 2.10.1 Extraction and digestion of trehalose
 - 2.10.2 Glucose assay
- 2.11 Intracellular oxidised glutathione determination
 - 2.11.1 Extraction of intracellular oxidised glutathione
 - 2.11.2 GSSG assay
- 2.12 Thiobarbituric acid reactive substances (TBARS) determination
 - 2.12.1 Extraction of TBARS
 - 2.12.2 TBARS assay
- 2.13 Fluorimetry studies
- 2.14 Oxidative stress

2.1 Yeast strains

The *Saccharomyces cerevisiae* wild type yeast strain used was the haploid segregant of the diploid strain 842 (*a/α*, *ade2-1/ade2-1*, *trp1-1/trp1-1*, *leu2-3/leu2-112*, *his3-11/his3-15*, *ura3/ura3*, *can^r1-100/CAN*). The Δ *hsp12* gene disruption mutant (*a/α*, *ade2-1/ade2-1*, *trp1-1/trp1-1*, *leu2-3/leu2-112*, *his3-11/his3-15*, *ura3/URA3*, *canr1-100/CAN*, *hsp12::URA3*) was constructed and tested as previously described [62]. Both strains were a gift from Dr P. Meacock at the Department of Genetics, University of Leicester, Leicester, U.K. The Hsp12p over-expressing strain [110] is isogenic to the wild type but contains the pYES2-*ENO1*^P-HSP12 plasmid, which enables the constitutive production of Hsp12p under control of the *ENO1* promoter.

2.2 Culture conditions

Yeast cells were inoculated into sterile YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) medium and grown to either mid-exponential (10 h cultures, 2.6 ODU for wild-type, 2.9 ODU for Hsp12p over-expresser and 3.5 ODU for Δ *hsp12*) or stationary phase (38 h cultures, 7.6 ODU for wild-type, 8.1 for the Hsp12p over-expresser and 7.7 ODU for Δ *hsp12*). Growth of the cells was monitored by measuring the absorbance at 600 nm on a Beckman DU530 Life Science UV/Vis spectrophotometer. One optical density unit (1 ODU) is equivalent to 3×10^7 cells ml⁻¹. Cultures were routinely inoculated with 2 ODU of starter culture per 50 ml YPD and incubated at 30°C with continuous agitation.

2.3 Rapid total yeast protein extraction

Mid-exponential phase cells were collected by centrifugation (6000 x g, 10 min, 4°C), washed once in ice-cold Milli Q water and transferred to sterile Eppendorf tubes. Total yeast protein was extracted by suspending the cell pellets in 430 µl sterile cold 50 mM phosphate buffer, 150 mM NaCl pH 7.4 (PBS), adding 170 µl cold extraction buffer (75 mM Tris-HCl pH 6.8 containing 2% v/v SDS, 10% v/v glycerol and 1.5% v/v 2-mercaptoethanol) and vortexing with 200 µl glass beads (0.5 mm diameter) for 5 min at maximum speed at 4°C to perforate the cell wall. Cellular debris was pelleted by bench top centrifugation (10000 x g, 5 min, 4°C). The supernatants (protein extracts) were transferred to clean Eppendorf tubes and stored on ice.

2.4 Protein concentration

Protein concentrations in cell lysates were quantified using the Bradford method for protein determination [111]. The amount of protein in each sample was determined by extrapolation from a calibration curve constructed with bovine serum albumin (BSA, Boehringer Mannheim) solutions ranging from 0 – 8 µg/ml BSA.

2.5 Gel electrophoresis (SDS-PAGE)

Total yeast proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [112]. The stacking and separating gels were 6.7% v/v and 20% v/v respectively. The marker used was a 1 mg/ml solution of chicken erythrocyte histones, with approximate molecular masses (kDa): H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0 and H4, 11.2. The samples were loaded in duplicate onto the gel and electrophoresed overnight at 4°C at a constant voltage of 80 V. To facilitate visualisation of total protein in the samples, one half of the gel was stained with Coomassie brilliant blue and then destained using 7% v/v acetic acid and 25% v/v technical ethanol. The other half of the gel was used for Western blotting.

2.6 Western blotting

2.6.1 Transfer of proteins onto nitrocellulose

SDS-PAGE gels were thoroughly washed in transfer buffer (25 mM Tris-HCl pH 8.3, 0.192 M glycine, 20% v/v methanol) before being laid onto pre-soaked nitrocellulose paper (pore size: 0.45 µm; Schleicher and Schull) supported by pre-soaked Whatmann 3MM paper. The above was placed on top of a stack of damp absorbent cloth, which was supported by the carbon anode block. Additional pre-soaked Whatmann 3MM paper was placed on top of the gel, followed by another stack of damp absorbent cloth and the carbon cathode block. Transfer was allowed to proceed for 1 h at RT using a constant current of 400 mA. To ensure that efficient protein transfer had taken place, the proteins on the membrane were reversibly stained with Ponceau S (0.5% w/v Ponceau S powder; 1% v/v acetic acid) and destained using sterile Milli Q water.

2.6.2 Visualisation of transferred proteins

The dry blot was incubated in blocking buffer (PBS containing 5% w/v skimmed milk powder) for 30 min at RT to block all unbound sites. A 1/100 dilution of a rabbit anti-Hsp12p antibody [58] was added before incubation at 4°C for 16 h. The blot was thoroughly washed in 0.05% v/v Tween-80 in PBS and then incubated for 1 h at RT in 5% w/v skim milk powder in PBS containing a 1/5000 dilution of the secondary goat-anti-rabbit antibody conjugated to alkaline phosphatase. The blot was again thoroughly washed in 0.05% v/v Tween-80 in PBS and then in 10 mM Tris-HCl, 150 mM NaCl pH 7.4. The blot was then incubated at RT in 100 mM NaCl, 100 mM MgCl₂, 100 mM Tris-HCl pH 9.5 to which 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo 4-chloro 3-indoyl-phosphate (BCIP) were added. Once the colour had developed, the blot was washed in water and air-dried.

2.7 Desiccation conditions

Cultures were harvested by centrifugation (6 000 *x g*, 10 min, 4°C) and washed once in cold sterile Milli-Q water. Aliquots (100 µl) of cell suspensions of equal cell density were transferred into the cut-off lids of sterile Eppendorf tubes. The lids were placed into sterile petri dishes and stored at 30°C. After 0, 22, 46 and 70 h of desiccation, the samples were rehydrated by placing the Eppendorf lids in 2 ml of sterile PBS heated to 45°C to avoid cell leakage. The samples were left to rehydrate for 5 min at 45°C, vortexed to minimise cell clumping and then washed and resuspended in 2 ml sterile Milli-Q water.

2.8 Cell viability

Colony-forming units (CFU) assays were used to determine the viability of the strains after desiccation. Aliquots (100 µl) of appropriately diluted cell suspensions were spread onto YPD agar plates and incubated at 30°C for 3 days before counting the number of colonies. The percentage of viable cells after desiccation was calculated by expressing the counts obtained for the desiccated samples relative to the counts obtained before desiccation.

2.9 Membrane integrity

Flow cytometry using the membrane exclusion dye propidium iodide (PI) was used to determine the membrane integrity of the strains. PI only enters cells with permeabilised plasma membranes (PI positive cells) [113]. PI staining was performed according to Chen *et al.* 2003 [114] with slight modifications. Cells were washed and resuspended in PBS pH 7.4 to a density of 0.03 ODU after which 3 μ l of 1 mg/ml PI stock solution (Sigma) was added to the suspensions. The suspensions were incubated for 5 - 10 min in the dark to allow the dye to permeate any membrane-impaired cells. Analysis of PI staining was performed on a Cytomics FC 500 (Beckman Coulter) flow cytometer using an excitation wavelength of 488 nm emitted from a 15 mW Argon-ion laser. PI emission signals were measured at 585 nm using the FL-2 detector. A discriminator of 50 was set on the FS axis to avoid small particle detection, while a minimum of 10 000 events were detected for each sample using a flow rate of less than 200 events per second to avoid the detection of coincident events. Unstained and heat treated (80°C for 60 min) cells were used as PI negative and PI positive controls respectively. Data were analysed using the CXP software program.

2.10 Intracellular trehalose determination

2.10.1 Extraction and digestion of trehalose

Trehalose was extracted and digested according to the method of Parrou *et al.* 1997 [115]. Briefly, trehalose was extracted from the cells by resuspending the cell pellets in 250 μ l 0.25 M Na₂CO₃, followed by heating for 2 h at 95°C with periodic vortexing. Samples were adjusted to pH 5.2 by adding 150 μ l 1 M acetic acid and 600 μ l 0.2 M sodium acetate buffer (pH 5.2). Trehalose was digested to glucose by incubating the suspensions overnight with 3 mU of trehalase (Sigma) at 37°C with agitation.

2.10.2 Glucose assay

The amount of glucose liberated in each sample was assayed using the hexokinase and glucose-6-phosphate dehydrogenase rate determination method [116]. Each 517.5 μ l assay contained 125 μ l of sample (digested trehalose), 24 mM triethanolamine, 0.92 mM ATP, 9.7 mM MgCl₂, 1.3 mM β -NADP, 1.2 U of glucose-6-phosphate dehydrogenase and 0.024 U of hexokinase (final concentrations). The maximum rate (Δ OD_{340nm}/min) of each reaction was used to determine the amount of glucose in each sample after extrapolation

from a calibration curve using glucose solutions ranging from 0 – 60 µg/ml glucose. The total protein content in each sample was determined using the Bradford method. The intracellular trehalose content in the cells was expressed as µg trehalose/ µg total protein.

2.11 Intracellular oxidised glutathione determination

2.11.1 Extraction of intracellular oxidised glutathione (GSSG)

GSSG was extracted from the cells by resuspending the cell pellets in 100 µl cold potassium phosphate buffer pH 6.5 and vortexing at maximum speed with 200 µl glass beads (0.5 mm diameter) for 8 periods of 40 s with storage on ice between vortexing events. After an additional 100 µl of the same buffer was added to the lysates and mixed, the samples were centrifuged (10000 \times g, 4 min, 4°C) and the supernatants transferred to clean Eppendorf tubes to be used in the GSSG assay.

