CHAPTER I

INTRODUCTION

1. BACKGROUND

Yeasts are one of the most important groups of microorganisms exploited by humans and serve a steady and beneficial purpose. Yeasts are also relatively safe for human consumption and easily modified. The ability of yeast to produce alcohol in the form of beer was known by the Sumerians and Babylonians before 6000 B.C. Later, in 4000 B.C., the Egyptians discovered that the carbon dioxide generated by the action of brewer's yeast could leaven bread (Demain and Solomon, 1981).

In the modern era, yeasts of which *Saccharomyces* is by far the most widely used genus, are used in a variety of key industrial processes: in baking for leavening and as a flavouring agent; in alcoholic beverage production such as beer, wine and sake; in the production of industrial alcohol; in biomass formation and also in the production of vitamins and enzymes (Rose, 1980; Reed and Nagodawithana, 1991).

The activities of yeast are affected by the environment in which they live. Environmental changes, both physical and chemical, influence the growth of yeast due to effects on metabolic processes. Temperature is one of the most important environmental parameters influencing the yeast life cycle. On the one hand, temperature can cause rapid growth by accelerating the activities of some enzymes and, on the other hand, can cause damage to cells by denaturation of proteins and other essential cellular constituents.

Molecular oxygen is another very important factor governing the growth of yeast. However, oxygen can be toxic when the concentration is greater than the normal requirement. The damaging effects of oxygen can be attributed to the formation of oxygen-derived free radicals (Halliwell and Gutteridge, 1989). All classes of biological molecules are potential targets for free radical attack

and these include lipids, proteins and nucleic acids (Sies, 1986; Halliwell and Gutteridge, 1989).

Much recent attention has been focussed on the mechanisms whereby biological systems deal with toxic forms of oxygen-derived free radicals. The role of antioxidants, both enzymatic and non-enzymatic, in reacting with and removing free radicals is a area of considerable interest and intense research, not least of all from the viewpoint that free radicals have been implicated as causative agents of major human diseases including cancer, atherosclerosis and various inflammatory disorders (Halliwell and Gutteridge, 1989). Although the characterisation of antioxidant enzymes, especially superoxide dismutase (SOD), have been long studied in yeast, dating from the original observations of Gregory *et al.* (1974), only a handful of studies have focussed on the effects of non-enzymatic antioxidants on yeast metabolism. This is extremely surprising considering the extensive use of non-enzymatic antioxidants in the food industry and the importance of yeast in the human food and beverage industries.

The aims of this study were to determine the effects of environmental stresses, both physical and chemical, on the yeast *Saccharomyces cerevisiae* and the role of non-enzymatic antioxidants in stress protection. The environmental stresses used in these experiments were primarily temperature and oxygen. The naturally-occurring antioxidants used were ascorbate (vitamin C), glutathione, α–tocopherol (vitamin E), ergosterol and β–carotene (vitamin A) and the synthetic antioxidants: butylated hydroxytoluene and propyl gallate. Three other factors, namely, trehalose content, heat shock proteins and lipid composition were also examined in order to investigate their possible interactive effects on stress tolerance of the yeast cell.

2. LITERATURE REVIEW

2.1. Response of yeast to temperature

Yeasts are capable of growth over a relatively wide temperature range, from below 0°C to just below 50°C (Stokes, 1971). The vast majority of yeasts grow within the temperature range at or above 0°C to 48°C and are hence termed mesophilic. Yeasts which are unable to grow at or above 20°C have been classified as psychrophilic and, conversely, yeasts unable to grow at or below 20°C have been termed thermophilic (Watson, 1987). There are only a limited number of yeasts which fall into either of these two categories and the vast majority of yeasts are thus mesophilic.

The interested reader is referred to reviews on the effects of temperature on yeast growth and metabolism by Stokes (1971), Van Uden (1984) and Watson (1987). In recent years, the heat shock response in which exposure of cells to a mild non-lethal temperature induces resistance to a higher, generally, lethal temperature, has attracted considerable attention (Watson, 1990; Piper; 1993; Parsell and Lindquist, 1993). In this respect, the role of heat shock proteins, trehalose and lipid composition in yeast thermotolerance appear to be of special importance. It should be pointed, however, that at this point in time, the precise mechanism of thermotolerance (and other stresses) is not known.

Heat shock protein

Heat shock induction of specific proteins in yeast was first reported in detail by Miller *et al.* (1982) and McAlister *et al.* (1979). Shortly thereafter, McAlister and Finkelstein (1980) observed that exposure of cells for a short period to mild heat shock (37°C) protected against subsequent challenge to a normally lethal temperature. Watson and Cavicchioli (1983) extended this observation to include heat shock induced tolerance to high concentrations of ethanol and, conversely, Plesset *et al.* (1982) established that a mild ethanol shock protected against a heat challenge. These initial observations have since been extended to include cross-tolerance correlations and the detailed

molecular biology of the stress response (Lindquist and Craig, 1988; Watson, 1990; Parsell and Lindquist, 1993).

