THE ISOLATION AND CHARACTERIZATION OF HEAT SHOCK PROTEIN HSP12 IN *LIPOMYCES STARKEYI*

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ABSTRACT

The stress response protein Hsp 12 is induced in *S. cerevisiae* cells upon exposure to salt stress, heat shock, ethanol, and upon entry to stationary phase (Mtwisha et al., 1998). In this study, the occurrence of proteins related to Hsp12 was investigated in a number of yeasts (namely, *Saccharomyces cerevisiae* S288C, *Schizosaccharomyces pombe*, *Debaromyces hansenii*, *Lipomyces starkeyi* Y-2024, *Saccharomyces cerevisiae* IFO 23X7 (Kaokai), *Zygosaccharomyces rouxii* and *Pichia sorbitophila*. This was performed by selective protein extraction followed by SDS-PAGE and western blotting using a *S. cerevisiae* anti-Hsp 12 antibody. The results showed that almost all the yeasts investigated possessed a protein that had an identical migration to that of Hsp 12 with the exception of *S. pombe*, which contained a 9 kDa protein. Western blotting using the anti-Hsp 12 antibody cross-reacted only with the two *S. cerevisiae* species in addition to the 12 kDa protein from *Lipomyces starkeyi* of all the species investigated. MALDI-TOF peptide mass analysis after tryptic digestion of the *L. starkeyi* 12 kDa protein showed that a close sequence similarity existed to that of *S. cerevisiae* Hsp 12 and none to rest of the 12 kDa proteins isolated from all the other species investigated.

In order to determine the sequence of the Hsp 12 protein, the *L. starkeyi* Hsp 12 gene was amplified using *S. cerevisiae* Hsp 12 primers. Gene sequencing of both *S. cerevisiae* and *L. starkeyi* Hsp 12 genes revealed three nucleotide differences existed between them. *L. starkeyi* Hsp 12 was found to be present in relatively small amounts during early growth stages but increased during log phase with a slight further increase during stationary phase. Increasing the salt concentration in the growth medium was found to induce Hsp 12. Increased levels of Hsp 12 appeared to confer a degree of protection during desiccation and subsequent rehydration of both *L. starkeyi* and *S. cerevisiae*.

ABBREVIATIONS:

A_{600}	absorbance at 600nm
BSA	bovine serum albumen
BCIP	5-bromo-4-chloro-3-indolyl phosphate
$CaCl_2$	calcium chloride
°C	degrees centigrade
D. hansenii	*debaryomyces hansenii*
E.coli	*escherichia coli*
fig	figure
g	gram
HCl	hydrochloric acid
h	hours
HSF	heat shock factor
Hsp	heatshock protein
IPTG	isopropyl β-D-thiogalactopyranoside
kDa	kilodalton
LEA	Late Embryogenesis Abundant
Lip	*Lipomyces starkeyi*
LB	luria broth
L. starkeyi	*Lipomyces starkeyi*
mA	milliamperes
MALDI-TOF	matrix assisted laser desorption/ionisation-time of flight
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
$MgCl_2$	magnesium chloride
Mw	molecular weight
min	minutes

NBT	4-nitroblue-tetrazolium chloride
NaOH	sodium hydroxide
nm	nanometer
NaCl	sodium chloride
PMSF	phenylmethanesulfonylchloride
PCR	polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
TEM	transmission electron microscopy
TWEEN 20	polyoxyethylenesorbitan monolaurate
Tris-HCl	Tris-(hydroxymethyl)aminomethane
TBS	Tris buffered saline
µl	microlitre
µm	micrometer
µg	microgram
V	volts
YEPD	yeast extract peptone dextrose
YPD	yeast peptone dextrose

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CHAPTER 1

1. GENERAL INTRODUCTION

An organism has to continuously adapt to various kinds and degrees of change to ameliorate adverse environmental stress factors to their natural environment. The ability of an organism to respond rapidly to fluctuations in temperature, nutrients, and other environmental changes is important for competitive fitness and cell survival. Understanding the response of cells to environmental changes is of interest because it can provide clues to the molecular apparatuses that enable cells to adapt to new environments and the molecular mechanisms that have evolved to regulate the remodelling of gene expression that occurs in those new environments (Causton et al., 2001). Significant clues to the mechanisms involved in adaptation to a new environment have come from studies of the genes that are expressed in response to specific stresses. For example, cells exposed to elevated temperature induce transcription of genes encoding heat shock proteins. The heat shock proteins represent a family of approximately a dozen proteins that are evolutionarily conserved. Studies of heat shock proteins led to a realization that many function as molecular chaperones (Ellis, 1999). Molecular chaperones are involved in regulation and maintenance of protein structure and function and have roles in almost every cellular process. Some molecular chaperones may even facilitate evolutionary processes (Rutherford and Lindquist, 1998).

Molecular chaperones or heat shock proteins (Hsps) occur in all organisms and are indispensable for the survival of the cell. Hsps were identified by the strong induction of protein expression under heat stress or other stress conditions (Schlesinger, 1986; Lindquist and Craig, 1988). As more information about Hsps functions became available, it turned out that they are involved in or even required for many cellular functions under normal growth conditions. The cellular activities of chaperones both in housekeeping tasks and stress protection are based on their ability to interact with proteins that are either unfolded or have not acquired their native conformation (Voos and Rottgers, 2002).

Generally, substrates for chaperone interaction are mainly hydrophobic proteins segments that are exposed to the surrounding solution. Aptly designated as chaperones, Hsps stabilize and protect bound polypeptides by preventing irregular interactions leading to denaturation and aggregation. Reactions like ribosomal translation or intracellular protein transport are prone to expose either unfolded protein segments to the environment or require posttranslational folding and/or unfolding events. Hence, most essential cellular functions of chaperones are connected with the biogenesis of proteins or organelles. The importance of molecular chaperones suggests that it will be valuable to identify and further study the complete set of stress inducible genes. If the number of stress-responsive genes is substantial, their identification could make a significant contribution to functional annotation of an important set of previously uncharacterised genes (Voos and Rottgers, 2002; Schmidt et al., 2001).

Cells must co-ordinate adjustments in genome expression to accommodate changes in their environment. Despite our lack of knowledge about the complete set of genes involved in these changes, investigators have identified transcriptional activators and repressors that likely contribute to co-ordinate remodelling of genome expression (Parker and Topol, 1984; Wu, 1985; Kingston et al., 1987; Sorger and Pelham, 1987; Causton et al., 2001). During heatshock, heatshock factors (Hsfs) are activated by different kinds of activators. In the absence of heat shock, Hsf1 (an activator) is inactive; the molecular chaperone Hsp 90 is thought to contribute to this inactivation by binding and sequestering the activator (Ali et al., 1998; Duina et al., 1998; Zou et al., 1998). Another set of activators, Msn2 and Msn4, act in concert to induce expression of genes under almost any stress condition. Msn2 and Msn4 normally reside in the cytoplasm and are transported during stress into the nucleus, where they bind to stress response elements (STREs) in promoters (Estruch and Carlson, 1993; Marchler et al., 1993; Martinez-Pastor et al., 1996; Schmitt and Mc Entee, 1996; Gorner et al., 1998; Alexandre et al., 2001).

The complete set of genes induced by various environmental changes has not been established; therefore it is not yet clear that their activators for stress are responsible for co-ordinate induction of their genes. Because the cell membrane is the site of the primary contact of the cell with its environment, its flexibility and adaptation capability largely determines the survival ability of the cell. The membrane structural lipids form a part of a complex and still insufficiently known adaptation system. The basic adaptation mechanisms are widely different in different cell types: a lowering of temperature below an optimum level brings about in both mammalian tissue and in micro-organisms a restructuring of the acyl residues of phospholipids, which alter the membrane fluidity to be compatible with basic physiological functions of the cell. It should be noted, however, that the extent of adaptation decreases with increasing specialization and differentiation of the cells (Sajbidor, 1997).

1.1 LEA-proteins

Recent data suggest that LEA-like proteins are also found in fungi, and they seem to be also involved in pathways that respond to water deficit. That is the case for Hsp 12 and GRE1 from *Saccharomyces cerevisiae*, whose amino acid compositions show a high content of hydrophilic amino acids distributed throughout their primary structure. In both cases it has been shown that their corresponding transcripts accumulate in response to hyperosmotic stress (Garay-Arroyo et al., 2000).

Stress response is a general property of all living organisms and survival of cells critically depends on their ability to sense alterations in the environment and then to respond appropriately to the new situation through the induction of protective stress responses. Yeast has turned out to be a good model system to study phenomena related to stress and the consequences of different types of stress can be deciphered. Novel mechanisms inaugurated by stress have been intensively studied (Garay-Arroyo et al., 2000).

Basic knowledge about the fundamental mechanisms of yeast responses to abiotic stress including salinity, freezing, heat, drought, ethanol and osmotic stresses are crucial to future industrial and pharmaceutical production of food and diagnostic products respectively. Plants, algae and bacteria have evolved a common set of adaptive responses to ameliorate the damaging effects of dehydration, salinity, cold, heat and osmotic stress. These adaptations include the accumulation of low molecular weight compounds known collectively as compatible solutes or osmoprotectants and glycine-rich, hydrophilic proteins known as late embryogenesis abundant (LEA) proteins. These LEA proteins in plants accumulate in developing seeds prior to maturation drying and are presumed to help protect embryos from desiccation stress. The unusual solubility properties of these proteins, such as resistance to heat coagulation, have led to suggestions that they alter the hydration properties of cellular constituents. Hydration characteristics and the water potential range at which wheat heat-soluble LEA proteins were expressed have been determined. However, the exact mechanisms by which LEA proteins confer stress tolerance are poorly understood. Representative genes from different LEA families have been described (Baker et al., 1988). The Group 1 LEA proteins are characterised by their very hydrophilic nature, and their high degree of similarity between species over the entire coding region. The greatest variation between Group 1 proteins are in the number of highly hydrophilic internal repeats present in protein family members (Espelund et al., 1992; Stacy and Aalen, 1998). Conserved glycine residues are present inside and outside of the repeat motif, and are predicted to represent turns in the secondary polypeptide structure. The copy number of the internal motif in Group 1 genes from a wide range of plant species varies from one to four (Stacy et al., 1995; Stacy and Aalen, 1998). While little evidence exists so far to confirm the proposed role of Group 1 LEA protein in protections against water stress, the high degree of random coil structure (and therefore likely enhanced water-binding capacity of the protein) suggests it could prevent embryos from experiencing lethal levels of desiccation (McCubbin et al., 1985; Stacy and Aalen, 1998). Complete genome databases are enabling novel approaches to determine if different organisms have evolved similar biochemical solutions to environmentally stressful conditions by using DNA microarrays (Garay-Arroyo et al., 2000; Alexandre et al., 2001).

1.2 Heat shock response

Heat shock response is a universal phenomenon observed in all organisms. Exposure to elevated temperature or other types of stress, such as anoxia and starvation, leads to the synthesis of so-called heat shock proteins (Hsps) (Lindquist, 1986). The accumulation of Hsps is associated with the acquisition of thermotolerance, an increased ability to survive short exposures to otherwise lethal temperatures (McAlister and Finkelstein, 1980). However there is no direct evidence for the protective function of Hsps during heat stress (Schlesinger, 1986). The yeast *Saccharomyces cerevisiae* has multigene families encoding the large Hsps (Werner-Washburne et al., 1987; Borkovich et al., 1989). Some small Hsps of Mw 26 kDa (Hsp 26) and Mw 12 kDa (Hsp 12) and others have also been identified (Petko and Lindquist, 1986; Mtwisha et al., 1998).

The large Hsps are highly conserved between species. Mutational analysis in yeast has shown that they perform vital functions during normal growth (Normington et al., 1989; Chirico et al., 1988; Deshaies et al., 1988; Ostermann et al., 1989). In spite of their massive accumulation after heat shock, their role of acquisition of thermotolerance is not clear, since gene inactivations had no effect on the induction of thermotolerance by brief heat shock (Craig and Jackobsen, 1984; Borkovich et al., 1989). Consistent with these observations, work with *Drosophila melanogaster* and *Dictyostelium discoideum* has shown a strong correlation between the accumulation of the small Hsps and the acquisition of thermotolerance (Berger and Woodward, 1983; Loomis and Wheeler, 1982). There are several Hsps known like Hsp 104, Hsp 90, Hsp 70, Hsp 60 and Hsp 27, which carry out similar functions in protein refolding from different cellular compartments. In addition, a number of smaller molecular weight Hsps of unknown function has been described. This includes the hydrophilic Hsp12 protein (Praekelt and Meacock, 1990; Mtwisha et al., 1998).

1.2.1 Small heat shock proteins

Heat shock proteins are classed as Group VI LEA-proteins (Parsell and Lindquist, 1993). Exposure of nearly of all organisms to an increased growth temperature causes the induction of the heat shock response (Parsell and Lindquist, 1993). These proteins are believed to assist the organism in coping with elevated effects of elevated temperature. Heat shock protein expression is either constitutive with induction by heat or expressed under developmental control. It is suggested that this response is important during various stages of development under normal growth temperature (Craig et al., 1993; Lee et al., 1994; Waters et al., 1996; Voos and Rottgers, 2002).