2.11.2 GSSG assay

The amount of GSSG in each sample was assayed using the glutathione reductase rate determination method based on the method of Mavis *et al.* 1968 [117]. Each 1500 µl assay contained 50 µl of sample, 37.5 mM potassium phosphate, 1.3 mM EDTA, 0.045 mM β -NADPH, 0.065% w/v BSA and 0.02 U of glutathione reductase (final concentrations). The maximum rate ($\Delta\text{OD}_{340\text{nm}}/\text{min}$) of each reaction was used to determine the GSSG concentration in each sample. GSSG solutions ranging from 0 - 3 mM GSSG were used as standards. The total protein content in each sample was determined using the Bradford method [111]. The intracellular GSSG content of the cells was expressed as µg GSSG/ µg total protein.

2.12 Thiobarbituric acid reactive substances (TBARS) determination

2.12.1 Extraction of TBARS

Quantification of TBARS was done according to Yang *et al.* 2001 [118] with slight modifications. Cell pellets were resuspended in 200 µl 10 mM potassium phosphate buffer pH 7 containing 0.4 mM butylated hydroxytoluene. Cells were perforated by vortexing at maximum speed with 200 µl of glass beads (0.5 mm diameter) for 6 periods of 20 s with storage on ice between vortexing events. After the addition of 800 µl of the same buffer and 120 µl of saturated trichloroacetic acid, the samples were vortexed and centrifuged at 4°C

for 5 min to precipitate cellular proteins. The supernatants were transferred to new Eppendorf tubes.

2.12.2 TBARS assay

After the background absorbance level of each sample was measured at 535 nm, 125 μ l of a 14.4 mg/ml 2-thiobarbituric acid solution prepared in 0.1 N NaOH was added to 1 ml of each sample, mixed and left to incubate at 75°C for 30 min. After the samples had cooled to RT, the absorbance at 535 nm was again determined. The difference in absorbance before and after acid hydrolysis indicated the extent of lipid peroxidation due to the formation of malondialdehyde (MDA). The concentration of MDA in each sample was extrapolated from a standard curve ranging from 0 – 5 μ M MDA. Standard MDA solutions were prepared from the hydrolysis of 1,1,3,3-tetraethoxypropane. The TBARS concentration was expressed as μ M MDA/mg cells.

2.13 Fluorimetry studies

The wild type strain transformed with the pYES2-HSP12-GFP2 plasmid [100] was grown to early exponential phase (0.3 ODU) and subjected to oxidative stress by adding H₂O₂ to a final concentration of 25 mM directly to the culture. Cell samples were taken at various time points, washed in 1 ml cold PBS pH 7.4 and resuspended in 2 ml of the same buffer. The fluorescence was measured on an Aminco SPF-500 fluorimeter (American Instrument Company, Silver Spring, MD) as previously described [119] using an excitation wavelength of 450 nm and emission wavelength of 507 nm. The ODU of the samples was determined for normalization purposes.

2.14 Oxidative stress

For oxidative stress studies in liquid media, H₂O₂ was added directly to the cell cultures (final concentrations indicated in text) and incubated at 30°C for a further 1 h with agitation before subsequent analysis. For oxidative stress studies on solid media, H₂O₂ was incorporated into YPD agar plates to a final concentration between 0 and 5 mM H₂O₂. Aliquots (10 μ l) of cell suspensions were spotted onto the plates. The plates were incubated at 30°C for 3 days before analysis.

Chapter 3: Results

- 3.1 Preliminary experiments
- 3.2 Investigation of the role of Hsp12p in desiccation tolerance
- 3.3 Investigation of the effect of increased levels of trehalose in the $\Delta hsp12$ strain under non-stress conditions
- 3.4 Investigation of the role of Hsp12p and trehalose in applied oxidative stress tolerance.

University of Cape Town

3.1 Preliminary experiments

Prior to any investigations being performed, growth curves (Fig. 1) were determined for the wild type, $\Delta hsp12$ and Hsp12p over-expressing strains in order to establish growth kinetics. Mid-exponential phase and stationary phase were found to occur in all three strains after 10 and 38 h of growth respectively. To ensure that the $\Delta hsp12$ strain had not reverted back to the wild type and to ensure that the Hsp12p over-expressing strain retained the pYES2-*ENO1*^P-*HSP12* plasmid and synthesised excess Hsp12p, total yeast protein was extracted from mid-exponential phase cells of all three strains and analysed by SDS-PAGE (Fig. 2A). To confirm Hsp12p expression levels, Western blot analysis was performed (Fig. 2B). The Western blot (Fig. 2B) showed that the $\Delta hsp12$ strain had not reverted back to the wild type and that the Hsp12p over-expressing strain was synthesising additional Hsp12p. The Western blot confirmed that all three strains were fully functional with regard to Hsp12p expression and were therefore suitable for use in subsequent comparative studies. The wild type and $\Delta hsp12$ strains were used in the entire study, while the Hsp12p over-expressing strain was used only in some investigations.

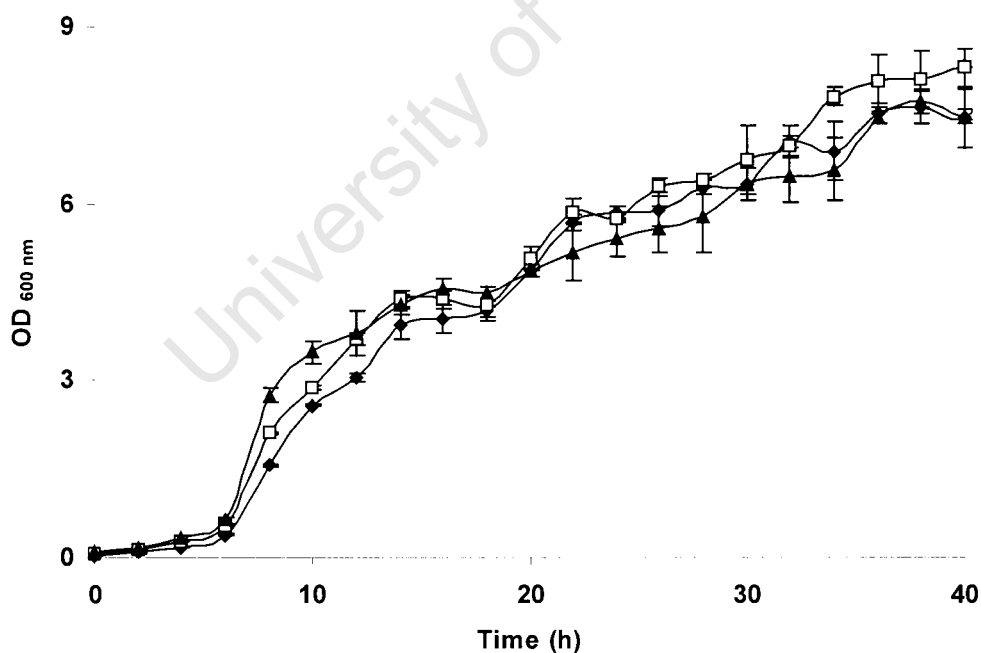


Figure 1: Growth of the wild type (♦), $\Delta hsp12$ (□) and Hsp12p over-expressing (▲) yeast strains in YPD media at 30°C. Growth was monitored by measuring the optical densities of the cultures at 600 nm every 2 h. Data represented are the mean \pm standard error of three independent experiments. Error bars not visible are within the data symbols.

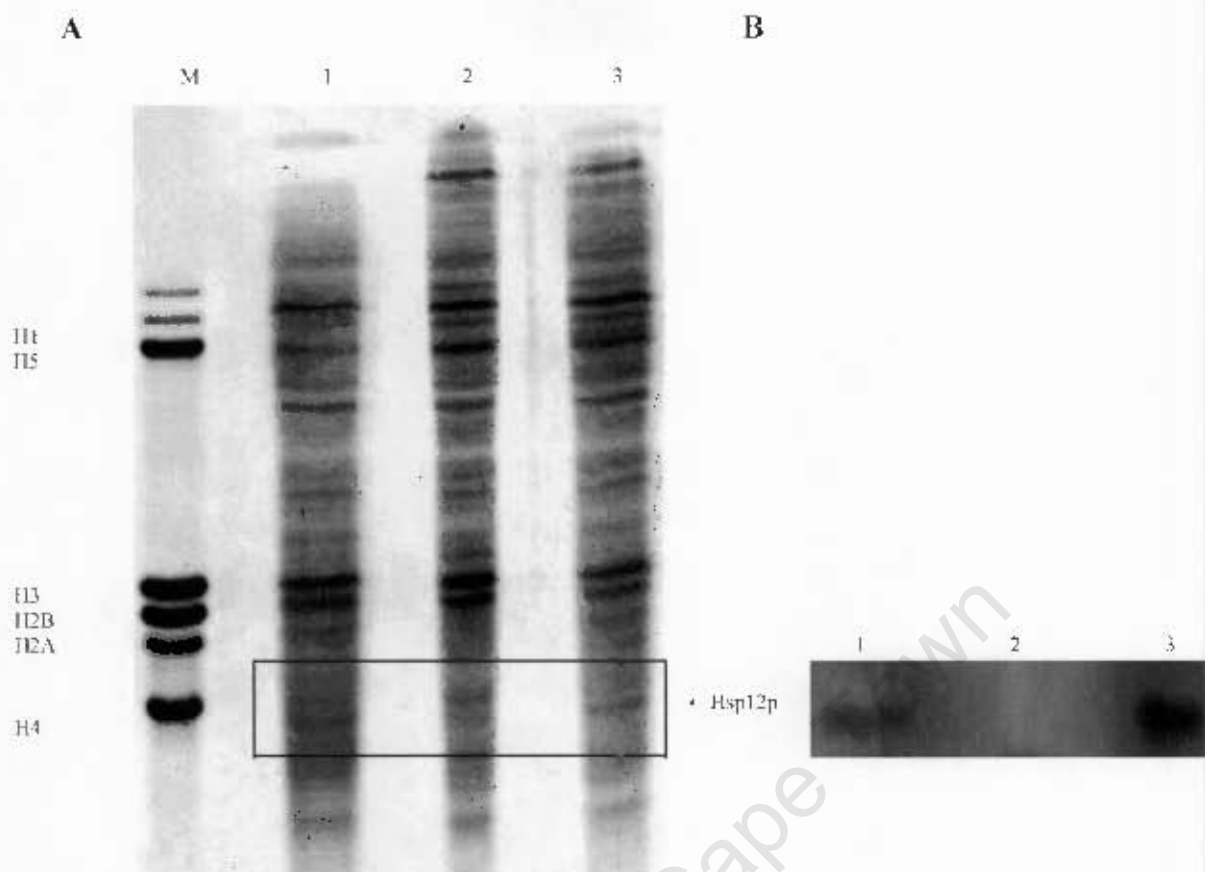
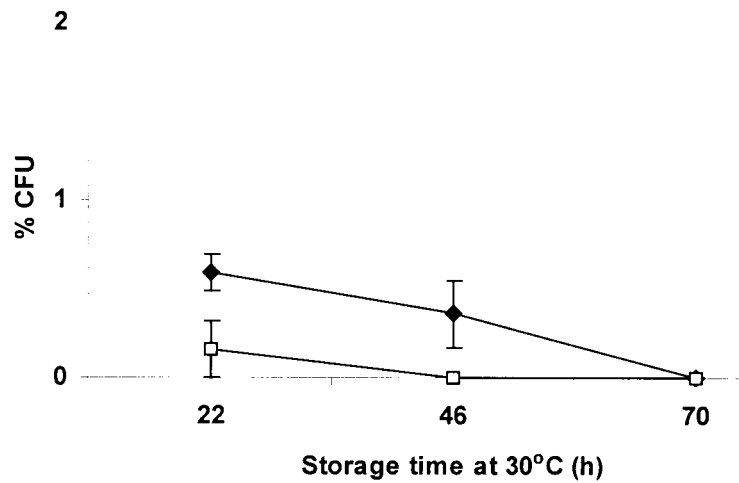