All organisms so far examined, ranging from Archae to plants and animals, induce the synthesis of specific and, in many cases, highly evolutionary conserved proteins on exposure to a heat shock. Many of these proteins are also induced upon exposure of cells to stresses other than heat and these include ethanol, heavy metals, amino acid analogues and oxidative stress (Nover, 1984; Watson, 1990), The more general term stress proteins has therefore been applied to describe this class of proteins (Morimoto *et al.,* 1990; Watson, 1990).

The heat shock or stress proteins of yeast can be broadly divided into different classes based on molecular mass as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The major groups belong to the heat shock protein (hsp) 100, hsp 90, hsp 70 and hsp 60 classes. In addition, a number of small molecular mass proteins have been described in yeast. These include hsp 26 (Petko and Lindquist, 1986), hsp 30 (Regnacq and Boucherie, 1992; Panaretou and Piper, 1992), hsp 12 (Praekelt and Meacock, 1990), hsp 10 (Hartl *et al.,* 1992) and ubiquitin (Finley *et al.,* 1987).

The latter protein (8.5 kDa) is highly conserved across all eucaryotic organisms. It is a stress-inducible protein in yeast for which there is some evidence that it may function, through its role in proteolysis, as a stress protectant (Finley *et al.,* 1987). More recently, hsp 30 has been localised as an integral yeast plasma membrane protein and it has been suggested to function to stabilise plasma membrane components, in particular the plasma membrane ATPase, during heat stress (Piper, 1993).

In most organisms, the hsp 70 family, of which yeast has at least nine members (Craig *et al.,* 1993), are among the most prominent proteins induced by heat. It is now well established that in the normal cell hsp 70 proteins, in conjunction with their binding to protein substrates, participate in a variety of

protein folding, unfolding and translocating processes (Gething and Sambrook, 1992; Craig *et al.,* 1993). In this respect, hsp 70 proteins function in various organelles and the endoplasmic reticulum as molecular chaperones.

Similarly, yeast hsp 60, also known as chaperonin 60, has been shown to participate in protein translocation and assembly associated with the mitochondria (Hartl *et al.,* 1992). The chaperonin activity of hsp 60 in yeast mitochondria requires interaction with hsp 10 (chaperonin 10), a homo-oligomer consisting of 10 kDa subunits found in mitochondria, chloroplasts and bacteria. Both hsp 60 and hsp 10 are essential proteins. There is evidence that these chaperonins, by virtue of their association with a wide variety of proteins, prevent protein aggregation at high temperatures and promote refolding when cells are returned to normal temperatures (Parsell and Lindquist, 1993). These proteins, therefore, may play an important role in the recovery of cells from stress.

It should be pointed out that many, if not all, hsp are constitutively expressed in the normal cell and play key roles in protein folding, assembly and translocation. Their fundamental role in normal cell physiology is thus well established. However, their precise function in the stressed cell and in recovery from stress is enigmatic and remains to be elucidated.

Earlier studies by Hall (1983) and Watson *et al.* (1984), in which heat shock protein synthesis in yeast was blocked by cycloheximide, indicated that hsp may not be required for heat shock induced thermotolerance. Furthermore, the kinetics of loss of induced thermotolerance do not correspond with levels of hsp (Cavicchioli and Watson, 1986). More recently, research from Lindquist and co-workers have shown that hsp 104 is important for yeast tolerance to different stresses, including heat and ethanol (Sanchez *et al.,* 1992). On the other hand, De Virgilio *et al.* (1991) examined thermotolerance in a deletion mutant of HSP 104 and the corresponding wild-type and found similar levels of induced

thermotolerance. The authors concluded that the acquisition of thermotolerance in these cells were in fact more closely related to levels of trehalose.

Trehalose as a stress protectant

Earlier studies indicated that trehalose, a non-reducing disaccharide of glucose, functioned as a storage compound in yeast (Lillie and Pringle, 1980). However, the concept of trehalose as a stress protectant was stimulated by the observation that heat shock induces the rapid synthesis of trehalose (Attfield, 1987; Hottiger *et al.,* 1987).

It is noteworthy that trehalose also accumulates in yeast during periods of starvation, on entry into stationary phase and an exposure to environmental stresses (Attfield, 1987; Van Laere, 1989; De Virgilio *et al.,* 1990; Wiemken, 1990; Hottiger *et al.,* 1992). Trehalose is also thought to influence membrane structure and in protecting membranes against desiccation (Crowe *et al.,* 1992), freezing (Coutinho *et al.,* 1988) and salt stress (MacKenzie *et al.*, 1988). It is also noteworthy that trehalose mobilisation has been reported to be controlled by the levels of specific hsp during heat shock. In particular, levels of hsp70 appear to influence trehalose mobilisation in the heat shocked cell (Hottiger *et al.,* 1992).