The small heat shock proteins (smHsps) generally range in masses from 12 kDa to 30 kDa (Helm et al., 1997) and are thought to be involved in the acquisition of thermotolerance (Lee et al., 1995). The smHsps in plants constitute about 1.0 % to 1.5 % of the total protein induced by the heat shock response (Jinn et al., 1995; Lee et al., 1995). The smHsps have been categorised into four multigene families based on the sequence similarity. Class 1 and class 2 are localised to the cytoplasm, class 3 to the chloroplast and class 4 to the endoplasmic reticulum (Vierling, 1991; Helm et al., 1993). A fifth class has been identified in several species and found to be localised in the mitochondria (Lenne and Dounce, 1994; Waters et al., 1996). A sixth class associated with membrane structures is also proposed to exist (Waters et al., 1996). There is evidence that the smHsps play a role in the acquisition of thermotolerance of cells (Berger and Woodward, 1983; De Groot et al., 2000)

1.2.2 Induction of heat shock proteins

Sublethal heat shock and ethanol stresses induce practically identical changes to protein synthesis. Above a critical threshold level, both stresses strongly induce heat shock protein synthesis, with simultaneous suppression of synthesis of most proteins made prior to the stress (Piper, 1993; Piper et al., 1994; Parsell and Lindquist, 1993; Mager and Moradas-Ferreira, 1993; Plesset et al., 1982).

1.3 Osmotic shock

In response to hyperosmotic environments, most eukaryotic cells activate a specialized mitogen-activated protein (MAP) kinase pathway. In *S. cerevisiae*, the key protein kinase Hog1 coordinates the transcriptional induction of variety of genes devoted to osmoadaptation and general stress protection (Alepuz et al., 2001).

Exposure of living cells to a solution with high osmolarity causes a rapid efflux of water. In order to adapt to the reduced water activity in the environment, and the consequent decrease in turgor pressure, cells respond by synthesizing or accumulating osmoprotective compounds (Brown, 1978; Brown and Edgley, 1979; Le Rudulier et al., 1984; Higgins et al., 1987; Booth and Higgins, 1990; Csonka, 1989;Wiemken, 1990; Varela et al., 1992). Upon osmotic upshift of exponentially growing *S. cerevisiae* cells, glycerol seems to be the major compatible solute (Brown, 1978; Brown and Edgley, 1979; Trollmo et al., 1988). A common response of organisms to drought, salinity, and low-temperature stresses is the accumulation of sugars and other compatible solutes. These compounds serve as osmoprotectants and, in some cases, stabilize biomolecules under stress conditions. One such compound as trehalose, a nonreducing disaccharide of glucose, plays an important physiological role as an abiotic stress protectant in a large number of organisms, including bacteria, yeast, and invertebrates. Trehalose has been shown to stabilize dehydrated enzymes, proteins, and lipid membranes, as well as protect biological structures from damage during desiccation (Sales et al., 2000). Apart from adaptation processes, a stress response is likely to be evoked in salt-treated cells to protect them against the unfavourable conditions (Varela et al., 1992; Garg et al., 2002).

Yeasts differ considerably in their tolerance to osmotic stress, the most tolerant species (e.g *Dipodascus australiensis*) commonly growing on substrates highly enriched in sugar or salt (Tunblad-Johansson and Adler, 1987; van Zyl et al., 1993; Yagi, 1988; Larsson and Gustafsson, 1993; Luxo et al., 1993). One of the mechanisms that has evolved to counter the water stress caused by osmotic dehydration involves synthesis and accumulation of an intracellular osmolytes which does not give rise to enzyme perturbations or other toxic symptoms (Edgley and Brown, 1978; Kayingo et al., 2001).

1.4 Ethanol stress

Yeast cells have developed a panel of stress response (transient) and adaptation mechanisms (long-term response) to cope with deleterious effects of ethanol. Heat shock proteins (Hsps) are synthesized during ethanol stress although their role in ethanol stress is still not well understood (Piper, 1995). It remains to be determined whether they play a similar role during heat shock, i.e. a stabilising effect, preventing aggregation of proteins, and assisting the refolding of proteins (Singer and Lindquist, 1998).

No systematic studies have looked at the molecular processes involved in the ethanol stress response (Piper, 1995). Although some genes, such as the Hsp genes, have been shown to respond to ethanol stress, the pleiotropic effects of ethanol suggest that a large number of genes involved in this specific stress response are still to be discovered. Furthermore, although specific stress response pathways exist for osmotic, heat, and oxidative stress, it is still not known how ethanol signalling occurs. A few years ago it was difficult to carry out a global gene expression study to identify important genes regarding ethanol stress. DNA microarrays now allow gene transcription in response to ethanol to be assessed (Alexandre et al., 2001; Causton et al., 2001).

1.4.1 Gene promoter elements responsive to heat and ethanol

Two gene promoter elements, the heat shock element (HSE) and the general stress responsive element (STRE), have been shown to direct activation of yeast heat shock with heat stress. The HSE consists of alternating repeats of the sequence nGAAn at each half-turn of the DNA helix while the STRE consensus sequence is AGGGG or CCCCT. These two elements differ in the diversity of stresses that cause their activation. The HSE is primarily activated only by heat shock in vegetative cells, losing its heat-inducibility at stationary phase. Ethanol induction of the STRE is readily demonstrable, as also is STRE activation by several other stresses, including heat shock and osmotic stress. Another major difference between the HSE and the STRE is that the latter element is under negative regulation by a cAMP-dependent protein kinase, while HSE activity is unaffected by the activity of this kinase. It is conceivable that ethanol may partly induce STRE activity through the oxidative stress that it causes, since oxidative stress is now known to influence the activity of certain STRE-regulated promoters (Piper, 1995). Recent data have shown that the addition of acetaldehyde induces several Hsps such as Hsp104 by 4 to 16 fold (depending on the strain), Hsp12 by 7 fold and Hsp26 induction by up to 26 fold. Ethanol stress also produces an increase in the expression of these Hsp genes (up to 2 fold for Hsp12, 9 fold for Hsp26, and 28 fold for Hsp104). Thus in most cases higher induction levels were detected with acetaldehyde than with ethanol (Aranda et al., 2002).

1.5 Stress responses.

Stress responses are ubiquitous among living cells. Chemical agents, temperature, osmotic shock, and nutrient depletion are among a highly diverse group of environmental conditions that alter patterns of gene expression in prokaryotic and eukaryotic cells. Among the most thoroughly studied of these stress responses is the transcriptional activation of heat shock genes following transient increase of growth temperature. The stress response differs from the classical heat shock regulatory response in that gene transcription is efficiently stimulated by a remarkable variety of stresses that includes in addition to heat shock, DNA alkylation, osmotic shock, oxidative damage, heavy metal

exposure, and certain types of nutrient limitations (Marchler et al., 1993; Kobayashi and McEntee, 1990, 1993).

1.6 Saline stress

Yeast cells have been considered an excellent model for the study of the mechanisms underlying tolerance to saline stress. This is because fungi and higher plants not only have similar ion transport systems in their plasma membranes but also share similar cation detoxification mechanisms (Gaxiola et al., 1999) and, most probably, signal transduction pathways (Lee et al., 1999; Pardo et al., 1998; Goossens et al., 2002).

Exposure of yeast cells to saline stress implies both exposure to specific cation toxicity and to osmotic stress. High salinity represents a stress for organisms because the excess of Na^+ or other monovalent cations imbalances the osmotic potential and generates water deficit, and the influx of Na^+ may also lead to metabolic toxicity (Hasegawa et al., 2000). Protective biochemical reactions range from the synthesis of osmolytes to increased chaperone activity, enhanced oxygen radical scavenging, changes in redox control, increased proton pumping activity, adjustments in carbon/nitrogen balance, and altered ion and water deficits (Hasegawa et al., 2000; Gaxiola et al., 1999). Certain ions, such as Na^+ or Li^+, are toxic to cells due to their ability to inhibit specific metabolic pathways, probably through inhibition of specific targets. This has been shown in the case of the yeast Hal2 protein and certain RNA-processing enzymes (Murguía et al., 1996; Dichtl et al., 1997). Therefore, regulation of intracellular ion contents represents an important response to ion stress. For instance, exposure to Na^+ increases the expression of the ENA1/PMR2A gene, encoding a P-type ATPase responsible for Na^+ and Li^+ ion efflux (Garciadeblas et al., 1993; Wieland et al., 1995; Hasegawa et al., 2000).

Increase in extracellular osmolarity results in transient induction of the expression of stress protective genes. A major outcome from this response is the accumulation of intracellular glycerol, which relies on the activation of the Hog1 (high osmolarity glycerol response) MAP kinase pathways (Brewster et al., 1995; Albertyn et al., 1994; Siderius et al., 1997). MAP kinases play a key role in regulation of stress responses in

many organisms from mammals to yeast (Waskiewicz and Cooper, 1995). Hog1 MAP kinases are essential for the survival of yeast in high osmolarity environments (Brewster et al., 1995) and are activated under osmotic-stress conditions by two independent osmo-sensors, a two-component system and the transmembrane protein Sho1 (Maeda et al., 1994; Maeda et al., 1995; Posas et al., 1996). These sensing mechanisms activate a kinase cascade that involves the Ssk2, Ssk22, and Ste11 MAP kinase kinase kinases (Maeda et al., 1995; Posas et al., 1996; Posas and Saito, 1997), the Pbs2 MAP kinase kinase, and finally, the Hog1 MAP kinase. Once phosphorylated, the Hog1 MAP kinase is translocated into the nucleus, where it induces diverse stress responses. It is worth noting that both phosphorylation and nuclear localization of Hog1 are very rapid and transient (Maeda et al., 1995; Posas et al., 1996; Posas and Saito, 1997; Ferrigno et al., 1998). The mechanism of gene regulation through activated Hog1 is still unknown because transcription factors under the control of these MAP kinases have not yet been well characterised. These are the transcriptional factors Msn1, Msn2, and Msn4 (Schmitt and McEntee, 1996; Rep et al., 1999), the bZIP-type protein Sko1 (Proft and Serrano, 1999), and Hot1 (Rep et al., 1999). Although the requirement for the Hog1 kinase has been demonstrated for the osmotic upregulation of a number of genes, an exhaustive list of the genes required for osmotic-stress adaptation is far from complete.

1.7. Research questions: Hsp12 like protein in yeasts.

Lipomyces starkeyi was used in this study for investigation of the small heat shock protein, Hsp 12. I had used this species before and wished to study the Hsp 12 protein from this interesting yeast.

The finding of a Hsp 12-like protein in *Lipomyces starkeyi* prompted an investigation into the sequence and physiological response of this protein to stress. The following areas of study were the focus of this work:

1. The occurrence of Hsp12-like in different yeast species.
2. The sequence homology between *L. starkeyi* Hsp 12-like protein and *S. cerevisiae* Hsp 12.
3. The physiological response of *L. starkeyi* Hsp 12 to stress.

CHAPTER 2

MATERIALS AND METHODS

2.1 Yeast strains

The yeast strain used for this study was *Lipomyces starkeyi* (Y-2024) obtained as a kind gift from Prof. J.L.F. de Kock (University of Orange Free State), South Africa. Comparative studies were performed using *Saccharomyces cerevisiae* S288C diploid strain and *Schizosaccharomyces pombe*, *Debaromyces hansenii*, *Saccharomyces cerevisiae* IFO 23X7 (Kaokai), *Zygosaccharomyces rouxii* and *Pichia sorbitophila*. The stock cultures were maintained at 4 °C on slants containing the following: yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l; glucose, 10 g/l; and agar 20 g/l. The stock cultures were transferred after every three months to fresh slants. Cultures were stored at –70 °C until required.

2.2 Growth media

The YEPD (yeast extract peptone dextrose) medium for yeast growth contained the following constituents: yeast extract, 10 g/l; peptone, 20 g/l; and glucose, 20 g/l (Van Rensburg et al., 1995). For nitrogen growth limiting medium peptone was not included to the above described growth medium constituents. Solid media (plates and slants) were made by adding agar to 2 % to the described growth broth. The medium was sterilized by autoclaving at 121 °C for 20 min.

2.3 Growth conditions

Cells from the stock cultures were spread on YEPD 2 % agar plates and incubated at 30 °C for 48 h for colony formation. Precultures were generated from a single colony which was inoculated into 5 ml of YEPD medium and grown for 48 h with agitation. 0.5 ml of the preculture was then inoculated into 100 ml of the YEPD in a conical flask and incubated at 30 °C with shaking between 200-300 rpm in a controlled environment

Incubator (New Brunswick, USA). Growth rate analysis was performed on control and stressed cells by incubating yeasts cultures overnight at 30 °C and measuring cell densities by light scattering at 600 nm. Experiments were performed in duplicate.

2.4 Protein isolation

L. starkeyi cells were grown as described above and pelleted by centrifugation. Equal amounts of this pellet by wet weight and glass beads of size 710-1,180 microns (Sigma) were ball-milled (Braun) in cold extraction buffer 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.1 mM PMSF. The ball-mill was cooled with liquid CO_2. It was operated with 11 s shaking and 34 s cooling intervals (Sales et al., 2000). This cycle was repeated five times ensuring that the temperature remained between 1 °C and 5 °C. The homogenate was centrifuged at 27000 g (15000 rpm) using a Beckman JA 20 fixed head rotor at 4 °C for 10 min. The supernatant was incubated in a heating block at 100 °C for 10 min. An unheated sample was kept for analysis of total extracted proteins. The heated sample was briefly centrifuged at 12000 g for 5 min at 4 °C. The supernatant was then transferred into a new tube. Both the unheated and heated samples were analysed by SDS-PAGE (section 2.7).