Figure 2. A: SDS-PAGE of total proteins extracted from mid-exponential phase cultures of the wild type (lane 1), $\Delta hsp12$ (lane 2) and Hsp12p over-expressing (lane 3) strains. Hsp12 is the lower of the two visible bands present in the box region. Chicken erythrocyte histones, with molecular masses (kDa): H11, 22.5; H3, 15.3; H12B, 13.7; H2A, 14.0 and H4, 11.2, were used as molecular weight markers (M). Each lane was loaded with 5 μ g total protein. B: Section of Western blot showing relative amounts of Hsp12p in the wild type (lane 1), $\Delta hsp12$ (lane 2) and Hsp12p over-expressing (lane 3) strains.

3.2 Investigation of the role of Hsp12p in desiccation tolerance

Hsp12p has previously been shown to protect liposomes against desiccation [58]. In order to establish whether Hsp12p could protect *S. cerevisiae* against desiccation stress *in vivo*, the cell viability of the wild type and $\Delta hsp12$ strains was assessed at both mid-exponential (Fig. 3A) and stationary (Fig. 3B) phases. Mid-exponential phase wild type cells tolerated desiccation marginally better than $\Delta hsp12$ cells although both strains lost >99% viability after 22 h desiccation. In contrast, stationary phase cells tolerated desiccation better than mid-exponential

A



B

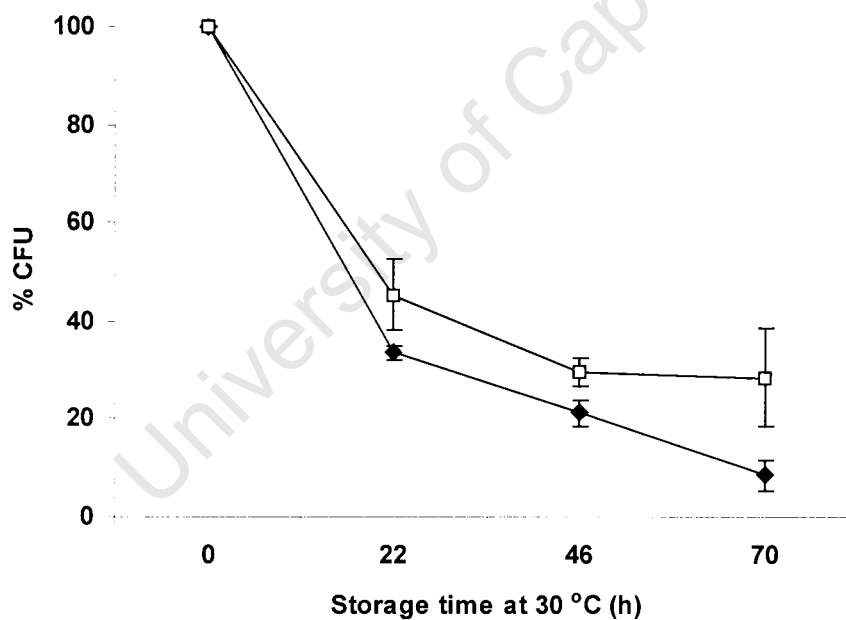


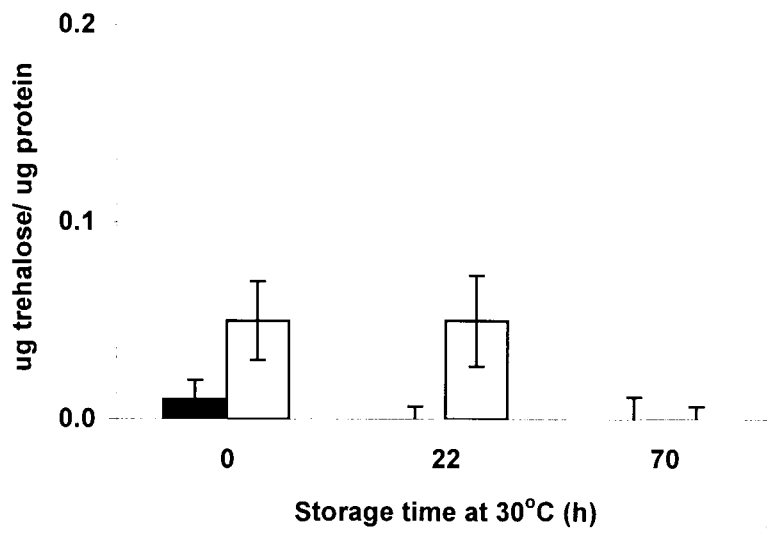
Figure 3: Percentage of colony forming units (CFU) of wild type (\blacklozenge) and $\Delta hsp12$ (\square) strains following rehydration after storage at 30°C for 0, 22, 46 and 70 h. Data for mid-exponential phase cells (A) and stationary phase cells (B) are shown. The error bars represent the standard error of the data derived from experiments performed in triplicate. Error bars not visible are within the data symbols.

phase cells with approximately 60% viability lost after 22 h. The $\Delta hsp12$ strain was now more resistant to desiccation than the wild type strain with approximately 30% of $\Delta hsp12$ cells viable after 70 h desiccation compared with approximately 10% of wild type cells.

The desiccation tolerance of *S. cerevisiae* has been shown to be dependent on the endogenous trehalose levels in the cells [120]. Additionally, it has been shown that trehalose could replace Hsp12p in protecting liposomes against desiccation [58]. We therefore investigated whether the $\Delta hsp12$ cells in stationary phase compensated for the lack of Hsp12p synthesis by over-expressing trehalose, thereby showing greater tolerance to desiccation than the wild type strain. The intracellular trehalose levels in the wild type and $\Delta hsp12$ strains were determined during the course of desiccation of mid-exponential (Fig. 4A) and stationary phase cells (Fig. 4B). Both strains had significantly higher trehalose concentrations at stationary phase compared with mid-exponential phase with that of the $\Delta hsp12$ strain approximately five times higher than that of the wild type strain in mid-exponential phase and approximately three times higher in stationary phase. Whereas the length of the desiccation process had little effect on the trehalose concentration in stationary phase cells, the trehalose concentration decreased to concentrations not measurable in mid-exponential phase cells. This result suggested that there was a correlation between the trehalose concentration and the residual viability observed after 70 h desiccation.

Since Hsp12p has been shown to be located in close proximity to the plasma membrane [30, 58], one of the main targets of desiccation-induced damage [121], the role of Hsp12p in maintaining plasma membrane integrity during desiccation was investigated. The membrane integrity of both strains at mid-exponential and stationary phase after desiccation was assessed by flow cytometry after incubation of the cells with PI. Representative histogram profiles of rehydrated cells following PI staining and flow cytometry are shown in Figure 5. The number of equivalent events and the cell fluorescence (log scale) are indicated on the Y and X axis respectively. The histogram profile of viable cells is centered at 10^0 on the X axis (Fig. 5A), while heat inactivated cells give a profile centered at 2×10^1 on the X axis (Fig. 5B). As the percentage of rehydrated cells with impaired membranes increases, the histogram profile shifts towards the right (Fig. 5C). This profile shift allows the number of cells with impaired membrane integrity to be determined. Plasma membrane integrity after desiccation, as determined from the percentage PI negative cells, was greater in wild type cells compared with

A



B

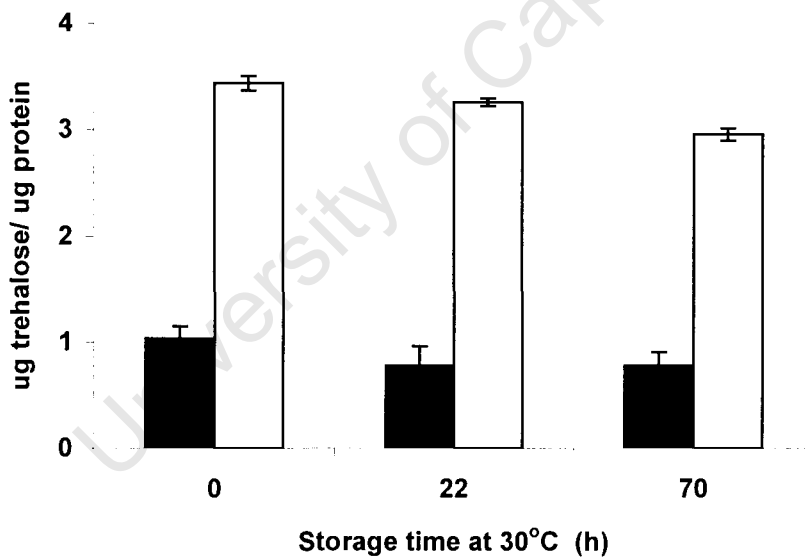


Figure 4: Trehalose concentrations present in wild type cells (filled bars) and $\Delta hsp12$ cells (empty bars) after 0, 22 and 70 h of storage at 30°C. Data for mid-exponential phase cells (A) and stationary phase cells (B) are shown. The error bars represent the average deviation of the data derived from experiments performed in triplicate.