As in the case of hsp, and despite the wealth of data supporting the concept that trehalose is a key stress protectant in yeast, there are numerous studies which indicate that, at least under certain conditions, levels of trehalose do not correlate with levels of stress tolerance (Gelinas *et al.,* 1989; Panek *et al.*, 1990; Winkler *et al.,* 1991; Attfield *et al.*, 1992). For example, Gelinas *et al.* (1989) reported a correlation between trehalose content and freezing tolerance only under certain growth conditions. A detailed study by Attfield *et al.* (1992) on heat and freezing tolerance in a number of closely related strains of *S. cerevisiae* concluded that trehalose content and stress tolerance were not related in a linear manner. A comprehensive study in this laboratory of fourteen baking strains of *S. cerevisiae* subjected to six distinct stresses (heat, ethanol,

rapid freezing, slow freezing, salt and acetic acid) did not show any statistically significant correlation between trehalose concentration and stress tolerance, except acetic acid (Lewis, 1994).

Membrane lipid composition

Heat and certain other stress agents such as ethanol act to increase membrane permeability (Ingram and Buttke, 1984). Ethanol has also been shown to induce changes in lipid composition of yeast and bacterial membranes as an adaptive response (Ingram and Buttke, 1984; Casey and Ingledew, 1986). A recent paper by Mansure *et al.* (1994) demonstrated a possible correlation between viability of yeast cells challenged with high ethanol concentration (10% w/v) and trehalose levels. These authors concluded that yeast cells can respond to heat and ethanol stress by increasing trehalose synthesis, resulting in increased survival. Similar conclusions were reached by D'Amore *et al.* (1991) using brewing strains of yeast. Mansure *et al.* (1994) further showed using model membrane systems (liposome) challenged with ethanol, that liposomes enriched in unsaturated phospholipids showed less leakage than those containing saturated phospholipids. Using a more biologically relevant system, namely yeast protoplasts, Alexandre *et al.* (1994) proposed a relationship among ethanol tolerance, lipid composition and plasma membrane fluidity. The latter was correlated with an increase in phospholipid unsaturation in response to an ethanol stress. However, detailed studies using a number of different strains and stressors (ethanol, heat, H_2O_2) in this laboratory have shown no simple correlation among membrane lipid composition, stress tolerance and membrane fluidity (Swan and Watson, personal communication).

In the case of temperature, it is well documented that, in common with most other organisms, yeast adjust their membrane lipid fatty-acyl composition with temperature (Watson, 1984). The lower the growth temperature the more unsaturated the phospholipid membrane. For example, psychrophilic yeast,

which are unable to grow above 20°C, have mono and polyunsaturated fatty acids, with the latter in relatively high concentrations. By contrast, thermophilic yeasts (unable to grow below 20°C) are rich in monounsaturated fatty acids and have little or no polyunsaturated fatty acids. On the other hand, mesophilic yeasts are rich in monounsaturated fatty acids and, depending on the species, with small amounts of polyunsaturated fatty acids.

It is therefore an attractive hypothesis to propose that membrane lipid composition, at least as determined by the fatty acyl composition, is an important parameter in determining heat sensitivity of organisms. Early studies on *E. coli* showed that cells enriched in oleic acid (C 18:1) were more thermoresistant than linolenic acid (C 18:3) enriched cells (Overath *et al.*, 1970). On the other hand, in mammalian systems, there appears to be no correlation between membrane lipid composition and thermotolerance (Konings, 1988; Konings and Ruifrok, 1985).

The yeast system is an attractive one in which to investigate possible relationships between membrane lipid composition and stress tolerance. The former can be readily manipulated by environmental factors which include growth temperature, membrane lipid composition and oxygen. The present studies address all three parameters with respect to tolerance of cells to heat, ethanol and oxidative stress.

2.2. Effect of oxygen

It is paradoxical that oxygen, which is essential for all aerobic life, is also one of the most toxic molecules known. This toxicity is due to highly reactive oxygen-derived free radicals, generated as by-products of normal cellular metabolism such as electron transport in mitochondria and chloroplasts. The major species of oxygen-derived free radicals are thought to be the superoxide anion and the highly reactive hydroxyl radical (Halliwell and Gutteridge, 1989). In addition, H_2O_2 formed in biological systems by dismutation of the superoxide

anion, is a most important oxygen-derived reactive species, although it is not a free radical.

Based on the requirement of or tolerance to oxygen, microorganisms can be broadly divided into aerobes, facultative anaerobes or anaerobes. Within each of these groups, there are subgroups depending on their requirement or tolerance to different levels of oxygen, for example obligate aerobes which absolutely require oxygen for maintenance and growth and obligate anaerobes, some of which are highly sensitive to low levels of oxygen.