2.5 Hyper-osmotic shock

The YEPD medium was supplemented with NaCl between 0 and 0.8 M. Cells were grown to stationary phase and heat soluble proteins were extracted as described. From the SDS-PAGE gel, protein concentrations were quantified using the TN-Image (Thomas-Nelson, 1995 version 2.19) computer programme.

2.6 Desiccation tolerance

Both *L. starkeyi* and *S. cerevisiae* species were grown on YEPD media with and without 0.3 M NaCl to stationary phase. Aliquots of yeast cultures were centrifuged and the pellets were resuspended in 50 mM Tris-HCl and 10 mM NaCl, pH 7.4 buffer. 50 µl aliquots were placed on an Eppendorf tube lid. This was then dried in a desiccator using

an airflow rate of 12 ml/min. At various times, cells were rehydrated using 950 µl of water and plated out on YEPD agar plates. Plates were incubated at 30 °C for two days. The number of colonies was determined.

2.7 SDS-PAGE gels

SDS-PAGE was performed out at room temperature using a 20 % separating gel with a 6.5 % stacking gel as described by Laemmli (1970). Gels were allowed to run for 4 h at a constant voltage of 250 V. The gels were stained with Coomassie Blue and destained using 45 % (v/v) methanol, 45 % (v/v) water, 10 % (v/v) acetic acid.

2.8 Western blotting

2.8.1 Transfer to Nitrocellulose

A nitrocellulose membrane of 0.45 µm pore size and Whatmann 3MM paper were cut to the size of the gel to be blotted using clean scissors and gloves. The anode side of cassette was assembled first with nappy liners pre-wetted in transfer buffer 25 mM Tris-HCl, 0.192 M glycine, 20 % (v/v) methanol, pH 8.3 drained and squeezed to remove excess buffer and placed on the anode carbon block. The SDS-PAGE gel was also dipped into the transfer buffer and laid flat on pre-wetted nitrocellulose paper, supported on pre-wetted Whatmann 3MM paper and placed on a thick wad of nappy liners. The gel was overlaid with pre-wetted Whatmann 3MM paper ensuring that no air bubbles remained trapped in the gel-paper sandwich. Another wad of nappy liners followed and thereafter the cathode block of carbon. Transfer was allowed to proceed overnight at 4 °C at 30 mA after which the nitrocellulose membrane was placed on a sheet of Whatmann 3MM paper and allowed to dry.

2.8.2 Visualisation of the transferred proteins

The dry nitrocellulose membrane was incubated in 10 ml blocking buffer (PBS) containing 5 % (m/v) skimmed milk powder for 30 min at room temperature. The antibody was added at a dilution of 1:100 and incubated for a further 2 h at room temperature. The membrane was washed 3 times with PBS containing 0.05 % (v/v) Tween, once with PBS, and then incubated for an hour at room temperature with 10 ml PBS containing 2 µl Goat-anti-Rabbit antibody coupled to alkaline phosphatase (Sigma). The membrane was washed 3 times with PBS containing 0.05 % (v/v) Tween and once with 10 mM Tris-HCl, 150 mM NaCl pH 7.4. The membrane was incubated in 10 ml of 100 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$ pH 9.5 to which 50 µl NBT (50 mg/ml in 70 % (v/v) DMF) and 50 µl BCIP (50 mg/ml in 100 % DMF) for secondary antibody detection. Incubation was allowed to proceed until bands appeared. The membrane was washed with water and dried.

2.9 In-Gel Tryptic Digestion

The band of stained protein was excised from the SDS-PAGE gel, cut into small pieces and placed in a 1.5 ml Eppendorf tube which had been washed 3 times with 500 µl $CH_3CN/H_2O/TFA$ (60: 39.9: 0.1 v/v). A blank section of the gel was used as a control. The cut gel pieces were washed sequentially with 250 µl CH_3CN/ H_2O (1:1 v/v) for 5 min, 250 µl $CH_3CN/$ 50 mM NH_4HCO_3 (1:1 v/v) for 30 min with agitation and finally with 250 µl CH_3CN/10 mM NH_4HCO_3 (1:1 v/v) for a further 30 min at room temperature without agitation. The gel pieces were dried to complete dryness before being rehydrated with 15 µl 10 mM NH_4HCO_3 containing 0.1 µg TPCK treated trypsin (Sigma) per 15 mm^3 of gel. After 10 min incubation, an additional 20 µl 10 mM NH_4HCO_3 was added and the digestion allowed to proceed at 37 °C for 24 h. A 1 µl aliquot was mixed with 1 µl α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in $CH_3CN/H_2O/TFA$ (60:37:3 v/v) and then subjected to MALDI-MS to obtain a peptide mass fingerprint.

2.10 Transmission electron microscopy

Yeast samples were fixed with 2 % formaldehyde 2 % glutaraldehyde in PBS and post-fixed with 1 % osmium tetroxide. The fixed samples were dehydrated using series of increasing ethanol concentrations before embedding in Spurr's resin (Spurr, 1969) and polymerized at 60 °C for 24 h. Thin sections (120 nm) were obtained using a Utracut S ultramicrotome (LEICA) and collected on copper grids. The samples were stained with 2 % uranyl acetate and 2 % lead citrate and were viewed and photographed at 120 kV using a JEM 200CX transmissiom electron microscope (JEOL, Tokyo, Japan).

MOLECULAR BIOLOGY

2.11 Isolation of total yeast DNA

5 ml culture of *L. starkeyi* Y-2024 was grown to stationary phase and harvested by centrifugation at 27000 g (15000 rpm) for 5 min at 4 °C. The pellet was suspended in 0.5 ml of homogenizing buffer 10 mM Tris-HCl, 50 mM NaCl, pH 7.4. 300 mg of 710—1180 microns (Sigma) glass beads were added to the suspension and the mixture vortexed at a high speed for 1 min. The homogenate was removed from the beads and 0.5 ml lysis solution 200 mM NaOH, 1 % SDS was added. The lysate was mixed gently by inversion and left at room temperature for 10 min. 0.5 ml of 5 M potassium acetate pH 5.4 was added and mixed by gentle inversion and left on ice. After 15 min on ice, the resulting precipitate was pelleted by centrifugation. The supernatant containing DNA was transferred to a new tube. 0.6 volumes of 100 % (v/v) isopropanol was used to precipitate DNA for 10 min at room temperature. The DNA pellet was recovered by centrifugation and purified by chloroform-isoamyl alcohol (24:1) after resuspension in 200 µl distilled water. The amount and purity of the DNA was determined spectrophotometrically.

2.12 PCR reactions

The *L. starkeyi* DNA was used as a template for amplification of the Hsp 12 gene. The Hsp 12 primers were designed from the *S. cerevisiae* Stanford gene database (SGD) and were used to amplify *L. starkeyi* DNA.

Primers and conditions used:

Forward- 5' ATGGATCCATGTCTGACGCAGGTAGAAAAG-3'
Reverse- 5' CTGAATTCTTACTTCTTGGTTGGGTCTTC-3'

The PCR programme used was:

Temp °C	Function	Time(s)	
95	Denaturation	3 min	
95	Denaturation	30 s	} 35 cycles
52	Annealing	30 s	
72	Extension	60 s	
72	Extension	5 min	

The optimum $MgCl_2$ concentration was determined experimentally and found to be 1.5 mM. The PCR product from agarose gel was extracted and purified using a Qiagen kit.

2.13 Ligation using the pGEM-T Easy vector and the 2X Rapid ligation buffer

All samples were briefly centrifuged or vortexed before use. pGEM-T Easy is a commercial vector which is provided together with its own positive control. The ligation reaction was performed as shown below:

	Reaction	Positive Control	Negative Control
Volume units	(µl)	(µl)	(µl)
2X Rapid Ligation Buffer	5	5	5
pGEM-T Easy Vector (50ng)	1	1	1
PCR product	3	–	–
Control Insert DNA	–	2	–
T4 DNA Ligase (3 Weiss units/µl)	1	1	1
Deionized water to final volume of	10	10	10

The reactions were mixed by pipetting and incubated for 42 h at 4 °C.

2.14 Transformation of competent cells

E. coli JM 109 strain competent cells were used for transformation. 5 µl of the ligation mixture was added to 95 µl of competent cells and incubated on ice for 10 min, at 37 °C for 5 min and again on ice for 5 min. 900 µl of LB broth was added to 100 µl transformed

cells, which were incubated with shaking for 90 min at 37 °C. 100 µl of the cell suspension was spread on LB-Amp-IPTG-XGal plates and incubated overnight at 37 °C.

2.15 Mini-prep method by alkaline lysis

Transformants were inoculated into 5 ml LB medium and grown overnight at 37 °C with ampicillin antibiotic selection. 2 ml were centrifuged at 12000 g in a 2 ml eppendorf tube (1 min) and the broth discarded.

The cells were resuspended into 0.2 ml 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA pH 8.0 and left on ice for 10 min after which 0.5 ml 0.2 M NaOH, 1 % SDS was added. After 10 min on ice, 0.3 ml pre-cooled 5 M potassium acetate, 60 ml; glacial acetic acid 11.5 ml; H_2O 28.5 ml was added and the cells left on ice for a further 10 min. The flocculent, which formed, were removed by centrifugation. 0.9 ml of supernatant was recovered and transferred to a 1.5 ml eppendorf tube. 0.6 ml isopropanol was added to precipitate the DNA. Samples were then centrifuged at 12000 g for 10 min and washed with 70 % (v/v) ethanol and air-dried.

2.16 Storage of bacterial cultures

Cells were stored at –70 °C by mixing 0.8 ml of the bacterial culture together with 0.15 ml of sterilized glycerol.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Growth characteristics of *L. starkeyi* and *S. cerevisiae.*

We initially compared the growth characteristics of the two species, *L. starkeyi* and *S. cerevisiae*. This was done to establish the time taken for yeast growth to reach stationary phase for each species. The growth phase has some effect on the yeast cell. Hsp 12 induction is known to be enhanced at stationary phase compared with lag phase.

Cells were grown in YEPD medium and cell densities were monitored for each species as a function of time. Each YEPD medium flask was inoculated with 0.5 ml of preculture with the same cell density, measured in a spectrophotometer at 600 nm. *L. starkeyi* was found to grow more slowly than *S. cerevisiae* with a prominent lag phase (fig. 1). *S. cerevisiae* growth reached stationary phase after 30 h compared with 48 h for *L. starkeyi.* The two species showed similar maximum growth rates. A possible reason for prolonged lag phase in *L. starkeyi* might be that an identical optical density may not guarantee an equal number of cells between two species, as a high percentage of lipids in *L. starkeyi*, which can be up to 70 % by weight, might impede light passing through the cells. *L. starkeyi* cells synthesise oils for energy and nutrient storage and these oils can be utilised during conditions of nutrient limitation. This might be the reason why *L. starkeyi* filled lipid containing organelles are present throughout growth (Uzuka, 1975). Since this was not our main focus of research, the reasons for the delayed lag phase in *L. starkeyi* were not investigated.

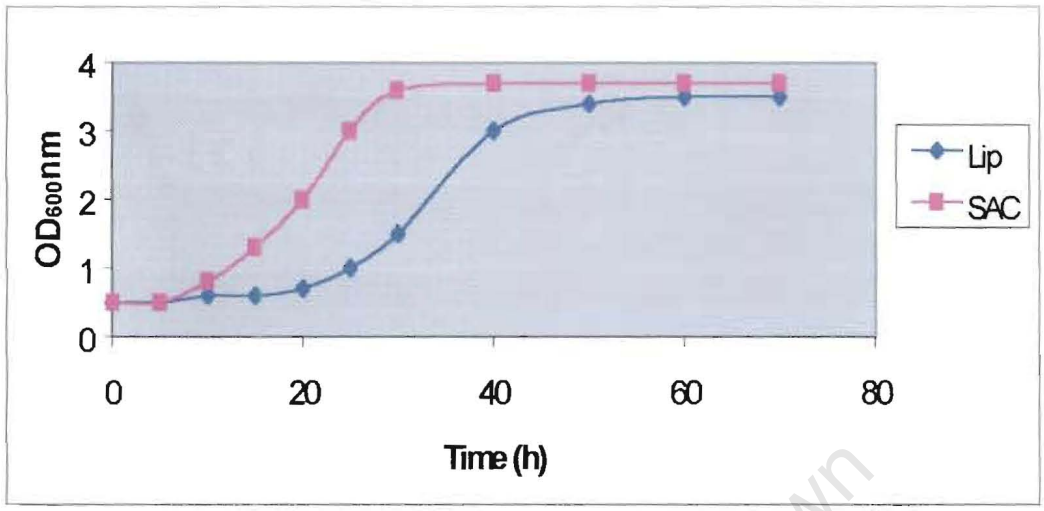


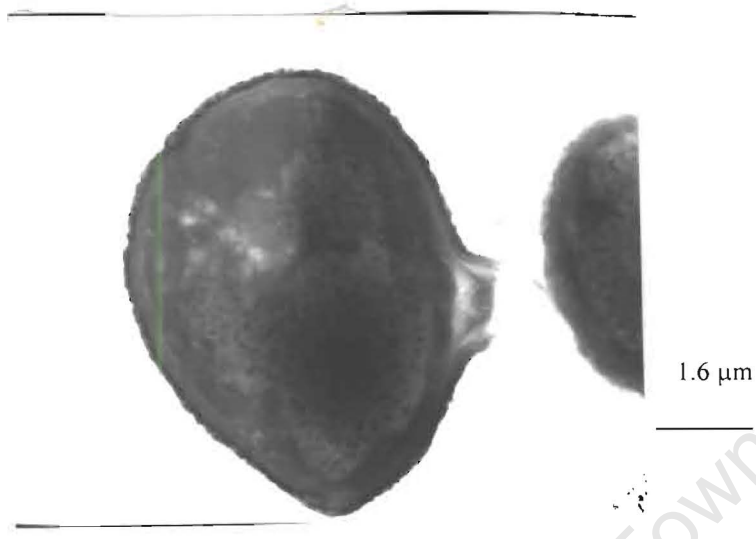
Figure 1: Growth rates of *L. starkeyi* (Lip) and *S. cerevisiae* (SAC) grown in YEPD medium under the same growth conditions. The same number of yeast cells based on the OD_{600} nm was used to initiate growth in both cultures.