$\Delta hsp12$ cells in both growth phases (Figs. 6A and 6B). After 70 h desiccation of mid-exponential phase cells, 25% of wild type cells excluded PI compared to 12% of $\Delta hsp12$ cells. These figures increased to 45% and 16% respectively in stationary phase. This result suggested that Hsp12p helps maintain the integrity of the plasma membrane *in vivo*, confirming the results obtained previously [58]. Although a positive correlation ($r^2 \geq 0.95$) (Table 2) exists between the percentage of cells with intact plasma membranes and the percentage viability, the integrity of the plasma membrane is not the sole factor that affects loss of viability during desiccation. Increased ROS during desiccation are also proposed to affect cell viability by increasing unfavourable oxidation reactions [105].

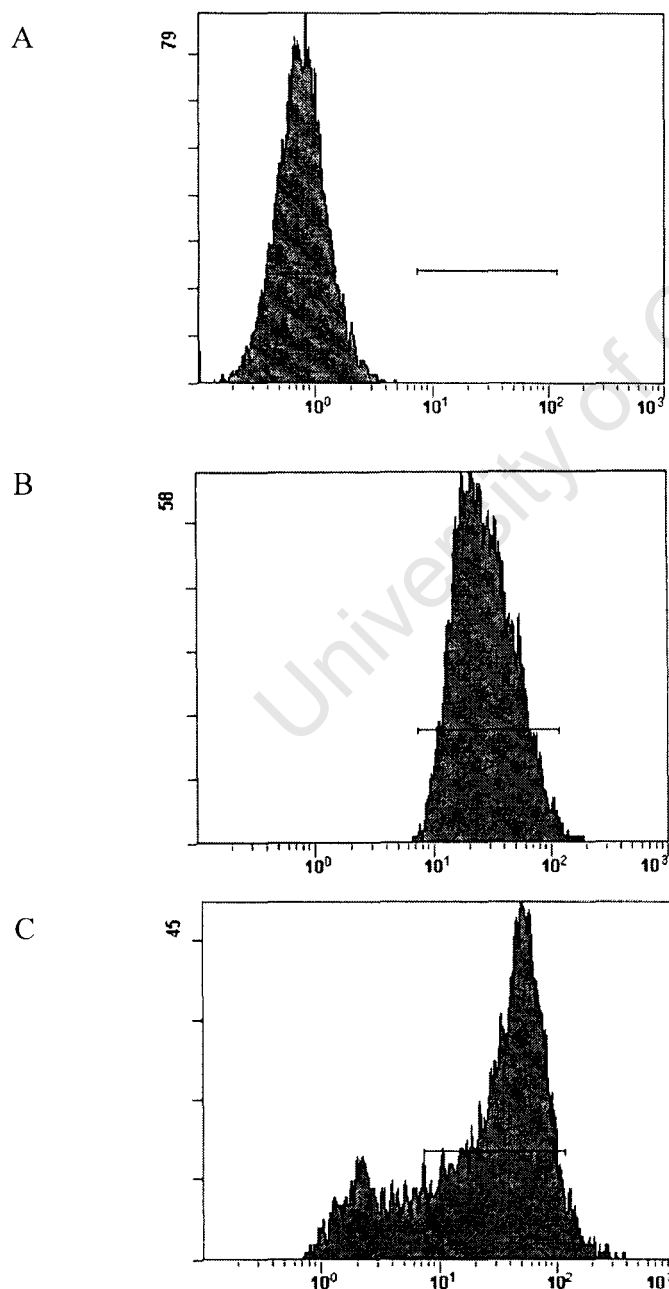
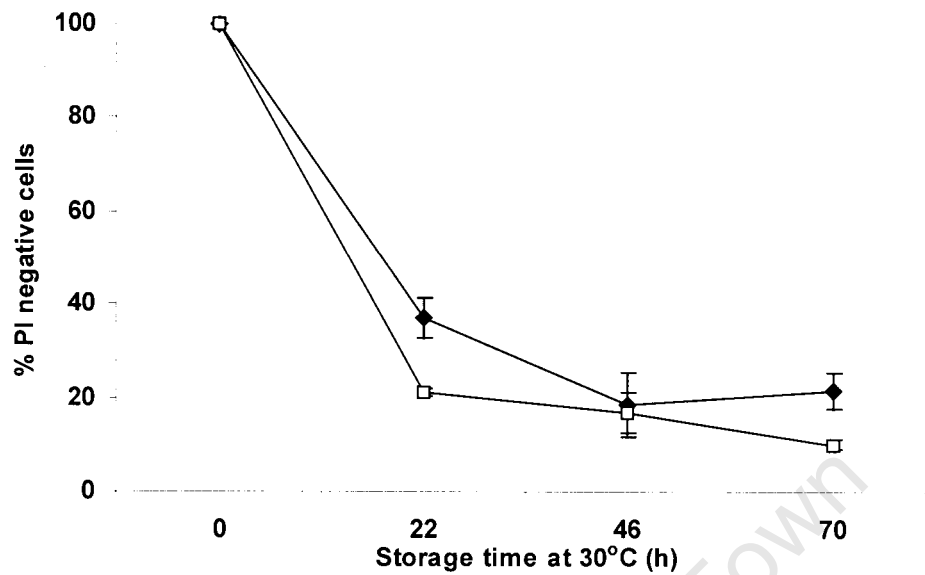


Figure 5: Representative histogram profiles obtained following PI staining and flow cytometry analysis of cells after rehydration. (A) Viable cells (negative control); (B) heat inactivated cells (positive control); (C) 22 h after desiccation. The profiles show fluorescence on the X axis and event number on the Y axis. The gate, set according to the positive control, represents PI positive cells.

A



B

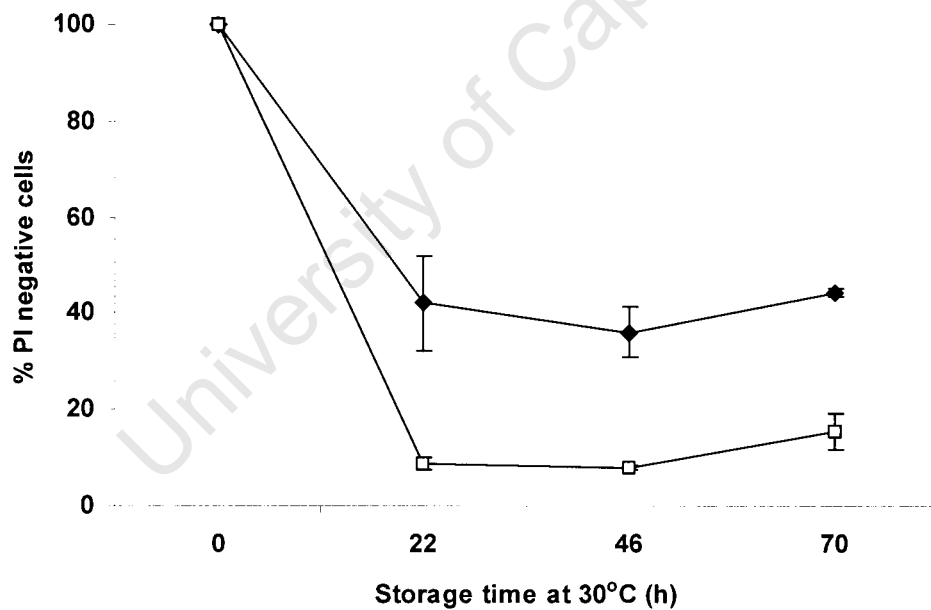


Figure 6: Percentage propidium iodide (PI) negative wild type (◆) and $\Delta hsp12$ (□) cells following rehydration after storage at 30°C for 0, 22, 46 and 70 h. Data for mid-exponential phase cells (A) and stationary phase cells (B) are shown. The error bars represent the standard error of the data derived from experiments performed in triplicate. Error bars not visible are within the data symbols.

Table 2: PI negative cells are a reliable indicator of cell viability.

Strain	Growth phase	Correlation Co-efficient
Wild type	Mid-exponential	0.98
	Stationary	0.95
$\Delta hsp12$	Mid-exponential	0.99
	Stationary	0.96

Correlation between percentage PI negative cells and percentage CFU. Wild type and $\Delta hsp12$ cells were grown to mid-exponential and stationary phases and desiccated by storage at 30°C for 0, 22, 46 and 70 h. The number of culturable and PI negative cells was determined after rehydration at the relevant time points.

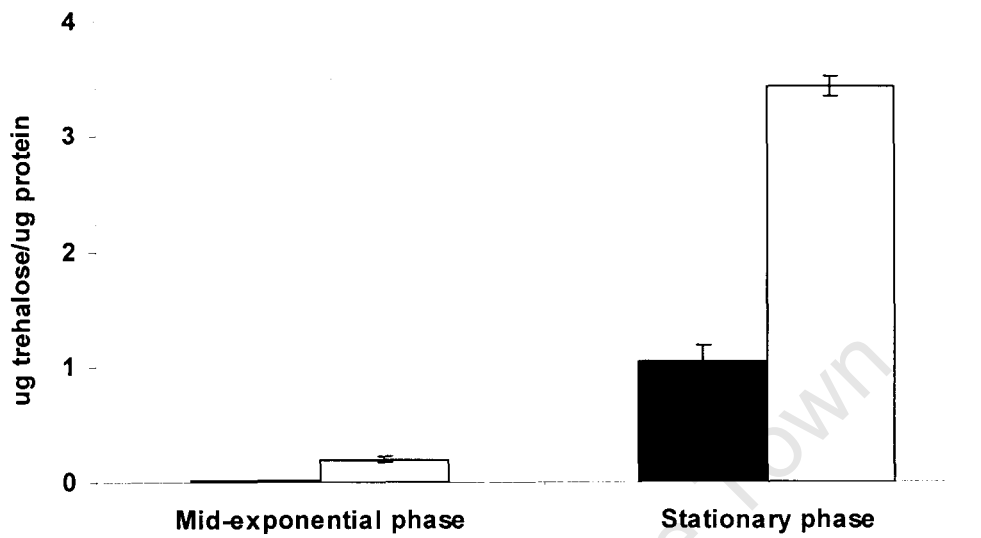
3.3 Investigation of the effect of increased levels of trehalose in the $\Delta hsp12$ strain under non-stress conditions

It has been proposed that trehalose might act as a scavenger of ROS [121]. Since ROS might contribute to decreased cell viability during desiccation, we investigated the relationship between the trehalose concentration, the oxidised glutathione (GSSG) concentration and the malondialdehyde (MDA) concentration in both strains in both mid-exponential and stationary phases. Reduced glutathione (GSH) has been shown to protect *S. cerevisiae* against oxidative stress by scavenging ROS, thereby being converted to GSSG [122]. Lipid peroxidation, caused by ROS-catalysed oxidation of cell membrane unsaturated fatty acids, results in the formation of MDA [57, 84].