Although it is generally assumed that yeast are facultative anaerobes and thus capable of growth aerobically or anaerobically, this appears to be a myth. A recent survey of type species of 75 genera, covering a broad spectrum of yeasts, revealed that only 18 species were capable of true anaerobic growth (Visser *et al.*, 1990). It was noteworthy that *S. cerevisiae* stood out as a rapid fermentor and grew well under strictly anaerobic conditions. It should be noted, however, that under strictly anaerobic conditions, *S. cerevisiae* does have a growth requirement for unsaturated fatty acid and ergosterol, both of which require oxygen for their biosynthesis (Andreasen and Stier, 1953; 1954).

The requirement for both sterol (Proudlock *et al.*, 1968) and unsaturated fatty acid (Meyer *et al.*, 1963) are fairly non-specific. It is possible, therefore, to manipulate the lipid composition of *S. cerevisiae* grown anaerobically (Watson and Rose, 1980) and thus, for example, examine the relationship between membrane lipid composition and stress tolerance, one of the aims of the present studies.

There is increasing knowledge as to gene regulation with respect to oxidative stress in bacteria, especially *Escherichia coli* and *Salmonella typhimurium* (Farr and Kogoma, 1991). There is less known about oxygen regulation in eucaryotic microorganisms. Zitomer and Lowry (1992) have recently reviewed the regulation of gene expression by oxygen in *S. cerevisiae*. The main emphasis of this review was on gene regulation by heme, which acts

as the prosthetic group in the cytochromes and other oxygen-binding proteins. On the other hand, there is surprisingly little information on genes (and their products) which may be regulated or induced by anaerobiosis in yeast, although Zitomer and Lowry (1992) did allude to the identification of genes which were expressed exclusively in the absence of oxygen. However, the nature and function of the gene products remains to be identified.

2.3. Antioxidants

The detoxification of highly reactive oxygen-derived free radicals is a prerequisite of all forms of life. Defense systems adopted by living organisms include non-enzymatic and enzymatic, collectively termed antioxidant defense systems. Halliwell and Gutteridge (1989) defined an antioxidant as "any substance which, when present at low concentration compared to that of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate".

Antioxidants are widely used in the food industry, primarily as protectants against oxidative degradation of lipids. In the past few years, considerable emphasis has been placed on the promotion and use of natural over synthetic antioxidants. The former include ascorbate (vitamin C), α–tocopherol (vitamin E) and β–carotene (vitamin A) and the latter include propyl gallate (PG) and butylated hydroxy toluene (BHT). There is continuing and considerable debate as to the relative merits of the two types of antioxidants. For example, the toxicity of BHT has been variously reported as non-toxic (Bomhard *et al.*, 1992) or to increase the rate of hepatic cancer in rats (Olsen *et al.*, 1986; Inai *et al.*, 1988). This debate has been further fuelled by the substantial body of scientific evidence pointing to involvement of oxygen-derived free radicals as causative agents of many human diseases, including cardiovascular, inflammatory disorders and cancer (Halliwell and Gutteridge, 1989). Moreover, the popularisation of the free-radical theory of ageing (Emerit and Chance, 1992) has lead to a demand by consumers for antioxidants (in the form of tablets

rather than food) and antioxidant therapy. There is a voluminous literature dealing with the interactions of antioxidants and free radicals in higher organisms (Halliwell and Gutteridge, 1989; Rice-Evans, 1993). This section will therefore be limited to a summary of previous relevant work on antioxidants in microbial systems. In this respect, and in marked contrast to studies on higher organisms, there are surprisingly few recent publications on the effects of non-enzymatic antioxidants on microbial physiology.

Synthetic antioxidants, used primarily in the food industry (Aruoma and Halliwell, 1991) such as BHT and PG, have been reported to inhibit the growth and survival of some bacteria (Chang and Branen, 1975; Davidson and Branen, 1980 a and b; Chung *et al.*, 1993), fungi (Fung *et al.*, 1977; Beuchat and Jones, 1978) and yeast (Beggs *et al.*, 1978; Eubank and Beuchat, 1982, 1983).

In two related papers, Eubank and Beuchat (1982, 1983) reported that heat stressed *S. cerevisiae* were significantly more susceptible to the synthetic antioxidants PG and butylated hydroxyanisole (BHA) than untreated controls. Furthermore, incorporation of the antioxidants into agar media also lead to lower survival for heat stressed cells, suggesting an effect on repair mechanisms of heat damaged cells.