3.2 Morphological studies

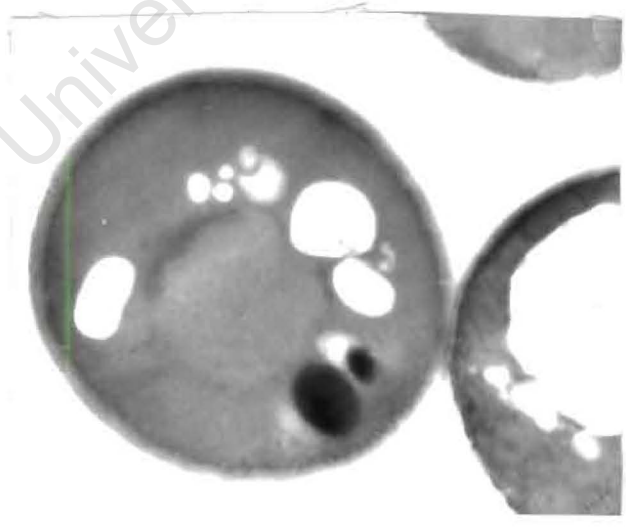
Since *L. starkeyi* and *S. cerevisiae* exhibited similar maximum growth rates, morphological studies were performed to ensure that the organisms used were indeed *L. starkeyi* and *S. cerevisiae*. The most striking difference between *L. starkeyi* and *S. cerevisiae* is that of *L. starkeyi* capable of forming large oil globules intracellularly when grown in excess of carbon but not with *S. cerevisiae*. *S. cerevisiae* divide by budding and *L. starkeyi* by fission.

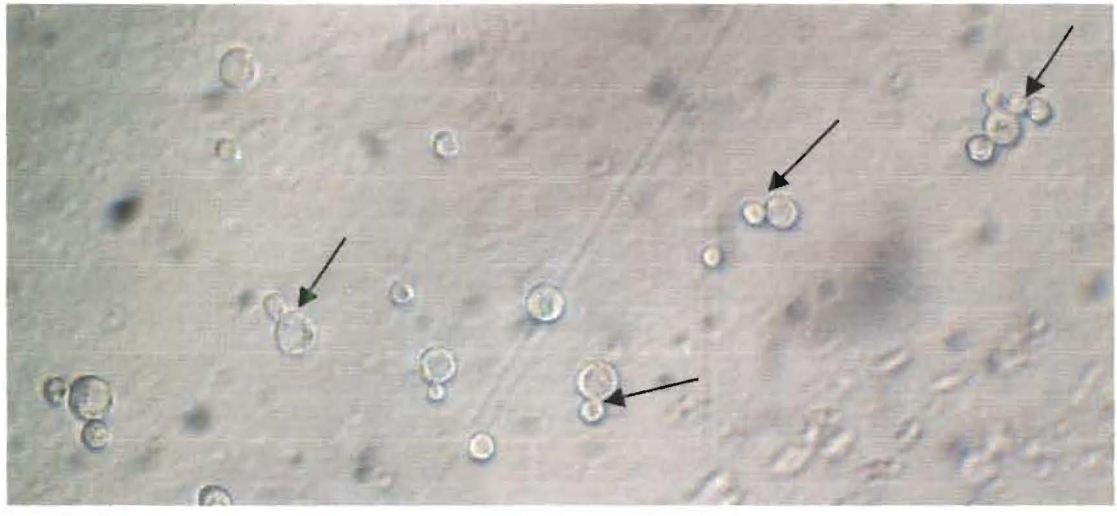
Cells grown to stationary phase were viewed by transmission electron microscopy (fig. 2 A & B) and also by phase contrast light microscopy (fig. 2 C & D). The micrographs of both transmission electron microscopy and phase contrast light microscopy showed some clear differences between the two investigated species. Budding was observed as in the case of *S. cerevisiae* (fig. 2 A & C), whereas no budding was observed with *L. starkeyi* (fig. 2 B & D). *L. starkeyi* cells under phase contrast light microscopy and transmission electron microscopy showed vesicles or vacuoles, which were presumed to be oil globules (fig. 2 B & D). These vesicles or vacuoles were not observed with *S. cerevisiae* cells (fig. 2 A & C). *L. starkeyi* cells were more round compared with the oval *S. cerevisiae* cells. An average yeast cell diameter ranges from 4 -12 μm. The diameter of *S. cerevisiae* and *L. starkeyi* was found to correspond to those reported in the literature (Kaiser, 2000). We concluded that the two species could be distinguished from one another morphologically and were indeed *L. starkeyi* and *S. cerevisiae*.

A



B





C

D

Figure 2: A and B represent transmission electron micrographs of *S. cerevisiae* and *L. starkeyi* respectively. The magnification used was X 10 000. The bar represents 1.6 μm. C and D represent Diaplan Light microscopy (Hoffman Modulation contrast) micrographs of *S. cerevisiae* and *L. starkeyi* respectively. The magnification used was X 100. The bar for C and D represents 13 μm and 6.5 μm respectively. The arrows indicate the budding of *S. cerevisiae* cell (C), some in bipolar and others in axial formation. *L. starkeyi* oil vesicles or vacuoles are also indicated by arrows (D).

3.3 Isolation of heat soluble proteins

In order to investigate the presence of hydrophilic or heat soluble proteins from *L. starkeyi,* total proteins were isolated from cells by ball milling and centrifugation to remove unbroken cells and particulate matter. The preparation was kept at 100 ^{0}C for 10 min to allow insoluble proteins to precipitate after which the preparation was centrifuged. Samples of the total protein extract and the proteins remaining soluble after heat treatment were analysed on SDS-PAGE (fig. 3). Many different proteins were present in the total extract (lane 1) but only a single protein, which migrated at approximately 12 kDa, was observed in the heat-treated preparation (lane 2).

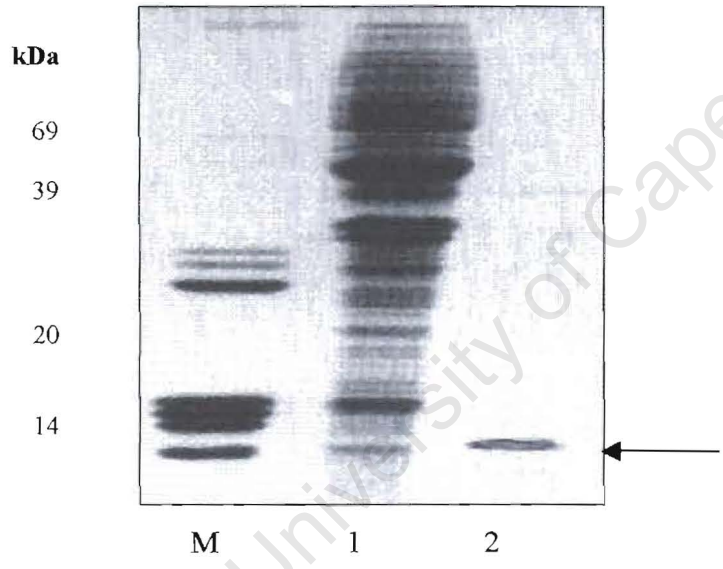


Figure 3: SDS-PAGE gel of *L. starkeyi* heat soluble proteins. M is a chicken erythrocyte histone marker; lane1 represents the total protein before heat treatment and lane 2 represents heat soluble protein. The arrow indicates a heat soluble protein of Mw 12 kDa.

Since Hsp12 from *S. cerevisiae* has similar properties to the 12 kDa protein from *L. starkeyi* in that both proteins are heat soluble and have a similar size, we examined whether the two proteins were indeed similar. The heat soluble proteins from both *L. starkeyi* and *S. cerevisiae* were electrophoresed on the same SDS gel in separate lanes as well as after mixing in the same lane. Should the two proteins be similar, it would be expected that they would co-migrate as a single band in this lane. The results (fig. 4) showed that when the two proteins were mixed and subsequently electrophoresed, they had the same migration and that the mixture of the two proteins (lane 3) migrated as a single band.

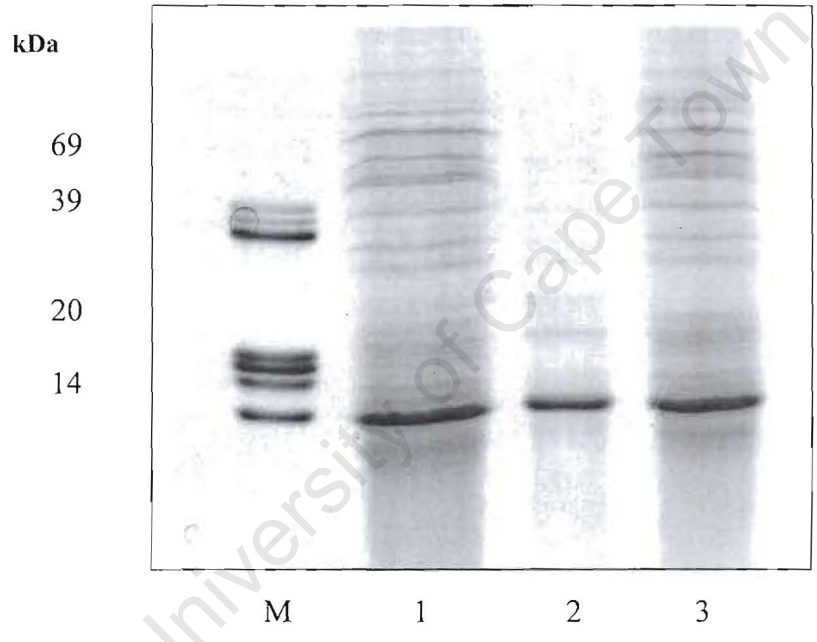


Figure 4: SDS-PAGE gel showing the migration of the heat soluble 12 kDa protein from *L. starkeyi* and *S. cerevisiae* Hsp 12. M represents the chicken erythrocyte histone marker. Lane 1 represents *L. starkeyi* 12 kDa protein; lane 2 represents the *S. cerevisiae* S288C Hsp 12 protein. The mixture of two proteins was loaded in lane 3.

3.3.1 Distribution of the heat soluble proteins among various yeasts.

In order to study the distribution of the 12 kDa heat soluble proteins, different yeasts were investigated. Yeast cells were cultured under similar growth conditions and the same protein isolation method was used for all species. After heating the total protein extracts, the heat soluble proteins were analysed by SDS-PAGE (fig. 5).

The results showed that a 12 kDa protein was present in all yeast species investigated with the exception of *S. pombe* in lane 7. This yeast had a faster migrating protein of approximately 9 kDa, which might be Hsp 9. Hsp 9 from *S. pombe* has been shown to have 42 % homology to Hsp 12 from *S. cerevisiae* and to be induced by similar factors to Hsp 12. There were also some less distinct bands present in many samples but our focus was on the 12 kDa proteins. This result suggests that most yeast have evolved a similar protein in response to adverse conditions as suggested previously (Ellis, 1999).