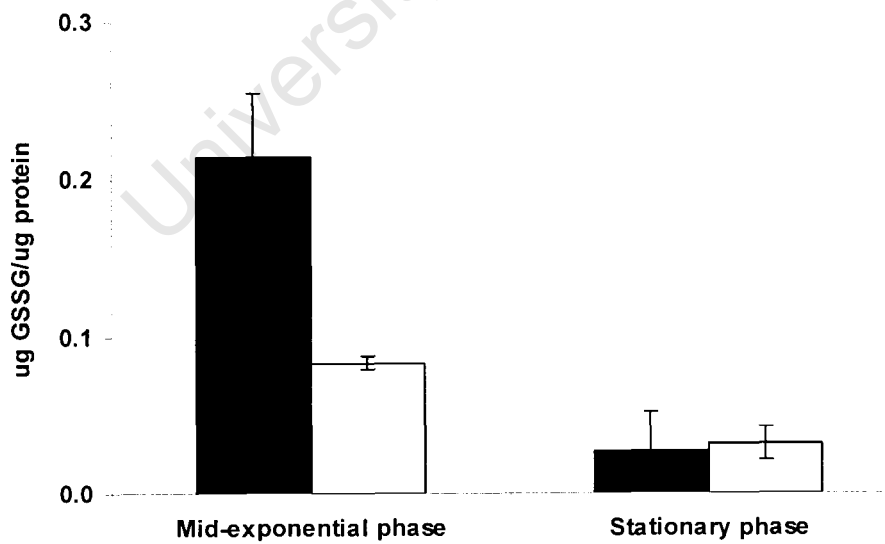
Both strains were grown to mid-exponential and stationary phase, at which points the trehalose, GSSG and MDA concentrations (Figs. 7A, 7B and 7C) were determined. At mid-exponential phase, the wild type strain displayed lower concentrations of trehalose and higher concentrations of GSSG and MDA than the $\Delta hsp12$ strain. At stationary phase, even though the $\Delta hsp12$ strain displayed a higher concentration of trehalose than the wild type strain, both strains had similar GSSG concentrations and the wild type strain displayed a higher MDA concentration. Our interpretation of these data is that, in mid-exponential phase, the increased trehalose concentration present in the $\Delta hsp12$ cells protect against ROS as shown by the decreased GSSG and MDA concentrations. In stationary phase, we propose that decreased metabolic activity resulted in less ROS reduction by lower levels of GSH, which in turn caused

increased lipid peroxidation. This effect was enhanced in wild type cells, which displayed lower trehalose concentrations.

A



B



Continues on pg. 40

C

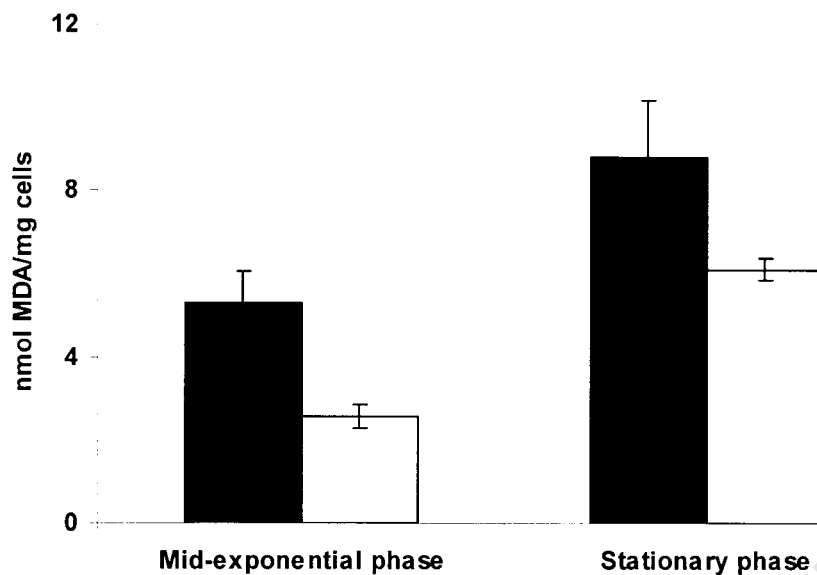


Figure 7: Trehalose concentrations (A), GSSG concentrations (B) and MDA concentrations (C) in the wild type cells (solid bars) and $\Delta hsp12$ cells (empty bars) at mid-exponential and stationary phase. The error bars represent the standard error of the data derived from experiments performed in triplicate.

3.5 Investigation of the role of Hsp12p and trehalose in applied oxidative stress tolerance

Micro-array studies have shown that *HSP12* transcription in *S. cerevisiae* was upregulated in yeast cells experiencing oxidative stress [123]. Additionally, 2-D electrophoresis has shown that Hsp12p synthesis was also upregulated within 15 min of exposure to H_2O_2 [48]. We initially confirmed these data by demonstrating that the wild type strain transformed with the pYES2-HSP12-GFP2 plasmid [100] exhibited increased fluorescence after 30 min exposure to 25 mM H_2O_2 (Fig. 8). Since Hsp12p biosynthesis increased in response to H_2O_2 exposure, we investigated whether the wild type strain would exhibit increased growth under oxidative conditions in relation to the $\Delta hsp12$ strain. Both strains were grown to mid-exponential and stationary phases and subsequently grown on agar plates containing up to 5 mM H_2O_2 . We found (Fig. 9) that cells grown to stationary phase generally tolerated growth in the presence of H_2O_2 better than cells grown to mid-exponential phase, confirming previous data that stationary phase cells have a degree of constitutive resistance towards applied oxidants [103]. In agreement with our previous observations (Fig. 3), the $\Delta hsp12$ strain tolerated H_2O_2 better

than the wild type strain in both phases. We therefore postulated that the $\Delta hsp12$ strain tolerates the H_2O_2 stress better than the wild type due to the trehalose that it overproduces.

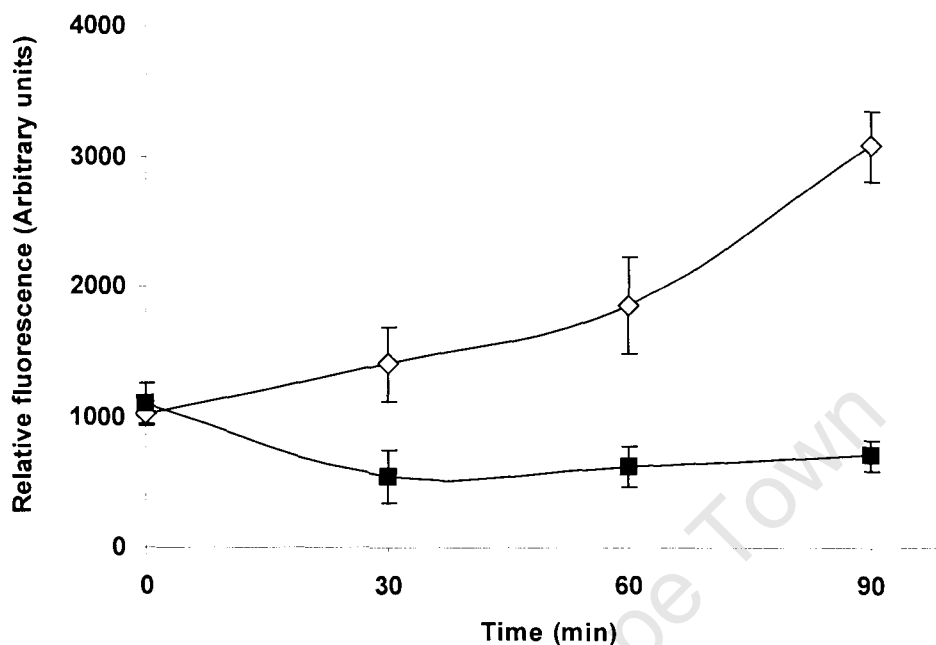


Figure 8: Relative fluorescence (450 nm_{excitation}, 507 nm_{emission}) of Hsp12-Gfp2p fusion protein expressed in the wild type strain, which contained the pYES2-*HSP12-GFP2* plasmid construct. Oxidative stress (25 mM H_2O_2) was applied to mid-exponential phase cells and the fluorescence measured over 90 min. ■, control cells (no H_2O_2 addition); ◇, stressed cells. The error bars represent the standard error of the data derived from experiments performed in triplicate.

Oxidative damage is known to occur when ROS levels exceed a toxic threshold [107]. To investigate the effects of added oxidant, both yeast strains were grown to mid-exponential and stationary phases, at which point 2 mM H_2O_2 was added to mid-exponential phase cells and 4 mM H_2O_2 was added to stationary phase cells. These H_2O_2 concentrations were chosen as they caused approximately equivalent reductions in yeast growth on solid medium (Fig. 9). After 1 h of treatment, the trehalose and MDA concentrations were determined (Fig. 10A and 10B). We found that in mid-exponential phase cells treated with 2 mM H_2O_2 , there was a marginal increase in the trehalose concentration in both strains. The MDA concentration increased approximately 2-fold in wild type cells with only a slight increase observed in $\Delta hsp12$ cells. In stationary phase cells, no effect of H_2O_2 was observed on either the trehalose or the MDA

concentrations of wild type cells. In contrast, exposure to 4 mM H_2O_2 resulted in an approximate 2-fold decrease in the trehalose concentration with no effect observed for the MDA concentration in the $\Delta hsp12$ cells.