In the case of naturally-occuring antioxidants there is a wealth of information on their occurrence, properties and proposed mode of action in protecting against oxygen-derived free radicals in higher organisms (see previous references). Here again, however, there is limited data on bacteria and eucaryotic microbes. Moreover, the antioxidants which have been extensively studied in higher organisms, especially humans, are rarely found in microbes. These antioxidants include ascorbate, α-tocopherol and β-carotene. The latter, however, is found in photosynthetic bacteria and in pigmented bacteria and pigmented yeasts.

It is particularly significant that in one of the few studies on antioxidants in yeast, Moore *et al.* (1989) reported that in the pigmented yeast, *Rhodotorula*

mucilaginosa, the presence of high concentrations of carotenoids protected against oxidative damage induced by hyperoxia or duroquinone-induced stress, the latter generating intracellular superoxide radicals. The duroquinone system was also used by Schroeder and Johnson (1993) who demonstrated the antioxidant role of carotenoids in the red-fermenting yeast *Phaffia rhodozyma*. An interesting observation of both studies was the reported presence of Mn-SOD but the absence of CuZn-SOD in the pigmented yeasts. It was proposed by both sets of authors that, in the absence of CuZn-SOD, carotenoids may function as the major cytosolic antioxidant. The occurrence and function of enzymatic antioxidants, including SOD, in yeast is discussed in the next section.

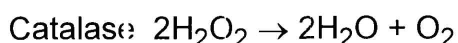
The tripeptide glutathione (L-glutamyl-L-cysteinyl-glycine) is found in most higher organisms and also occurs in many microorganisms, primarily facultative and aerobic microbes but apparently not in strict anaerobes (Penninckx and Elskens, 1993).

The role of glutathione, at least in higher organisms, appears to be well established as a substrate for various enzymatic antioxidants (eg. glutathione peroxidase) and as a free radical scavenger (Halliwell and Gutteridge, 1989). Deficiencies in glutathione metabolism have drastic, if not fatal, effects in higher organisms. There is good evidence in the literature that glutathione depletion sensitises mammalian cells to heat and oxidative stress (Andreoli *et al.,* 1986) and conversely, glutathione enhancement protects against stress (Lumpkin *et al.,* 1988; Harris *et al.,* 1991). The diverse role of glutathione, which include transport, enzyme activities and detoxification, in higher organisms suggests an essential function. By contrast, although the tripeptide is found widespread in microorganisms, it appears to play a less vital role (Penninckx and Elskens, 1993). Most relevant to the present studies is the report by Greenberg and Demple (1986) that glutathione depletion in *E. coli* does not lead to any change in resistance to heat, H_2O_2 or other oxidative stresses. In the case of yeast

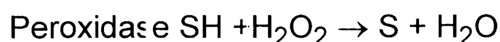
cells, work by Murata and Kimura (1987b) has shown that, although glutathione depletion was not lethal, growth was seriously impaired. Most importantly, glutathione offered some protection to cells against susceptibility to various toxic chemicals. The same group has recently extended these studies to include observations on glutathione-associated enzyme induction by oxidative stress in yeast (Inoue *et al.*, 1993 and Tran *et al.*, 1993).

Antioxidant enzymes

For the purpose of the present studies, only antioxidant enzymes which have been reported to be of importance in yeast will be discussed. A comprehensive review of antioxidant enzymes and their function in higher organisms can be found in Sies (1986) and Halliwell and Gutteridge (1989). These are a number of antioxidant enzymes which protect yeast against H_2O_2. These enzyme are the catalases and peroxidases which catalyse the following reactions:



Catalase $2H_2O_2 \rightarrow 2H_2O + O_2$



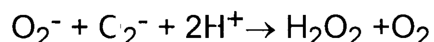
Peroxidase $SH + H_2O_2 \rightarrow S + H_2O$

In *S. cerevisiae*, two major catalases have been identified, cytosolic catalase T and peroxisomal catalase A (Seah *et al.*, 1973). The former enzyme levels are enhanced upon a heat shock (Wieser *et al.*, 1991). Catalase activity has been correlated with oxygen tension, with aeration of anaerobic cells resulting in increased catalase synthesis (Lee and Hassan, 1987; Clarkson *et al.*, 1991). Interestingly, Verduyn *et al.,* (1988) have shown that catalase-deficient mutants of *Hansenula polymorpha* were relatively resistant to H_2O_2 and capable of destroying H_2O_2 at a high rate. In addition, studies by Steels (1994) have indicated that catalase does not play a major role in thermotolerance or oxytolerance in *S. cerevisiae*. On the other hand, there is evidence that cytochrome c peroxidase plays an important role in oxytolerance in *S. cerevisiae*. Elevated levels of cytochrome c peroxidase are found in

deficient mutants exposed to H_2O_2 (Verduyn *et al.*, 1988) and the enzyme is thought to take part in H_2O_2 detoxification in mitochondria. Marked increases in cytochrome c peroxidase levels were detected when anaerobically grown *S. cerevisiae* were subjected to hyperoxic conditions (Steels, 1994).