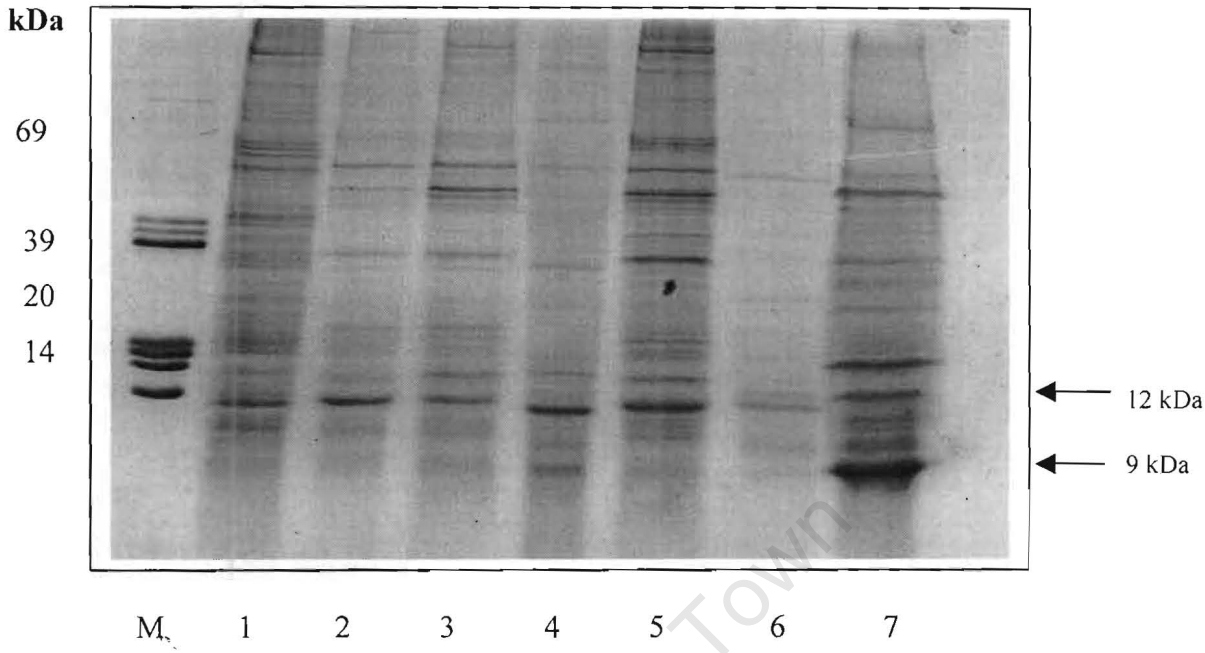


Figure 5: SDS-PAGE of heat soluble proteins from various yeast species. Lane 1 *Z. rouxii*; lane 2 *S. cerevisiae* S288C; lane 3 *L. starkeyi*; lane 4 *D. hansenii*; lane 5 *S. cerevisiae* IFO 23X7 (KAOKAI); lane 6 *P. sorbitophila*; lane 7 *S. pombe*. Lane M is the chicken erythrocyte histone marker.

3.3.2 Western blotting for different yeast species

To investigate whether the 12 kDa heat soluble proteins from different yeast species were similar, SDS-PAGE from (fig. 5) was used to perform a Western blot using an antibody raised against *S. cerevisiae* Hsp 12 (fig. 6). The Hsp12 antibody recognized the 12 kDa protein from the *S. cerevisiae* S288C and *S. cerevisiae* IFO 23X7 (KAOKAI) strains (lanes 2 & 5) as well as the 12 kDa protein from *L. starkeyi* UOFS Y-2024 (lane 3).

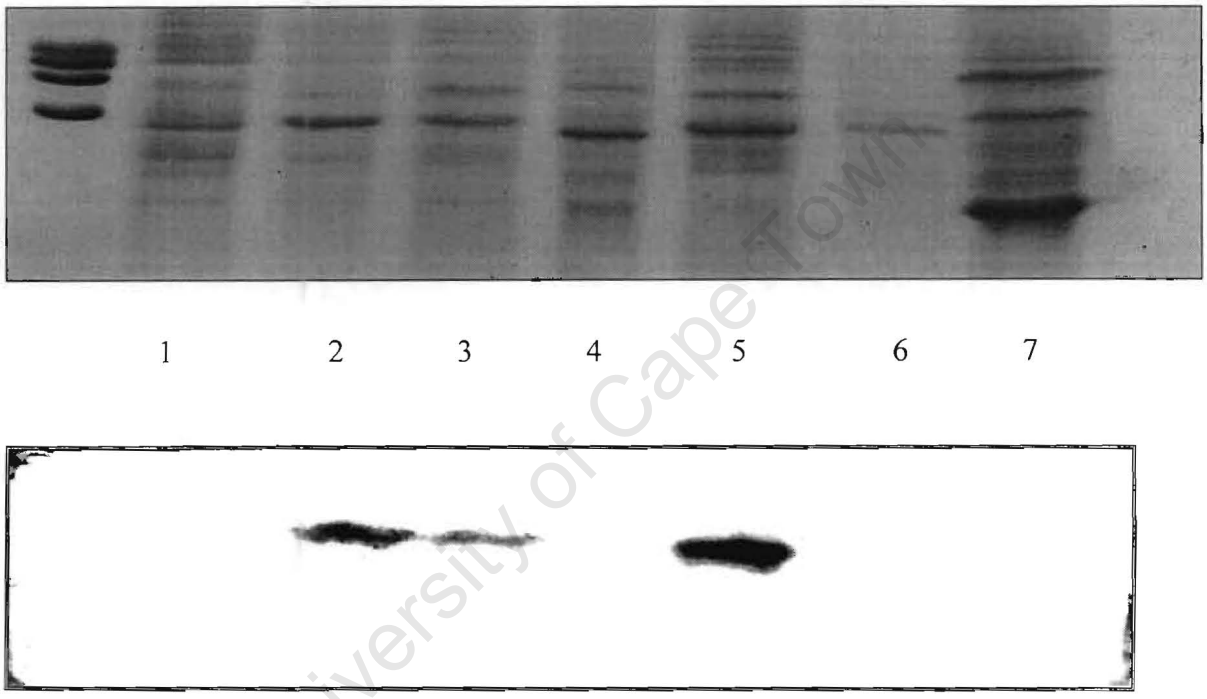


Figure 6: Western blot (B) performed with the SDS-PAGE gel (A) using *S. cerevisiae* anti-Hsp 12 antibody. Lane 1 *Z. rouxii*; lane 2 *S. cerevisiae* (S288C); lane 3 *L. starkeyi*; lane 4 *D. hansenii*; lane 5 *S. cerevisiae* IFO 23X7 (KAOKAI); lane 6 *P. sorbitophila*; lane 7 *S. pombe*. The two *S. cerevisiae* species (lanes 2 & 5) were recognised by the antibody as was *L. starkeyi* (lane 3).

The above result suggested that the two 12 kDa proteins were similar. We therefore determined the ancestral lineage for these species. This showed that *Lipomyces* share the same ancestor as *S. cerevisiae*. *Pichia sorbitophila* also shares the same ancestor as these two species but the anti-Hsp 12 antibody did not recognise its 12 kDa protein. *S. pombe* Hsp 9, which has been shown to have 42 % homology to the Hsp 12 *S. cerevisiae* gene was also not recognised by this antibody. The ancestral lineage of this yeast, *S. pombe* was found to be different. The abbreviated lineages of the four yeasts below are taken from the NCBI (National Centre for Biotechnology Information) website (www.ncbi.nlm.nih.gov).

Saccharomyces cerevisiae: *Eukaryota; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetaceae; Saccharomyces*

Lipomyces starkeyi: *Eukaryota; Fungi; Ascomycota Saccharomycotina; Saccharomycetes; Saccharomycetales; Lipomycetaceae; Lipomyces*

Schizosaccharomyces pombe: *Eukaryota; Fungi; Ascomycota; Schizosaccharomycetes; Schizosaccharomycetales; Schizosaccharomycetaceae; Schizosaccharomyces*

Pichia sorbitophila: *Eukaryota; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetaceae; Saccharomyces*

3.4 MALDI-TOF mass peptide analysis.

Mass spectrometry is a more specific than Western blotting method for the comparison of proteins. The former method determines peptide masses after enzymatic digestion and the latter method identifies proteins due their immunological identity based on the related epitopes. The mass fingerprints of the three 12 kDa heat soluble proteins from *L. starkeyi, S. cerevisiae* and *D. hansenii* and the 9 kDa Hsp from *S. pombe* were compared. Each protein was excised from the stained SDS gel, digested with trypsin, mixed with an equal volume of α-cyano-4-hydroxycinnamic acid matrix and subjected to MALDI-TOF mass spectrometry for peptide mass analysis.

The spectra (fig. 7) indicated that the 12 kDa proteins from *L. starkeyi* and *S. cerevisiae* were similar, with the spectra of the 9 kDa protein from *S. pombe* and the 12 kDa protein from *D. hansenii* different. This confirmed the result obtained from Western blotting, namely that the anti-Hsp 12 antibody did not recognize any proteins from these yeasts. *S. pombe* Hsp 9 has been shown to have 42 % homology to *S. cerevisiae* Hsp 12. *S. pombe* Hsp 9 was therefore not expected to possess identical mass peptides with *S. cerevisiae* Hsp 12. These two yeasts do not share a common ancestry unlike *L. starkeyi* and *S. cerevisiae.* A comparison of the peptide masses revealed significant homology between *S. cerevisiae* Hsp 12 and the 12 kDa protein from *L. starkeyi.* The list of some common significant peptides have been summarised in Table 1. Analysis of the *L. starkeyi* peptide masses by MS-FIT (Ludwig) suggested the presence of a Hsp 12-like protein. At least three peptides from the 12 kDa protein of *L. starkeyi* had different masses compared with peptides from *S. cerevisiae* Hsp 12. These differing peptide masses might be due to incomplete tryptic digestion or amino acid substitution. A single amino acid substitution would usually result in the mass shift of a relevant peptide since most amino acids possess distinctive molecular masses. The presence of peptides with identical masses, therefore suggests the presence of an identical amino acid sequence. The two 12 kDa proteins from *S. cerevisiae* and *L. starkeyi* had several peptide masses which were identical. This 12 kDa protein from *L. starkeyi* is therefore closely related to the Hsp 12 protein from *S. cerevisiae.*

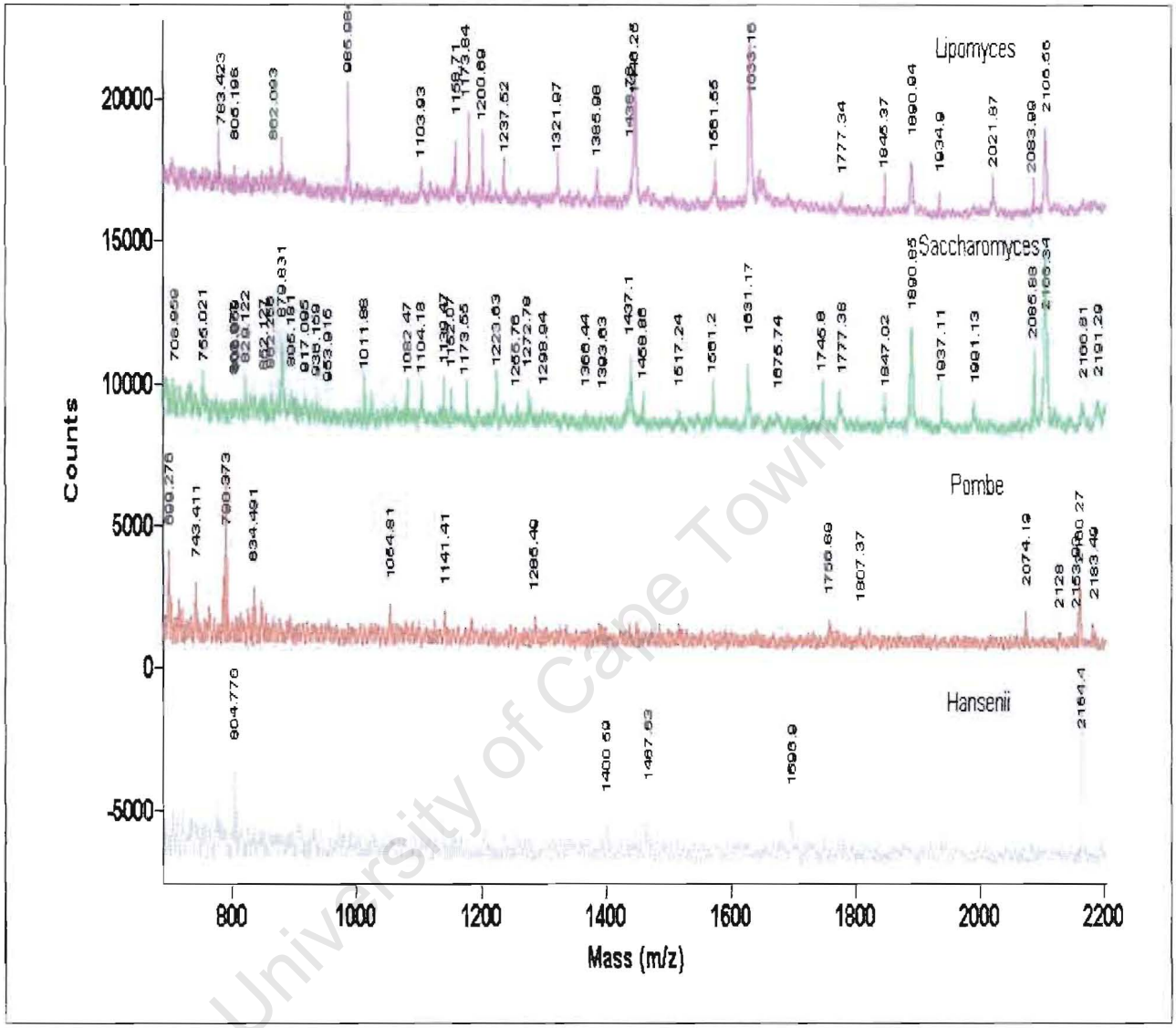


Figure 7: MALDI-spectra analysis of three 12 kDa and a 9 kDa heat soluble proteins from different yeasts digested with trypsin. From top to bottom: *L. starkeyi*, *S. cerevisiae*, *S. pombe* and *D. hansenii*. A mass range of 700 to 2200 daltons is shown.