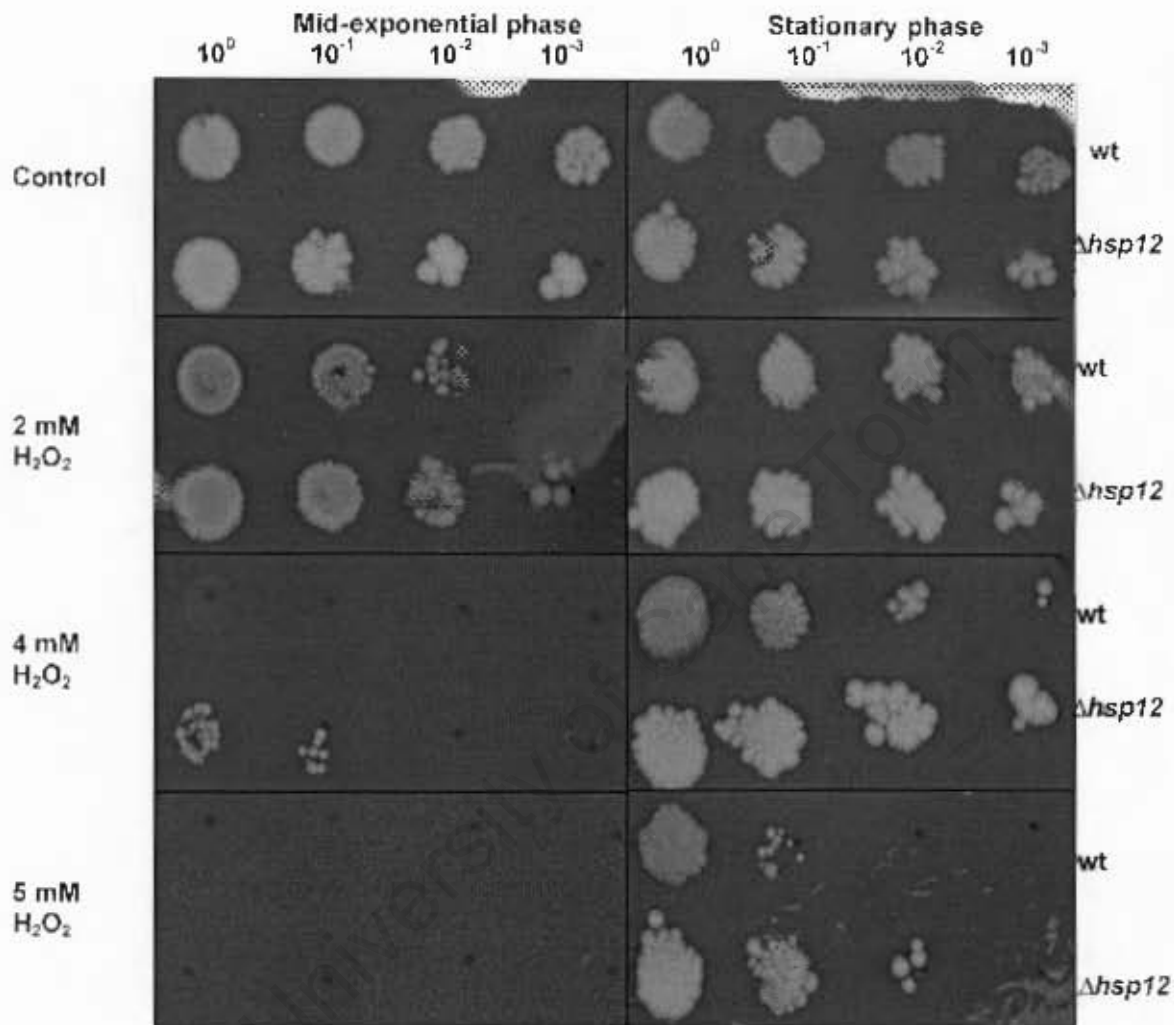
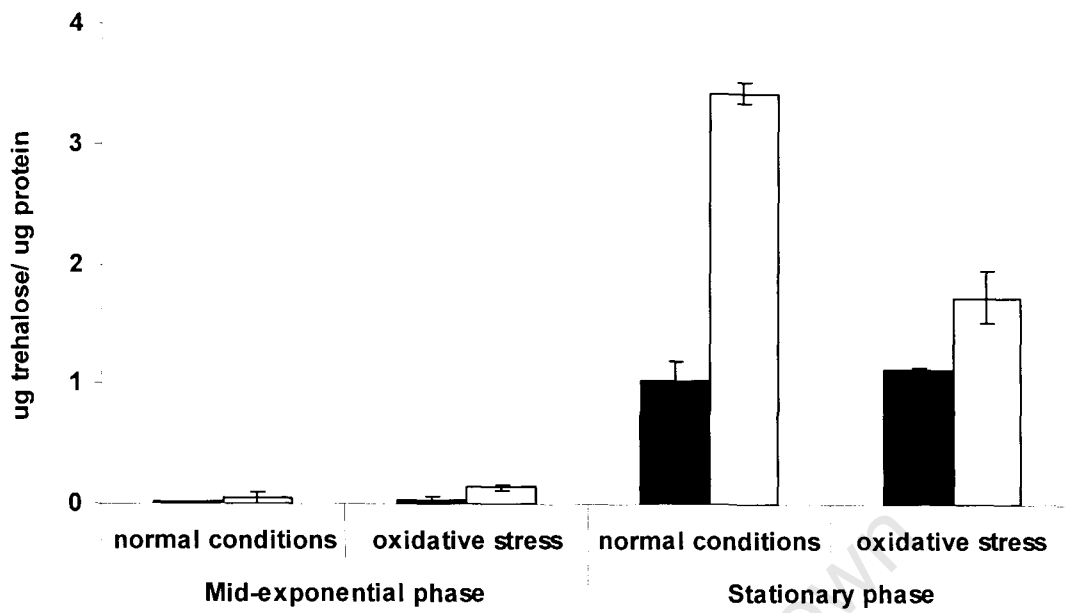


Figure 9: Growth at 30°C for 72 h of mid-exponential and stationary phase cultures of wild type and $\Delta hsp12$ yeast at various H_2O_2 concentrations. Values above the panels indicate the dilution of the yeast cultures used. Note that some reflection artefacts have resulted during photography.

The role of Hsp12p in maintaining the integrity of the plasma membrane during oxidative stress was investigated using propidium iodide staining. Representative histogram profiles following oxidative stress application are illustrated in Figure 11. Exposure for 1 h at 30 °C of mid-exponential phase cells to low concentrations of H_2O_2 resulted in no measurable damage (not shown). However, exposure to 750 mM H_2O_2 (Fig. 12) resulted in approximately 70% of

A



B

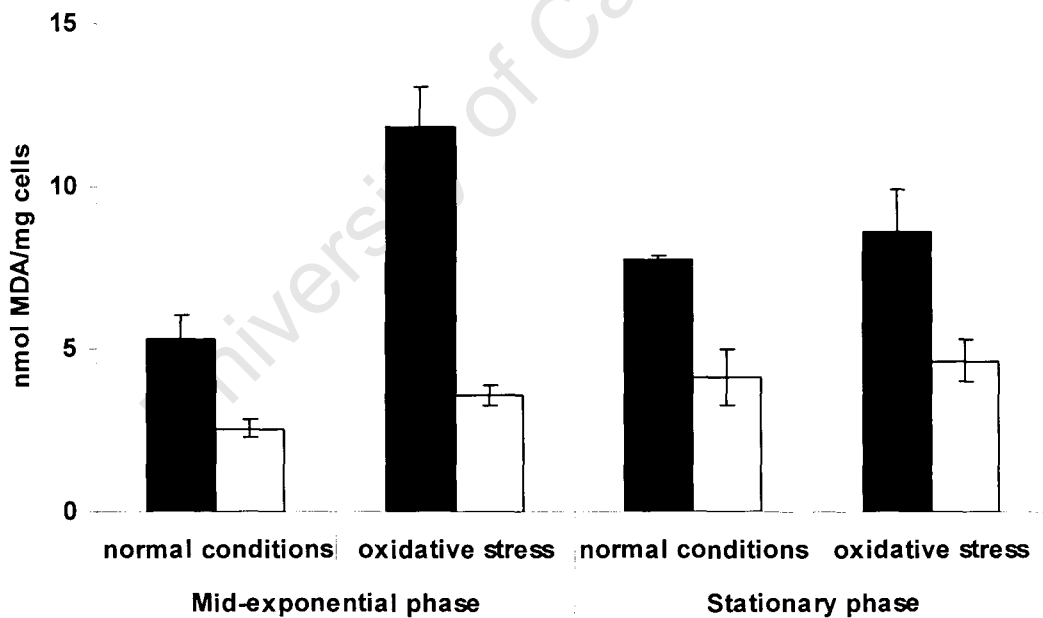


Figure 10: Trehalose concentrations (A) and MDA concentrations (B) in wild type cells (solid bars) and $\Delta hsp12$ cells (empty bars) at mid-exponential and stationary phase under the indicated conditions. Oxidative stress was 2 mM and 4 mM H_2O_2 at mid-exponential and stationary phases respectively. The error bars represent the standard error of the data derived from experiments performed in triplicate.

wild type cells exhibiting increased membrane permeability compared with almost 90% of $\Delta hsp12$ cells. Approximately 75% of mid-exponential phase cells over-expressing Hsp12p had more permeable membranes (not shown). Neither the wild type nor $\Delta hsp12$ strain showed enhanced membrane permeability when stationary phase cells were used (Fig. 12).