It is only relatively recently that glutathione peroxidase activity was reported in yeast (Galiazzo *et al.*, 1987; 1988). The group of Kimura and coworkers (Inoue *et al.*, 1993; Tran *et al.*, 1993) have recently shown that glutathione peroxidase plays a key defensive role in protecting the yeast *H. mrakii* against oxidative stress. The enzyme is inducible on exposure of cells to reactive oxygen species (lipid hydroperoxides). Recent studies from this laboratory have shown that glutathione peroxidase levels in yeast are regulated by oxygen tension with a 3 to 5-fold elevation on aeration of anaerobically grown cells (Steels, 1994).

The superoxide dismutases (SOD) are a class of enzymes which function to remove catalytically the superoxide radical according to the reaction:



$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Three distinct superoxide dismutases are found in biological systems, all of which catalyse the same reaction of superoxide dismutation but differ in the metal moiety. The CuZn-SOD is found in almost all eucaryotic cells, localised in the cytoplasm, and in a few species of bacteria (Fridovich, 1989; Halliwell and Gutteridge, 1989). The Fe-SOD is found primarily in procaryotes and in a few plant species and the Mn-SOD is found in both procaryotes and eucaryotes. In the case of the latter, the Mn-SOD is found associated with the mitochondria. This may be a reflection of the endosymbiotic theory of evolution based on the concept that mitochondria of eucaryotic cells originated from a procaryotic ancestor.

The pioneering observations of Fridovich and coworkers (Gregory *et al.*, 1974; Fridovich, 1989) established a key role for SOD as an antioxidant

enzyme for detoxification of H_2O_2 and related oxygen-derived free radicals. The yeast CuZn-SOD (Lee and Hassan, 1987; Clarkson *et al.*, 1991) and the Mn-SOD (Steels, 1994) are oxygen-inducible and it thus now appears well established that these enzymes play an important part in protecting cells against oxidative stress. Particularly relevant are studies on mutants defective in Mn-SOD which are hypersensitive to high oxygen concentrations (Van Loon *et al.*, 1986; Westerbeek-Marres *et al.* 1988). Moreover, CuZn-SOD-deficient mutants are particularly sensitive to exogenous H_2O_2 (Bilinski *et al.*, 1985). Finally, the Mn-SOD is also heat shock inducible and therefore a possible link between heat and oxidative stress is suggested (Steels, 1994).

Overall, it is evident that SOD in yeast is a most important antioxidant enzyme which functions to protect cells against the damaging effects of H_2O_2 and highly reactive oxygen-derived free radicals.

CHAPTER 2

MATERIALS AND METHODS

1. MATERIALS

1.1. Yeast strain

Saccharomyces cerevisiae ATCC 26422 (American Type Culture Collection, Rockville, U. S. A.), a diploid sake yeast, was used in all the experiments described in this work.

1.2. Chemicals

All reagents were AnalarR grade or better. Specific chemicals were obtained as follows:

Merck/BDH (Melbourne): KH_2PO_4, Glucose, Trichloroacetic acid (TCA), Bis-acrylamide, H_2O_2, H_2SO_4, Ethanol, Methanol, Hexane, Chloroform, BF_3-methanol, Diethyl ether, Glycerol, 2-Mercaptoethanol, Bromo phenol blue.

Sigma (St. Louis, USA): Ascorbic acid, Butylated hydroxytoluene (BHT), Propyl gallate (PG), Oleic acid, Linoleic acid, Linolenic acid, Methionine, Ethylenediamine tetraacetic acid (EDTA), Anthrone, Cycloheximide, Glass beads 0.45-0.5 mm diameter.

Ajax Chemicals (Sydney): HCl, KCl, Acetic acid.

Boehringer-Mannheim (Sydney): Glutathione (reduced), Ergosterol, Phenyl methyl-sulfonyl fluoride (PMSF).

Pierce Chemicals (Sydney): Coomassie protein assay reagent.

Pharmacia (Sydney): Ficoll 400.

Riedel-dehaën: Thiourea.

ICN Biomedicals (Sydney): Acrylamide, Tris, Glycine, Sodium dodecyl sulphate (SDS), Ammonium peroxydisulphate (AMPS), Radiolabelled ^{35}S-methionine (containing 75% L-^{35}S-methionine and 15% L-^{35}S-cysteine,1037 Ci/mmole).

Oxoid (Sydney): Agar no. 1, Yeast extract, Bacteriological peptone.

Difco Laboratories (Sydney): Yeast nitrogen base without amino acids.

Bio-Rad (Sydney): Silver stain kit, protein molecular weight standards.

Kodak (Melbourne): N,N,N',′N -Tetramethyl-ethylenediamine (TEMED), Fixer, Developer.