TABLE 1: Comparison of the masses of tryptic peptides obtained by MALDI-TOF analysis of *S. cerevisiae* and *L. starkeyi* 12 kDa proteins. Ticks indicate the presence of common peptide masses in the range 1000 to 2200 daltons.

Masses	*S. cerevisiae*	*L. starkeyi*
1104 ±2	✔	✔
1173 ±2	✔	✔
1437 ±2	✔	✔
1560 ±2	✔	✔
1631 ±2	✔	✔
1777 ±2	✔	✔
1845 ±2	✔	✔
1890 ±2	✔	✔
1936 ±2	✔	✔
2084 ±2	✔	✔
2106 ±2	✔	✔

3.5 HSP12 GENE ISOLATION

3.5.1 Polymerase chain reaction.

Since MALDI-TOF analysis revealed that the *L. starkeyi* and *S. cerevisiae* 12 kDa proteins were similar, we compared the sequences of the two genes. It was hoped that this would allow identification of peptides containing amino acids altered between the two species. This was done to investigate if gene sequence differences would account for the peptide mass changes observed. A PCR from *L. starkeyi* total genomic DNA was performed using *S. cerevisiae* Hsp 12 primers. The forward primer represented the start of translation and the reverse primer represented sequence downstream of the stop codon (fig. 9) of the *S. cerevisiae* Hsp12 gene. PCR of the *L. starkeyi* genomic DNA resulted in a product of 330 base pairs (fig. 8). PCR of *S. cerevisiae* DNA, used as a control, resulted in a PCR product of the same size (fig. 8).

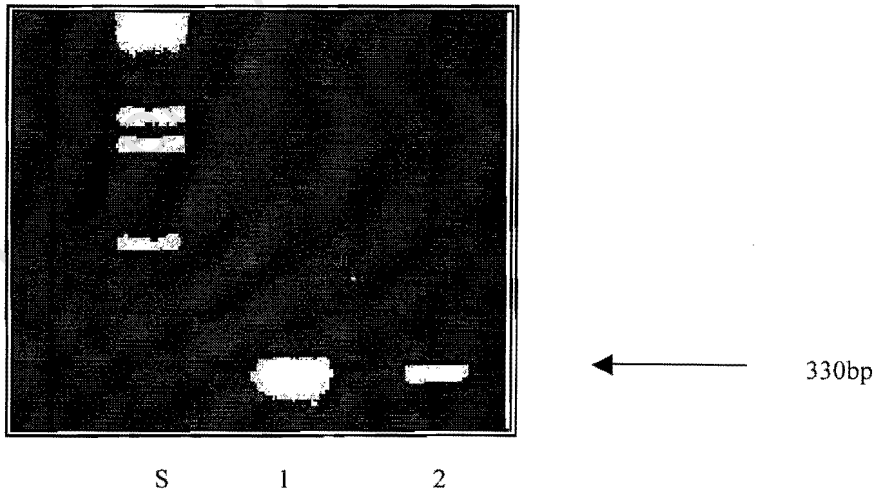


Figure 8: PCR amplification of *S. cerevisiae* (lane 1) and *L. starkeyi* DNA (lane 2). Lane S represents the DNA marker, Lambda DNA digested with PstI.

3.5.2 Nucleotide sequences of *L. starkeyi* and *S. cerevisiae* Hsp 12 gene.

The band representing the PCR product was excised and purified using a Qiagen gel extraction kit. The purified product from *L. starkeyi* was cloned into pGEM T-EASY and the recombinant DNA transformed into *E. coli* JM109 competent cells. Three out of twelve positive clones were sequenced. The sequences below were present in all three clones.

The results (fig. 9 a & b) showed a similarity in sequence existed between Hsp 12 from *S. cerevisiae* and the 12 kDa protein from *L. starkeyi*. As shown (fig. 9 a), the *L. starkeyi* gene had three nucleotide differences compared with the *S. cerevisiae* Hsp 12 gene. The *L. starkeyi* nucleotide sequence included the *S. cerevisiae* Hsp 12 primer region which removed the possibility of changes occurring in the first seven amino acids.

Hsp 12 gene sequences of *S. cerevisiae* and *L. starkeyi.*

S. cerevisiae on top and *L. starkeyi* below.

```
1       ATGTCTGACGCAGGTAGAAAAGGATTCGGTGAAAAAGCTTCTGAAGCTTTGAAGCCAGAC
1       ATGTCTGACGCAGGTAGAAAAGGATTCGGTGAAAAAGCTTCTGAAGCTTTGAAGCCAGAC
           forward primer
61      TCTCAAAAGTCATACGCTGAACAAGGTAAGGAATACATCACTGACAAGGCCGACAAGGTC
61      TCTCAAAAGTCATACGCTGAACAAGGTAAGGAATACATCACTGACAAGGCCGACAAGGTC

121     GCTGGTAAGGTTCAACCAGAAGACAACAAGGGTGTCTTCCAAGGTGTCCACGACTCTGCC
121     GCTGGTAAGGTTCAACCAGAAGACAACAAGGGTGTCTTCCAAGGTGTCCACGACTCTGCC

181     GAAAAAGGCAAGGATAACGCTGAAGGTCAAGGTGAATCTTTGGCAGACCAAGCTAGAGAT
181     GGAAAAGGCAAGGATAACGCTGAAGGTCAAGGTGAATCTTTGGCAGACCAAGCTAGAGAT

241     TACATGGGAGCCGCCAAGTCCAAGTTGAACGATGCCGTCGAATATGTTTCCGGTCGTGTC
241     TACATGGGAGTCGCCAAGTCCAAGTTGAACGATGCCGACGAATATGTTTCCGGTCGTGTC

301     CACGGTGAAGAAGACCCAACCAAGAAGTAATTACTTCTTGGTTGGGTCTTC
301     CACGGTGAAGAAGACCCAACCAAGAAGTAATTACTTCTTGGTTGGGTCTTC
                                    reverse primer
```

Figure 9 a: The *S. cerevisiae* and *L. starkeyi* Hsp12 gene sequences. Nucleotide changes in bold are also indicated by underlining. The forward and reverse primers are indicated by underlining.

S. cerevisiae on top and *L. starkeyi* below

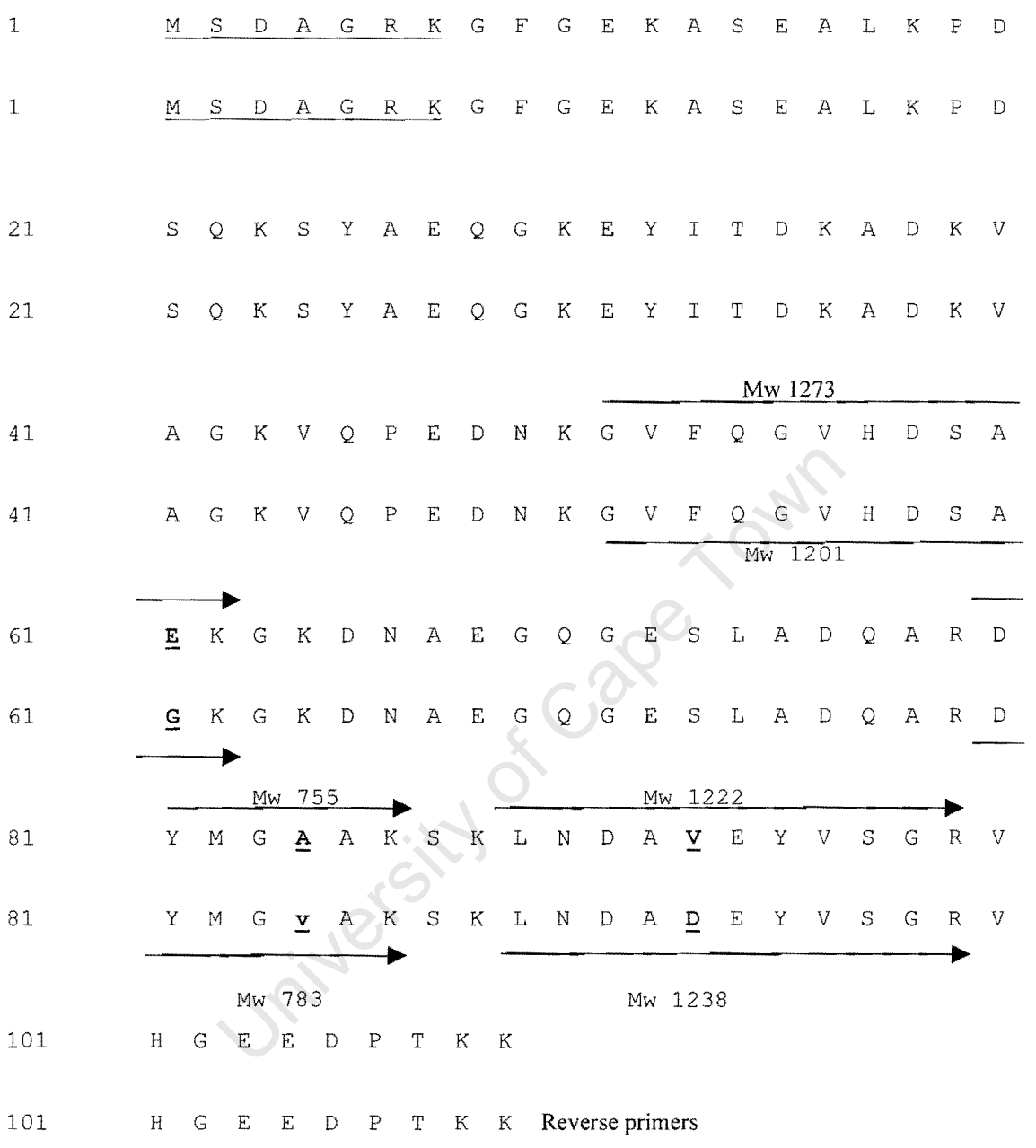


Figure 9 b: The sequence data for *S. cerevisiae* Hsp 12 and the *L. starkeyi* Hsp12-like protein. The forward primer is indicated by underlined amino acids. Nucleotide changes are indicated by the three underlined bold amino acids. The arrows show three tryptic peptides with the calculated masses shown above and below the sequences.

The nucleotide codon and corresponding amino acid changes from *S. cerevisiae* to *L. starkeyi* respectively were:

1. GAA to GGA (E to G) codon 181, mass change 129 to 57.
    - ❖ Peptide mass change expected = 72 daltons
2. GCC to GTC (A to V) codon 244, mass change 99 to 115
    - ❖ Peptide mass change expected = 16 daltons
3. GTC to GAC (V to D) codon 263, mass change 71 to 99 respectively.
    - ❖ Peptide mass change expected = 28 daltons

The MALDI-TOF spectra were therefore analysed for peptides that corresponded to these mass changes. Peptides with masses of 1273, 755 and 1222 daltons in *S. cerevisiae* were found to have corresponding peptides with masses of 1201, 783 and 1238 respectively in *L. starkeyi* (fig. 9 b). These peptides accounted for the changes deduced from the sequence data.

Since the first seven amino acids of the *L. starkeyi* Hsp 12-like protein were forced to be identical with that of *S. cerevisiae* due to designing the forward primer using the *S. cerevisiae* Hsp 12 gene sequence, we attempted to identify the peptides corresponding to this region in the *L. starkeyi* spectrum. The masses for tryptic peptides corresponding to residues 1-5 (503 Da) and 1-6 (633 Da) were too small to be observed in the portion of the spectrum analysed. However the mass corresponding to incomplete tryptic cleavage, residue 1-11 (1151 Da) excluding Met, was within the mass range analysed. This peptide was not present in *L. starkeyi* but instead a peptide with a mass of 1158 Da was present (fig. 10). Amino acid substitutions that could account for a mass change of approximately 7 Da were Met to His (6.0 Da), Arg to Tyr (7 Da) and Glu to His (8.0 Da). The most likely change was R to Y and not E to H change. M to H change, M has been removed which even if present would remain the same in both sequences as it is the start codon. We therefore propose that residue R 6 in *S. cerevisiae* Hsp 12 is Y 6 in the corresponding Hsp 12-like protein from *L. starkeyi*. These data would therefore suggest that the *L. starkeyi* 12 kDa protein is closely related to *S. cerevisiae* Hsp 12. We therefore propose that the *L. starkeyi* 12 kDa protein be referred to as *L. starkeyi* Hsp 12.