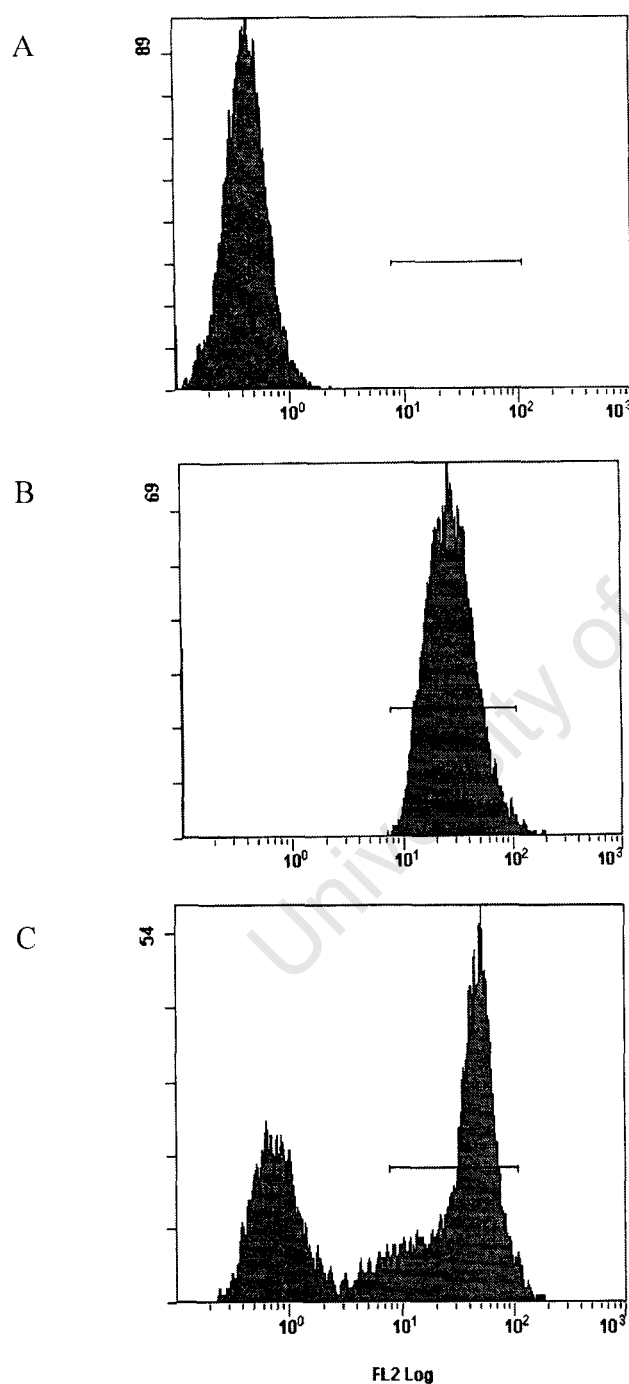


Figure 11: Representative histograms profiles obtained following PI staining and flow cytometry analysis of cells after oxidative stress treatment. (A) Viable cells (negative control); (B) heat inactivated cells (positive control; illustrating the usual PI staining of membrane permeable cells); (C) 1000 mM H_2O_2 for 60 min. The profiles show fluorescence on the X axis and event number on the Y axis. The gate, set according to the positive control, represents PI positive cells.

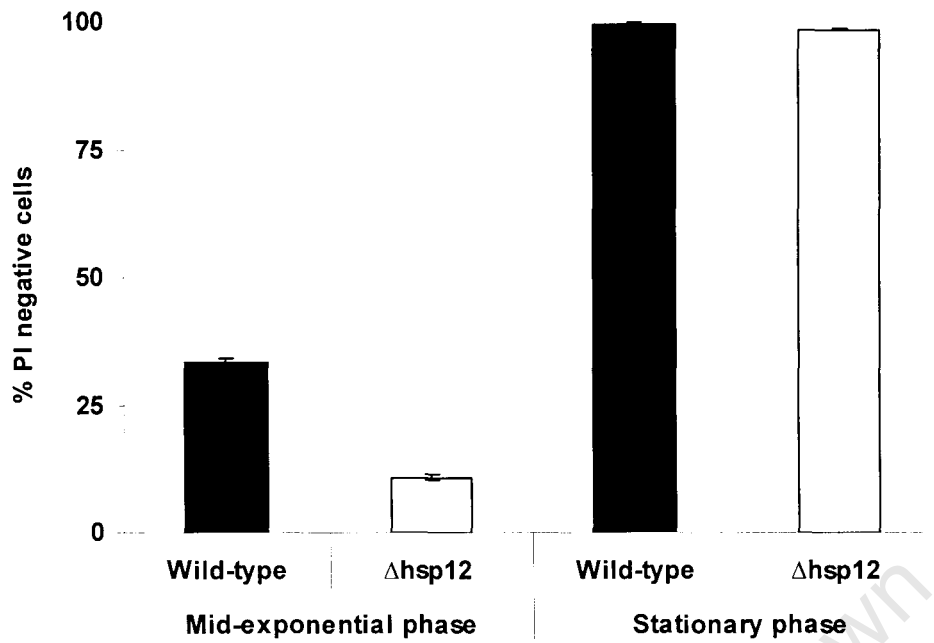


Figure 12: Percentage propidium iodide (PI) negative wild type (filled bars) and $\Delta hsp12$ (empty bars) cells after oxidative stress exposure of 750 mM H_2O_2 for 60 min. Data for mid-exponential phase cells and stationary phase cells are shown. Error bars represent the standard error of the data derived from experiments performed in triplicate.

Chapter 4: Discussion

The observation that Hsp12p was synthesised in response to heat treatment [62] suggested that it may play a role in yeast desiccation tolerance, as heat stress generally occurs prior to periods of desiccation in the natural environment. This fact and the observation that Hsp12p protects liposomal membranes against desiccation *in vitro* [58] prompted us to ascertain whether Hsp12p provides the same membrane protection *in vivo*. The ability of membrane protecting agents to access the plasma membrane and form hydrogen bonds has been proposed to determine the viability of yeast subsequent to desiccation [124]. The fact that Hsp12p has high hydrogen bonding potential and can access the phospholipid head groups in the plasma membrane, due to its amino acid and putative random coil structure, make it a likely membrane protective agent during desiccation.

Micro-array studies have revealed that the transcription of cell wall structure related genes changes during desiccation. A decrease in the transcription of *FKS1* ($\beta(1-3)$ glucan synthase) and numerous cell wall mannoprotein-related genes e.g. *PMT1-5* have been reported [66]. These changes result in the cell wall losing rigidity and becoming flexible during desiccation and suggest that both the composition and organisation of the cell wall are critical for yeast desiccation tolerance. Hsp12p may function in maintaining the cell wall organisation during desiccation, as *HSP12* transcription increases in response to desiccation and Hsp12p has been shown to increase the flexibility of the yeast cell wall [65, 96].

The observation that the stationary phase cells used in this study tolerated desiccation better than the mid-exponential phase cells was not unanticipated, as the physiological state of microbial cells is known to determine their stress tolerance [53]. Exponential phase yeast cells are known to be less desiccation tolerant. It has been proposed that this is because they contain barely detectable levels of trehalose and because the trehalose specific transporter in the plasma membrane is inactive. Stationary phase cells have increased intracellular trehalose levels and there is increased synthesis of the trehalose transporter [38, 39, 49, 51]. In addition, stationary phase cells have thicker cell walls which contribute to their stress tolerance [53].

The finding that the mid-exponential phase wild type strain displayed marginally improved desiccation tolerance compared to the $\Delta hsp12$ strain provided the first evidence that Hsp12p may well protect the plasma membranes of yeast against desiccation *in vivo*. However, the

converse occurred when the same experiment was performed on stationary phase cells, the $\Delta hsp12$ strain being more tolerant than the wild type strain. This result was unexpected and prompted us to investigate whether any additional protective mechanisms were employed by the $\Delta hsp12$ cells in an attempt to compensate for the loss of protection normally provided by Hsp12p. The prior observation that trehalose could replace Hsp12p in protecting liposomes against desiccation [58] and the knowledge that high levels of endogenous trehalose increase the desiccation tolerance of yeast [120], led us to investigate whether stationary phase $\Delta hsp12$ cells were compensating for the lack of Hsp12p by over-producing trehalose and thereby becoming more tolerant to desiccation than the wild type cells.

The intracellular trehalose levels were determined before and during desiccation in both strains at both growth phases. There were significantly higher trehalose concentrations in stationary phase cells compared to mid-exponential phase cells in both strains. The observation that the $\Delta hsp12$ strain contained more trehalose than the wild type strain at both phases, confirmed the hypothesis that the $\Delta hsp12$ strain is over-producing trehalose to compensate for the lack of Hsp12p. There appeared to be a direct correlation between the intracellular trehalose concentration and the residual viability of the cells following desiccation, consistent with previous studies [36, 39, 124]. In agreement with previous data [120], the length of the desiccation process had little effect on the intracellular trehalose levels of stationary phase cells. In contrast the trehalose concentrations in mid-exponential phase cells decreased to levels not measurable. We propose that the decrease in intracellular trehalose during mid-exponential phase is due to the cells consuming trehalose to obtain energy required to cope with the desiccation. It has been shown that there is no significant trehalose biosynthesis in desiccated yeast, as there is no available sugar to be transformed into trehalose and the cost of synthesising trehalose through gluconeogenesis or from glycogen is energetically too costly [66]. This finding prompted Singh *et al.* 2005 to propose that a small protein or peptide, such as a hydrophilin, may function in a similar manner to trehalose during desiccation [66]. This proposal and the fact that Hsp12p was identified as a hydrophilin [65], supports the idea that Hsp12p may participate in membrane protection during desiccation.

The accumulation of trehalose in the yeast cytoplasm effectively acts as a hypo-osmotic stress, in that two glucose molecules combine during the formation of each trehalose molecule. This hypo-osmotic stress results in plasma membrane stretch and the activation of the cell wall integrity pathway [31]. Given that the cell wall organisation and composition are critical for

proper cellular function and that Hsp12p plays an important role in maintaining cell wall structure, we propose that the $\Delta hsp12$ strain over-expresses trehalose to mimic a hypo-osmotic stress condition. This would cause plasma membrane stretch to activate the PKC pathway and result in the cell wall being remodelled to compensate for the loss of structural integrity normally provided by Hsp12p.

Standard microbiological methods to monitor cell viability, such as CFU assays, provide limited information as they only indicate viability. Sub-lethally injured or viable but non-culturable cells often go undetected with these methods, which provide no information regarding the physiological states of the cells [125]. Yeast populations are heterogeneous and several physiological states do exist. The use of flow cytometry allows a cell's physiological condition and hence the population's heterogeneity to be determined. Many studies have stressed the importance of using more than one viability indicator to obtain a comprehensive overview of the physiological states of cells being examined [113, 125, 126]. Since Hsp12p is located in close proximity to the yeast plasma membrane [30, 58], the role of Hsp12p in maintaining the plasma membrane integrity during desiccation was investigated using flow cytometry. Propidium iodide is a good indicator of membrane integrity, as it only enters cells with permeable cytoplasmic membranes [127, 128]. This molecule is only fluorescent when it stoichiometrically intercalates into double stranded DNA [113, 129]. It was found that the percentage of PI negative cells following desiccation was greater for the wild type strain than for the $\Delta hsp12$ strain at both growth phases. This result confirmed that Hsp12p protected cells during desiccation by maintaining the integrity of the plasma membrane.