2. METHODS

2.1. Culture conditions

2.1.1. Stock culture

Yeast cells were maintained on YEP agar plates containing 1% yeast extract, 0.5% bacteriological peptone, 0.1% KH_2PO_4, 1.5% agar and 2% glucose (all w/v). Subcultures were made every 3-4 weeks and stock plates stored at 4°C.

2.1.2. Starter culture

A starter was prepared from a single colony taken from a working plate, and inoculated into 20 ml of 2% glucose-YEP broth contained in a 100 ml conical flask. Starter cultures were generally grown overnight (14-18 hr) at 25°C in a Paton orbital shaker (Paton Industries, Adelaide) operating at 180 o.p.m.

2.1.3. Aerobic growth

Starter cultures were used to inoculate (generally 0.05-0.1 ml) experimental cultures (100 ml 2% glucose-YEP broth contained in a 250 ml conical flask) to an optical density of 0.3 to 0.4 at 640 nm (Novaspec 4049 spectrophotometer, LKB Biotechnology). Experimental cultures were grown as described above (25°C, 180 o.p.m). Cultures at this stage were in the respirofermentative growth phase as defined by Lewis *et al.* (1993), corresponding to mid-to-late logarithmic growth phase (approximately 1 x 10^7 cells/ml and 0.5% w/v glucose remaining in the culture).

2.1.4. Anaerobic growth

Anaerobic growth conditions were conducted in water-jacketted (for external temperature control) glass vessels of different capacities, generally containing 600 ml medium. The medium consisted of 2% glucose-YEP supplemented with ergosterol (5 mg/l) and either oleic acid (C 18:1, 30 mg/l) or linolenic acid (C 18:3, 30 mg/l) as lipid supplements. These latter

supplements were required for optimum anaerobic growth as yeast are unable to synthesise sterol and unsaturated fatty acids in the absence of oxygen (Andreasen & Stier, 1953, 1954). A steady stream of high purity nitrogen gas, supplied aseptically and through a sintered disc, was bubbled constantly through the medium to maintain anaerobic conditions.

Aerobic starter cultures (see 2.1.2) were used to inoculate (0.5-0.7 ml inoculum) the experimental cultures for anaerobic growth. Anaerobic growth was generally for 18-20 hr at which stage cell density was approximately 1×10^7 cells/ml, corresponding to mid-to-late logarithmic growth phase.

2. 2. Viable count

Cell viability was estimated by the spread plate technique by plating out (0.1 ml) a range of appropriate dilutions (in duplicate) in YEP broth (minus glucose) on to 2% glucose-YEP agar plate. Plates from aerobically grown cells were incubated for 2-3 days at 28°C. The cells were counted as colony forming units per ml (cfu) and plates with 20-300 cfu were counted. All plates from cells grown anaerobically were incubated at 28°C in an anaerobic cabinet (Model SJ-3, Kaltec, Edwardtown, South Australia) which was constantly flushed with CO_2:H_2 (96% :4%).

Colony counts were determined using a Stuart colony counter (Stuart colony scientific counter, Ringwood East, UK).

2.3. Percentage of budded cells

The percentage of budded cells was estimated with the aid of a haemocytometer (Neubauer improved, bright line) using an Olympus Microscope (Olympus CH-2, Japan) and a magnification of 400.

% of budded cells = (number of budded cell / total of cells) x 100

Daughter and mother cells were considered as two cells, if the size of the daughter cell was similar to the mother cell, or as one cell, if the size of the daughter cell was significantly smaller than the mother cell.

2.4. Dry weight

Twenty to forty ml of washed cells were filtered onto Millipore filters (0.22 µm) and filters dried in a 60°C incubator for about 24 hr. Filters were transferred into a desiccator, allowed to cool and weighed.

2.5. Induced and intrinsic thermotolerances

For thermotolerance studies, cells (20-100 ml culture) grown at 25°C were centrifuged at 3000 rpm for 5 min and the pellet resuspended in YEP broth minus glucose. Cells were heat shocked by incubation at 37°C for 45 min in an oscillating water-bath. Following the heat shock, cells were then immediately incubated at 48°C (heat stress) in an oscillating water-bath for various times. Cells were then rapidly cooled to 25°C by immersion (30 s) of the flask in ice-water before dilution plating for viable count determination (section 2.2).

Intrinsic thermotolerance was determined by heating cells directly from 25°C to 48°C over a time course. Cooling and cell viability determination were as previously described.

Percentage survivors were expressed as the number of colony forming units (cfu) after heat treatment divided by the number of colony forming units before heat treatment.

% survivors = (no. cfu after heat treatment / no. cfu before heat treatment) x 100.

2.6. Determination of oxidative and ethanol stresses

Oxidative stress was imposed by addition of H_2O_2 at different concentrations (5 mM and 10 mM) to appropriate cultures. Ethanol stress was measured by addition of ethanol to give a final concentration of 16% v/v. Cells

were incubated at 25°C with H_2O_2 or ethanol over a time course and viability and percentage survivors determined as in 2.2.