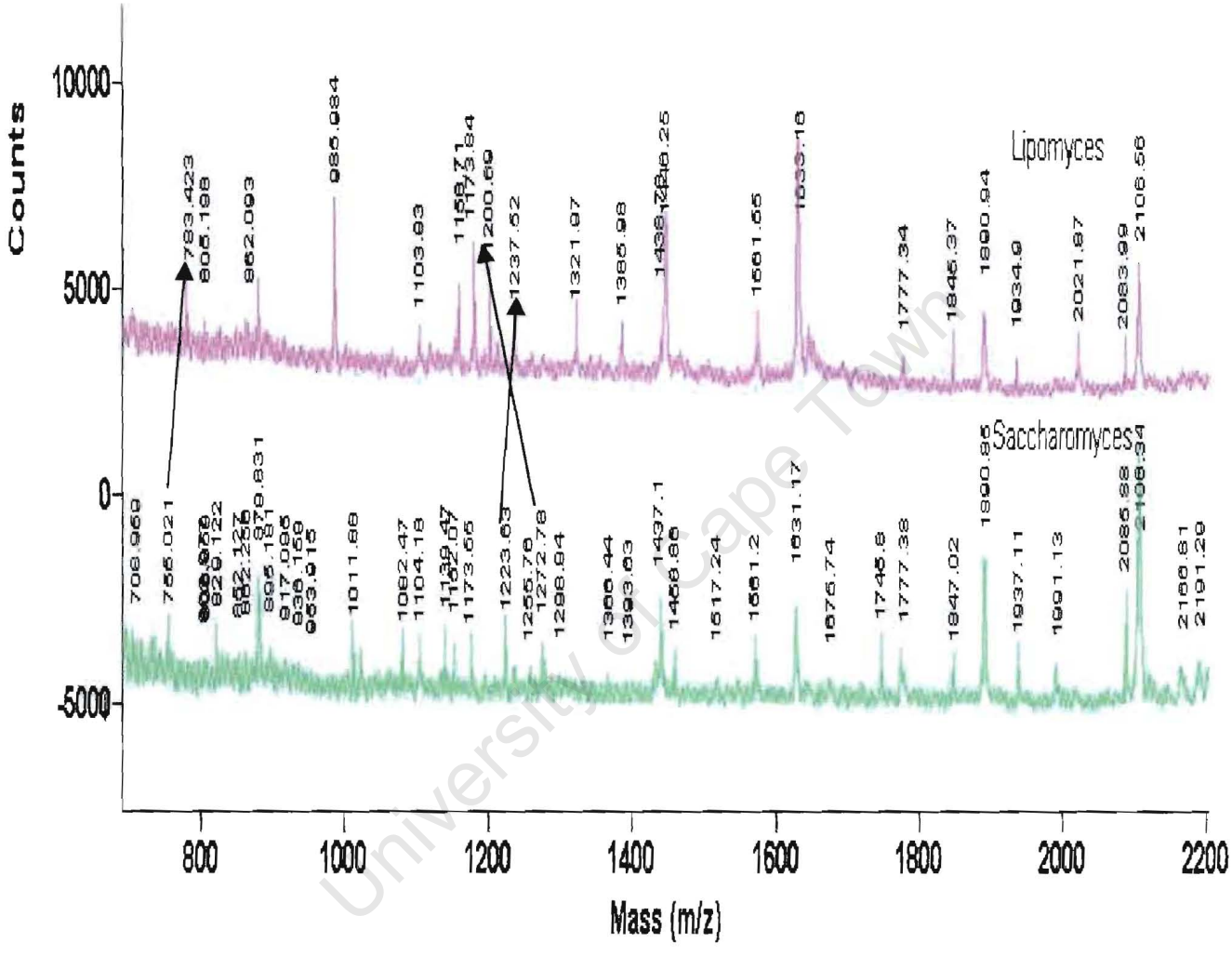


Figure 10: MALDI-spectra analysis of 12 kDa heat soluble proteins from two different yeasts digested with trypsin. From top to bottom: *L. starkeyi* and *S. cerevisiae*. The mass range of 700 to 2200 daltons is shown.

3.6 Induction of the heat soluble protein by different stimuli and its expression.

Since Hsp 12 in *S. cerevisiae* has been classified as a stress response protein, induced by a variety of stresses including heat shock and osmotic stress (Aranda et al., 2002; Piper, 1995; Lindquist, 1986; Praekelt and Meacock, 1990; Mtwisha et al., 1998; Hasegawa et al., 2000), we therefore investigated whether the *L. starkeyi* Hsp 12 was induced by similar conditions.

3.6.1 The growth effect under nitrogen rich and limiting growth medium

We investigated the growth of *L. starkeyi* under nitrogen rich and under nitrogen limiting growth medium. Nitrogen is an integral part of nucleic acid and protein biosynthesis.

Cells were grown in nitrogen rich medium (normal YEPD) and in nitrogen limiting growth medium by excluding the peptone from this medium. Growth was monitored as a function of time. The results (fig. 11) showed that under nitrogen limiting growth medium, *L. starkeyi* cells grow faster compared to cells grown under nitrogen rich medium. Fission yeasts are known to grow well without peptone though they can still grow in YEPD medium which is known to be favourable to *S. cerevisiae* a budding yeast (Moreno et al., 1991).

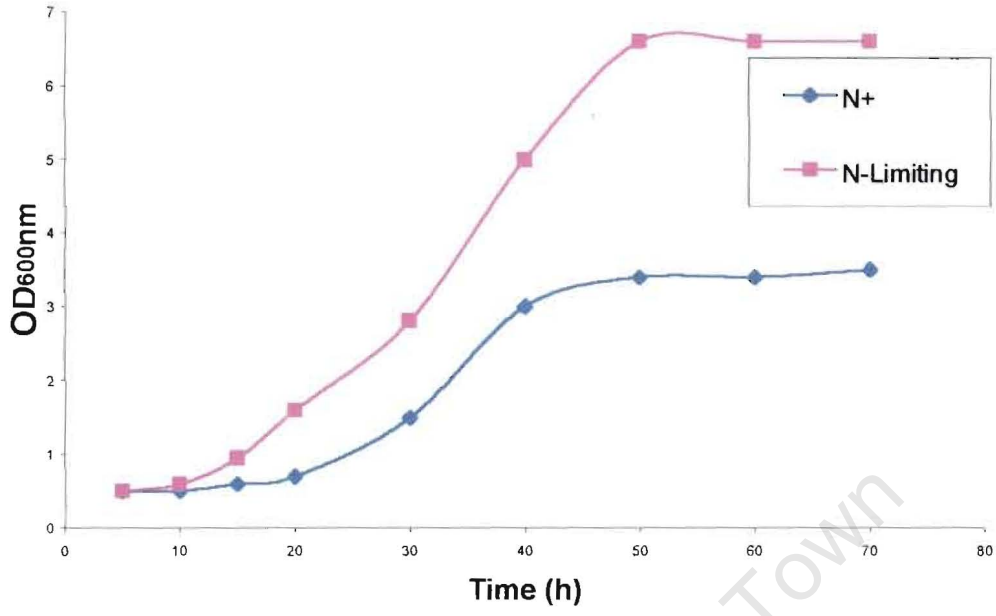


Figure 11: The rate of growth of *L. starkeyi* under Nitrogen rich (N+) and Nitrogen limiting (N-Limiting) growth medium as a function of time.

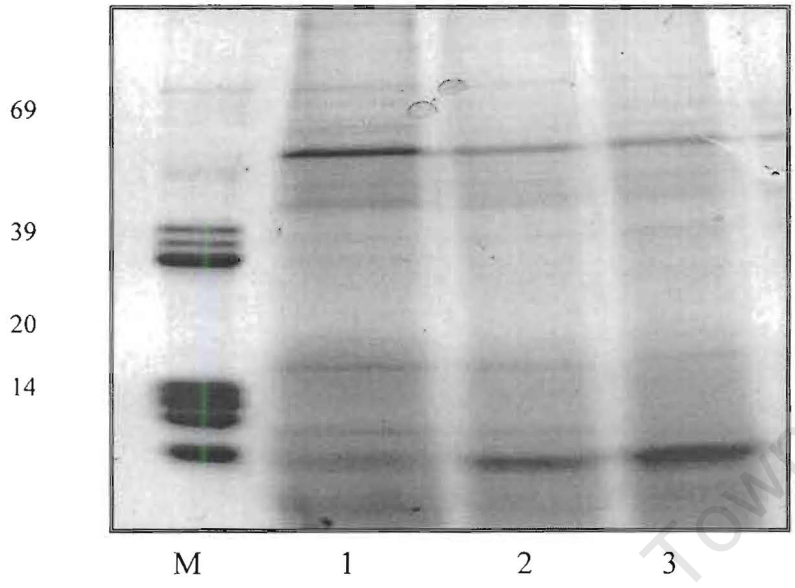
3.6.2 Induction by growth phases.

Since the growth characteristics of *L. starkeyi* under nitrogen rich and limiting growth medium were studied, we investigated the relationship between the concentration of *L. starkeyi* Hsp 12 and the growth phases. Using the established growth characteristics (fig. 1), cells were grown and protein extracted from lag phase, exponential phase and stationary phase cells. An equal number of cells were used at each point based on the OD 600nm.

SDS-PAGE analysis (fig. 12) demonstrated that there was an increased amount of Hsp 12 as growth proceeded. Lag phase cells showed low amounts of Hsp 12 but this increased during log phase with a slight further increase in stationary phase. There was no significant difference observed in the amounts of Hsp 12 extracted after growth in nitrogen limiting and nitrogen rich media. Nitrogen is not a stress that induces Hsp 12 biosynthesis.

A

kDa



B **kDa**

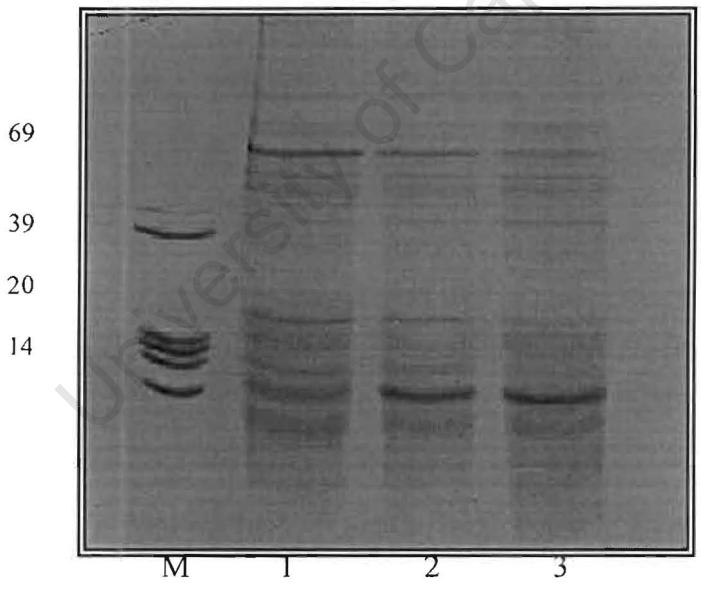


Figure 12: SDS-PAGE gel showing the heat soluble protein extracted from different growth phases of *L. starkeyi.* A represent proteins extracted in cells growing under nitrogen limiting medium and B represent proteins extracted in nitrogen rich medium. M represents the chicken erythrocyte histones. Lane 1 represents lag phase, lane 2 represents log phase and lane 3 represents stationary phase in both A and B.

In order to quantify the concentration of Hsp12 at different growth stages (fig. 12 b), the lanes were scanned and quantified using the TN-Image software. This showed that the relative amounts of Hsp 12 were in the ratio of: 1:3.8: 4.0 at lag phase, log phase and stationary phase respectively (fig. 13). These increased Hsp 12 concentration might be brought about by glucose depletion since it has been shown that the glucose effects transcription of the Hsp 12 gene in *S. cerevisiae* (Bisson et al., 1999; Alexandre and Charpentier, 1998).

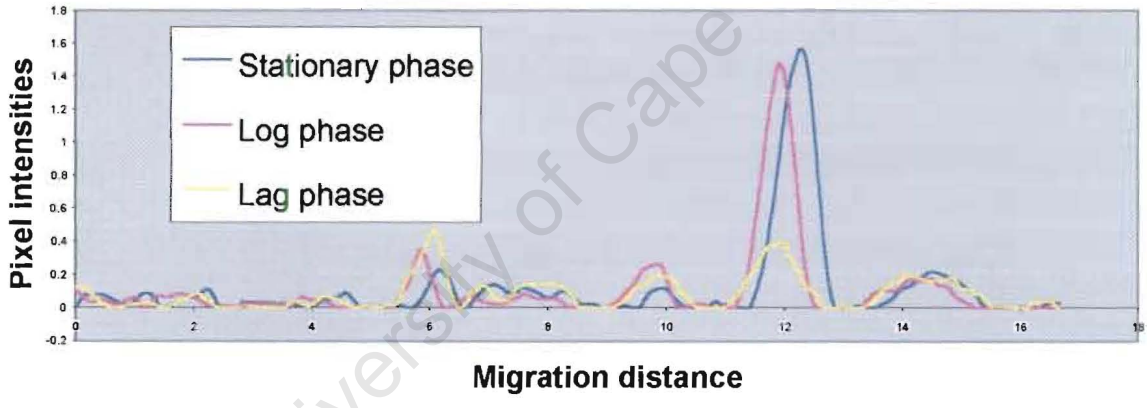


Figure 13: Quantification of Hsp 12 at various growth stages of *L. starkeyi* from the SDS-PAGE gel shown in fig.11. The concentration of Hsp 12 was estimated from the computer image using TN-Image software (1995).

3.6.3 Salt stress analysis

3.6.3.1 Effect of salt concentration on Hsp 12 induction.

Salinity is a major environmental stress which has drawn the attention of biologists because the protective mechanisms underlying salt-tolerance may have commercial applications. Salt stress in yeast has been reported to be accompanied by an increase in the intracellular level of glycerol, free amino acids, and sodium, as well as by changes in the lipid and fatty acid composition (Galinski and Truper, 1994).