Strains with high levels of endogenous trehalose have been shown to have low levels of lipid peroxidation, which suggests that trehalose may participate in protecting against oxidative damage [121]. This suggestion is supported by the fact that trehalose decreases the rate of unsaturated fatty acid oxidation *in vitro* [59]. Furthermore, trehalose is synthesised in response to heat, desiccation and oxidative stress, all of which contribute to increased intracellular oxidation. It was therefore proposed that trehalose might act as a ROS scavenger [121]. The proposal that a sugar can act as a free radical scavenger is not unfounded, as both glycerol and mannitol are known to be effective ROS scavengers [59]. We decided to confirm whether trehalose might be acting as a ROS scavenger by exploiting the fact that the $\Delta hsp12$ strain contains higher endogenous trehalose levels than the wild type strain. The effect of trehalose on the maintenance of redox homeostasis and on the extent of lipid peroxidation was

determined in both strains at both growth phases under non-stress conditions. The redox status of the cells was determined from the intracellular GSSG content, while the extent of lipid peroxidation was determined from the intracellular MDA content. MDA is a toxic by-product of ROS-catalysed oxidation of unsaturated fatty acids [40, 57, 105]. GSH is normally present in high concentrations in the cells; it has a redox active sulphhydryl group that reacts with ROS and becomes oxidised to GSSG [122]. The increased trehalose content of the $\Delta hsp12$ strain at mid-exponential phase seemed to protect against ROS. This was concluded from the fact that the $\Delta hsp12$ strain had lower levels of GSSG and MDA than the wild type strain, indicative of decreased ROS and lipid peroxidation respectively. Both strains had similar GSSG levels in stationary phase, which we propose is due to decreased metabolic activity in these cells, and is a consequence of lower levels of ROS reduction by GSH. Cells that are deficient in GSH have been shown to have increased lipid peroxidation levels [122]. Both strains exhibited increased lipid peroxidation in stationary phase, but the level was higher in wild type cells, which contained lower intracellular trehalose levels. These results suggest that trehalose may act as a ROS scavenger, thereby preventing oxidative damage [59, 121]. Trehalose is presumed to function synergistically with other oxidative defence mechanisms in the cell to provide protection against oxidative damage. It is suggested that trehalose prevents the initial damage caused by ROS, but does not assist in the long-term cellular response to oxidative damage [59]. It was recently suggested that trehalose scavenges ROS from yeast cells that possess a deficiency in one or more defence pathways and thus acts as a complementary defence mechanism [57]. This provides another plausible reason for the synthesis of elevated levels of endogenous trehalose in the $\Delta hsp12$ strain.

Hsp12p has been implicated in protection of yeast cells against copper ions, which at elevated concentrations result in the formation of ROS. Previous studies have suggested that Hsp12p may provide membrane protection during oxidative stress [70]. We initially decided to probe whether Hsp12p biosynthesis increased in response to oxidative stress. This was achieved by measuring the fluorescence emitted from cells harbouring a GFP2-HSP12 fusion construct in response to H₂O₂ exposure. H₂O₂ was chosen as the oxidant in this study, as it is the most abundant cellular ROS and crosses biological membranes freely [130]. H₂O₂ is sensed as an oxidative stress but its toxicity is primarily due to highly reactive hydroxyl radicals, which form during Fenton and Haber-Weiss reactions [102, 131]. H₂O₂ causes protein oxidation, lipid peroxidation and DNA lesions [51, 59, 102]. The increase in fluorescence in response to H₂O₂

was comparatively slow during the first 60 minutes of exposure. This was not unexpected, as gene expression in response to oxidative stress is known to occur slightly later compared with responses to other stresses [101]. Furthermore, the maximal induction of several heat shock proteins is relatively delayed, peaking after 45-60 min of stress exposure [48, 73]. The fact that Hsp12p biosynthesis occurred in response to oxidant exposure suggested that it might provide protection against oxidative stress.

We subsequently investigated whether the wild type strain would tolerate growth under oxidative conditions better than the $\Delta hsp12$ strain. Stationary phase cells of both strains grew better than the mid-exponential phase cells on media containing H_2O_2 . This finding corroborated the statement that stationary phase cells possess a measure of constitutive resistance towards oxidants due to the elevated expression of the genes encoding trehalose production, antioxidant enzymes and heat shock proteins [84, 103, 132]. Moreover, it was found that the cellular permeability of stationary phase cells to exogenous H_2O_2 was 5 fold lower than exponential phase cells, possibly due to the thickness of the cell walls. The ability to limit H_2O_2 diffusion into the cell has been shown to correlate with cell survival and is suggested to be a key protective mechanism against exogenous H_2O_2 [132]. The plasma membrane and cell wall have both been suggested to act as barriers that limit the diffusion of H_2O_2 into the cells. The fact that the cell wall can act as a barrier is not unfounded, as the cell wall is the direct site of contact with the environment and it is porous to low molecular weight molecules [130]. The cell wall of the $\Delta hsp12$ strain is more compact and less flexible than that of the wild type strain [96]. In fact the hydrodynamic permeability of the $\Delta hsp12$ strain is lower than the wild type strain, suggesting that Hsp12p increases the yeast cell wall permeability. This provides a feasible explanation as to why the $\Delta hsp12$ strain tolerated H_2O_2 better than the wild type strain in both growth phases - essentially their compact cell walls are limiting the diffusion of H_2O_2 into the cells.

The oxidative stress tolerance of both strains was assessed in order to give an indication of whether Hsp12p may provide tolerance towards applied oxidative stress. During oxidative stress the main target of ROS are the unsaturated fatty acids of the plasma membrane. The resulting lipid peroxidation causes loss of membrane integrity and the influx of undesirable substances [57]. The MDA concentration increased substantially in mid-exponential phase wild type cells during oxidative stress, but not in the $\Delta hsp12$ strain. This suggested that the wild

type strain was experiencing greater oxidative stress than the $\Delta hsp12$ strain. The intracellular trehalose concentrations were consequently determined before and after oxidative stress, to establish whether the over-production of trehalose in the $\Delta hsp12$ strain was perhaps increasing its oxidative stress tolerance. The role of trehalose as a protector against oxidative stress is however controversial, as the capacity of cells to survive severe H_2O_2 exposure and trehalose accumulation were found to be unrelated [133, 134]. Furthermore, a deficiency in trehalose synthesis did not impair the acquisition of H_2O_2 tolerance [103]. These studies both suggest that trehalose is not essential for tolerating H_2O_2 . In this study, it was found that the intracellular trehalose levels increased marginally in both strains at mid-exponential phase, which might suggest that the strains are synthesising trehalose in an attempt to tolerate the applied oxidative stress. But we further suggest that it is the initial trehalose concentration present in the cells that is likely to determine their tolerance towards applied oxidants, as the $\Delta hsp12$ strain had slightly higher initial endogenous trehalose levels and consequently experienced less oxidative stress than the wild type strain. In support of this, a previous study has shown that the initial trehalose concentration in the cells does correlate with the cells resistance to oxidative stress [59]. The MDA concentrations were not significantly altered in either of the strains during stationary phase, which suggests that both strains are tolerating the oxidative stress equally well. The finding that exogenous H_2O_2 had no effect on the intracellular trehalose levels of the wild type strain but caused a significant decrease in that of the $\Delta hsp12$ strain led us to suggest that the $\Delta hsp12$ strain is utilising trehalose to obtain energy needed to recover and grow in the presence of H_2O_2 . This finding also suggests that the presence of Hsp12p in stationary phase may allow the cells to tolerate exogenously applied oxidants.

The plasma membrane forms the second cellular defence against the influx of exogenous substances into the cells. The plasma membrane is hypothesised to protect cells against exogenous H_2O_2 . In fact it was suggested that the regulation of the plasma membrane permeability coefficient may be a new mechanism by which cells respond to H_2O_2 . The observation that $\Delta erg3$ and $\Delta erg6$ strains, both of which have increased membrane permeabilities, were more sensitive to H_2O_2 provided the initial supporting evidence for the hypothesis [130, 132]. We decided to establish whether Hsp12p might maintain the permeability of the plasma membrane during H_2O_2 exposure, due to the observation that Hsp12p maintains the integrity of the plasma membrane during desiccation. Exposure of mid-exponential phase cells to high H_2O_2 concentrations resulted in all cells displaying increased

membrane permeabilities. High H₂O₂ concentrations were used to elicit strong cellular responses and facilitate studies. It should be noted that such a severe stress almost certainly results in considerable damage and even death in a large part of the population under study [27]. It was found that a larger proportion of the $\Delta hsp12$ cells than the wild type cells became permeable. This shows that Hsp12p plays a role in maintaining the integrity of the plasma membrane during oxidative stress. The observation that over-expression of Hsp12p could not further increase membrane protection against H₂O₂ can be attributed to the fact that Hsp12p synthesis is maximally up-regulated in wild type cells during H₂O₂ exposure. The Hsp12p content consequently reaches the threshold level needed for protection against H₂O₂ and any additional Hsp12p synthesised would not further contribute to increased membrane protection. The role of Hsp12p in maintaining the integrity of the plasma membrane during stationary phase could not be conclusively established, as both the wild type and $\Delta hsp12$ strains showed little to no membrane permeability under the conditions used in this part of the study.

Chapter 5: Conclusions

The ability to resolve the function of a protein in a particular stress response is difficult when the only observed association is enhanced expression of the gene encoding the protein [27]. The creation of isogenic knockout strains was intended to allow a high degree of unprejudiced functional analysis of different genes [135]. However, genetic alterations in yeast do not always lead to altered phenotypes due to numerous reasons [27]. The most pertinent reason, applicable to this study, is the fact that compensatory protective mechanisms are often stimulated, such as in the $\Delta hsp12$ strain. The trehalose synthesised by the $\Delta hsp12$ strain masked the *HSP12* deletional mutation, making the role of Hsp12p in desiccation and oxidative stress tolerance difficult to establish. Since trehalose synthesis and *HSP12* expression are correlated in yeast, they may fulfil similar functions in the cell, but it is possible that the similarities are purely circumstantial. It is more likely that trehalose and Hsp12p act synergistically but provide different modes of protection against the imposing stress condition. The results of this study led us to suggest that the putative role of Hsp12p in *S. cerevisiae* is to stabilise the plasma membrane against membrane damaging stresses including desiccation and oxidative stress. Additionally, we conclude that trehalose may be one of the principal protective factors against oxidative stress by acting as a ROS scavenger.

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