Cells were grown in normal YEPD medium to stationary phase with the addition of NaCl between 0 M to 0.8 M. Heat soluble proteins were extracted and analysed by SDS-PAGE (fig. 14). The highest Hsp 12 concentration occurred with growth in the presence of 0.3 M NaCl. The decreased level of heat soluble protein at NaCl concentrations above 0.5 M (fig. 14) was probably due to inhibition of cell growth since proteins were apparent on the gel shown (fig. 14).

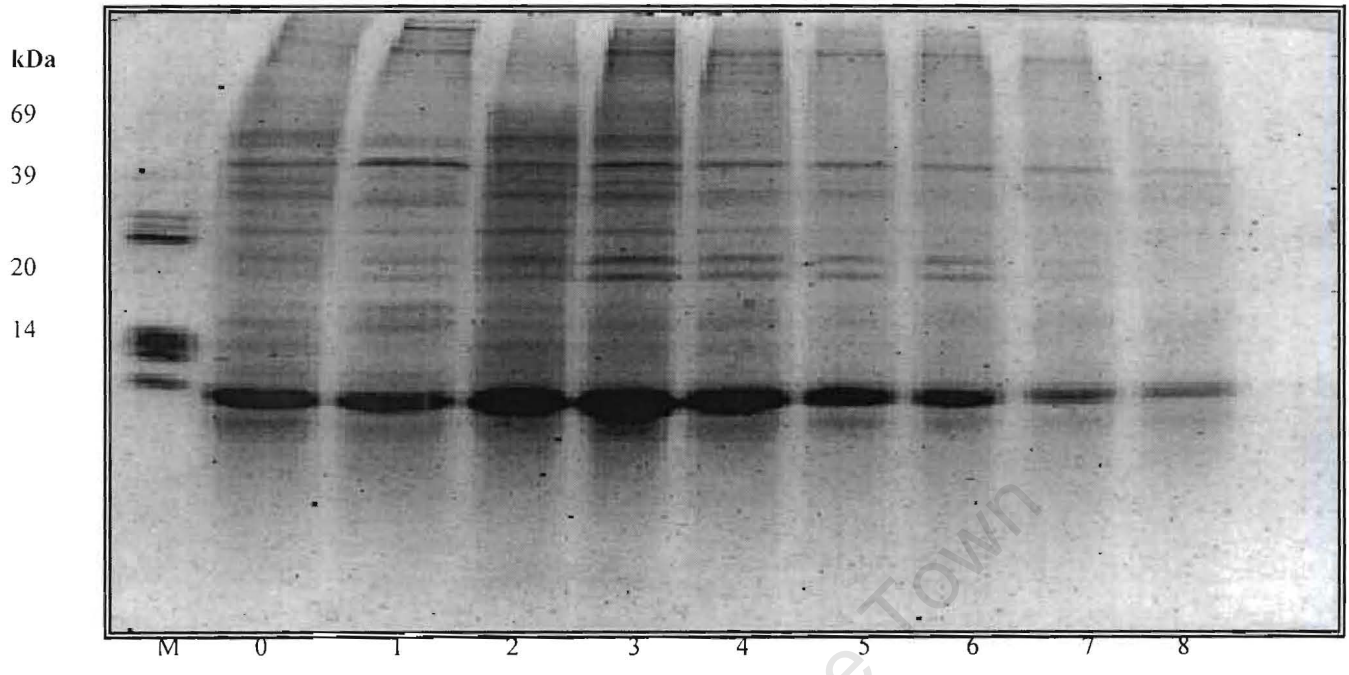


Figure 14: SDS-PAGE of heat soluble protein extracted from *L. starkeyi* grown using different NaCl concentrations. M represents the chicken erythrocyte histone marker and lanes 0 to 8 represent proteins extracted from *L. starkeyi* grown in YEPD containing 0 to 0.8 M NaCl in 0.1 M increments.

3.6.3.2 The effect of salt on *L. starkeyi* growth

Since considerably more Hsp 12 was present when *L. starkeyi* cells were grown in the presence of 0.3 M NaCl, the effect of 0.3 M NaCl on the growth rate was investigated. It was found that 0.3 M NaCl slightly inhibited *L. starkeyi* growth (fig. 15). Since water moves from high to low water potential, this would also apply in cells grown in presence of salt rendering them small. This might affect light scattering when compared to cells grown in the presence and absence of salt due to changes in cell size.

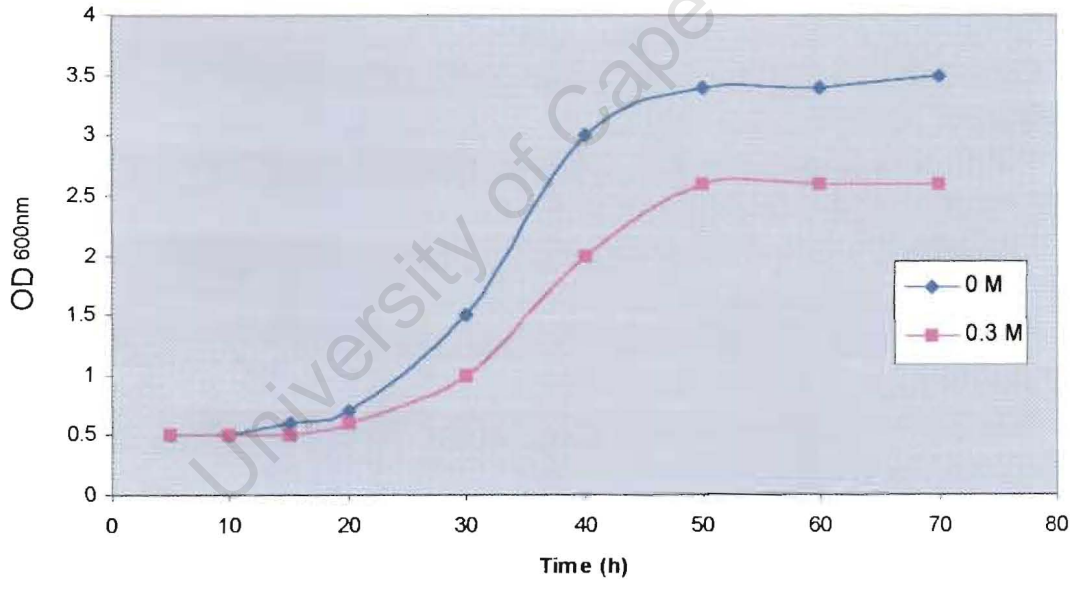
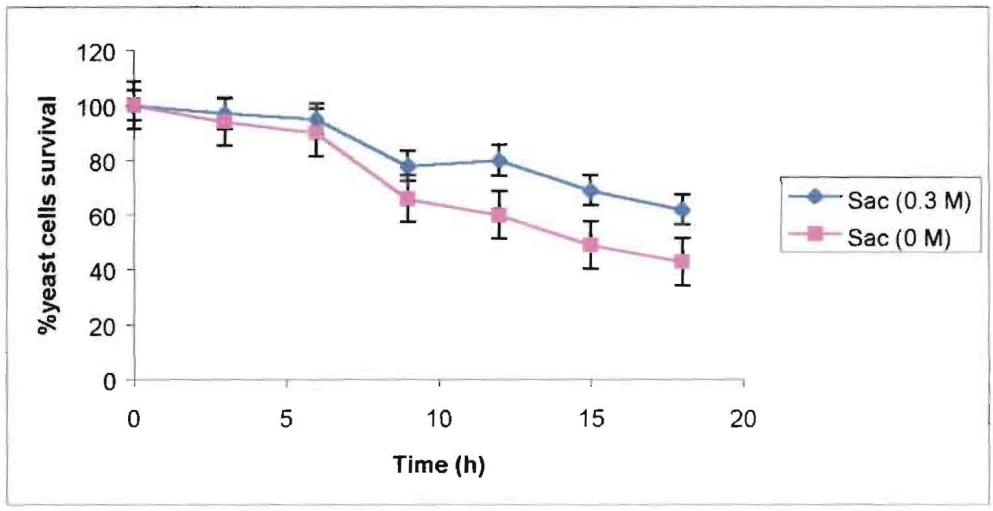


Figure 15: The rate of growth in *L. starkeyi* in the presence of 0 M and 0.3 M NaCl monitored as a function of time.

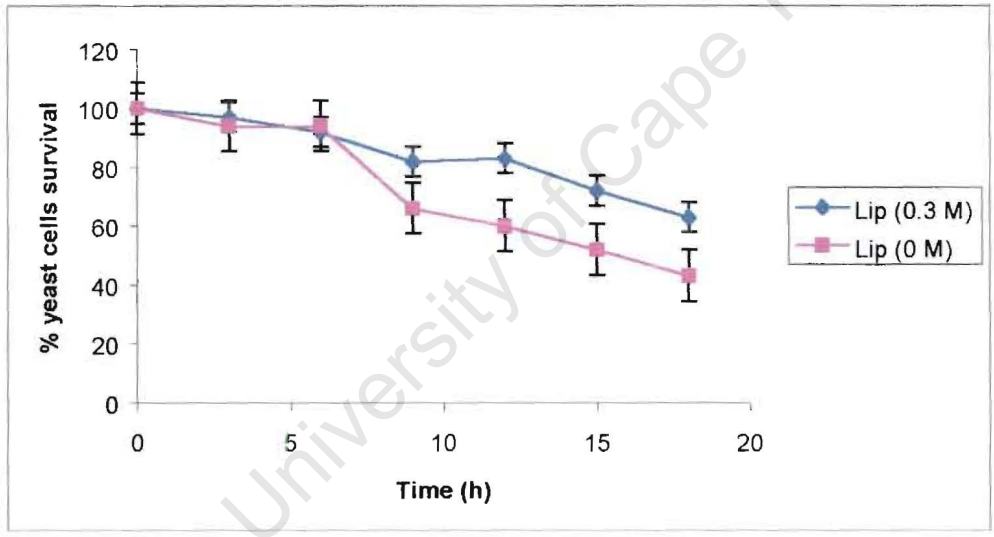
3.7 Desiccation tolerance of yeasts *L. starkeyi* and *S. cerevisiae*

It has been reported that Hsp 12 protected liposomes and *S. cerevisae* against desiccation induced stress (Sales et al., 2000). We therefore investigated whether the higher concentration of Hsp 12 brought about by growth in the presence of 0.3 M NaCl would protect the cells against desiccation stress. Cells were grown in YEPD medium with and without 0.3 M NaCl. This salt concentration was used as Hsp 12 was optimally induced under these conditions. 50 µl of *L. starkeyi* and *S. cerevisiae* cells from cultures grown to late log phase were dried in a desiccator for various times. The dried samples were resuspended in water, serial dilutions made and an appropriate dilution plated on agar YEPD plates. The survival rates were determined by counting the number of colonies after 48 h. The results shown are from two separate experiments with each measurement performed in triplicates.

The results (fig. 16) showed that the survival rates of *L. starkeyi* and *S. cerevisiae* cells were similar. Survival rates of both yeasts improved in the cells that had been prestressed by growth in the presence of 0.3 M NaCl. Approximately 50 % of non-salt stressed cells were still viable after 18 h dehydration whereas 60 - 65 % viability was observed with salt stressed cells. Results showed that there was a correlation between the period of desiccation and the viability, namely that the survival rate decreased as a function of time spent in the dehydrated state. Since it has been reported that Hsp 12 protects liposomes and *S. cerevisiae* against desiccation, these data demonstrate a correlation between Hsp 12 induction and enhanced survival. Other factors induced by NaCl, such as sugars and other proteins might also be involved.



A



B

Figure16: Yeast survival as a function of time of dehydration and rehydration. A: *S. cerevisiae* and B: *L. starkeyi.* 100 % survival represents undried cells. Error bars represent differences in plate counts of two separate experiments performed for each species.

4. CONCLUSIONS

This thesis reports the presence of a Hsp 12 protein from *Lipomyces starkeyi*. Western analysis, MALDI-TOF mass peptide analysis, gene cloning and sequencing revealed that the Hsp 12 from *L. starkeyi* has close homology to *S. cerevisiae* Hsp 12. This was interesting because *S. pombe* Hsp 9 which is known to have 42 % homology to Hsp 12 was not recognized by the anti-Hsp 12 antibody. *S. pombe* Hsp 9 has been reported to be induced by similar stresses to *S. cerevisiae* Hsp 12. Though other species (*Z. rouxii, D. hansenii, P. sorbitophila)* had heat soluble protein of about 12 kDa, these proteins were not recognized by the *S. cerevisiae* anti-Hsp 12 antibody.

The sequence of the *L. starkeyi* Hsp 12 gene was found to be similar to that of *S. cerevisiae* Hsp 12. Three nucleotide differences were found to exist compared with the *S. cerevisiae* Hsp 12 gene. These nucleotide changes resulted in the following amino acids codon changes, namely GAA to GGA, GCC to GTC and GTC to GAC. These amino acids substitutions altered the masses of the relevant tryptic peptides, which were detected by MALDI TOF mass spectrometry.

Analysis of the Hsp 12 concentration as a function of growth showed that the protein was present during lag phase and increased during log phase and increased slightly further in stationary phase. This was analogous to the known situation observed with *S. cerevisiae*. There was a correlation between Hsp 12 induction and the presence of NaCl in the growth medium with a maximum induction of Hsp 12 occurring in the presence of 0.3 M NaCl. Cells grown in the presence of 0.3 M NaCl showed an enhanced cell survival upon dehydration and subsequent rehydration.

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