2.7. Effect of glucose

The majority of experiments to determine the ability of cells to cope with stress were performed by re-suspending the cells in fresh YEP media without glucose (see 2.5). In some experiments to investigate the effects of glucose on thermotolerance, cells were stressed in the growth media (generally containing around 0.5% w/v glucose) without prior washing.

2.8. Effect of antioxidant

The effects of antioxidants on cell survival during stress were investigated in four ways. *Firstly*, by addition of antioxidants to cell cultures (20 ml) at 25°C for 15 min before heat and oxidative (10 mM H_2O_2) treatments. Antioxidants [10 or 20 mM ascorbic acid, 10 or 20 mM glutathione, 100, 200 or 750 ppm BHT (butylated hydroxytoluene), 100, 250 or 750 ppm propyl gallate (PG)] were added to aerobic cells 15 min prior to heat treatment. In experiments on the effects of antioxidants during oxidative stress (10 mM H_2O_2), 10 mM ascorbic acid or 10 mM glutathione was added 15 min prior to addition of H_2O_2.

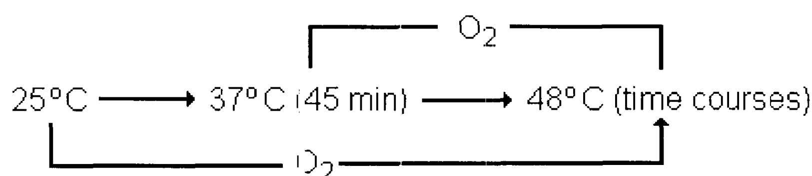
Secondly, the effects of antioxidants were investigated by incorporation of antioxidants in the agar plates. Twenty ml of culture were subjected to heat shock/stress and cells were then plated onto YEP agar containing the appropriate antioxidant (10 mM ascorbic acid, 10 mM glutathione, 10 μM vitamin E, 10μM β–carotene or 5 mg/l ergosterol) in order to investigate the effect of these antioxidants on cell recovery from stress. For vitamin E and ergosterol, it was necessary to dissolve the antioxidants in ethanol and for β-carotene in tween 80. For comparison, the same concentration of ethanol (0.5% v/v) and of tween 80 (0.1% v/v) were incorporated into control plates.

Thirdly, and in contrast to the previous experiments, the effect of antioxidants was examined by growing the cells in the presence of the antioxidant in the culture media. This was carried out by adding either 10 mM ascorbic acid or 10 mM glutathione to the growth media (2% glucose-YEP broth). Experimental cultures were then subjected to heat shock/stress as outlined in section 2.5.

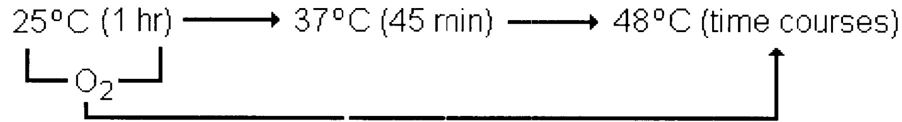
Forthly, the effect of antioxidants was investigated by means of liquid holding recovery i.e. by addition of antioxidants to the liquid media after heat treatment. Twenty ml of culture were exposed to heat shock/stress for 60 min or 120 min and then cultures were placed in YEP media containing antioxidant (10 mM ascorbic acid) for 5 or 10 hours. In some experiments, liquid holding was in YEP media plus 2% glucose with or without antioxidant.

2.9. Effect of oxygen and nitrogen

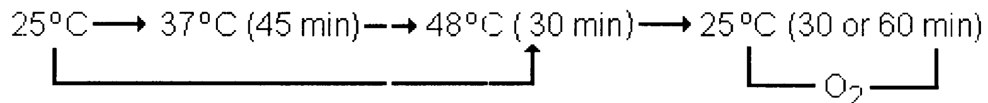
Three experimental procedures were carried out to determine the effect of oxygen on stress tolerance of cells. The first procedure was addition of oxygen during heat treatment (induced or intrinsic heat treatments). One hundred ml of aerobically or anaerobically grown cells were subjected to induced or intrinsic heat treatments (section 2.5) in the presence of oxygen. A steady stream of high purity oxygen was passed through the cultures during the heat treatments. The effect of oxygen during oxidative (5 mM H_2O_2) or ethanol (16% v/v) stresses were also studied by aeration with oxygen throughout the incubation at 25°C.



Secondly, 100 ml of aerobically grown cells were oxygenated by aeration as above for 1 hr prior to determination of induced and intrinsic thermotolerance.



Thirdly, cultures grown at 25°C were treated with heat (induced or intrinsic thermotolerance) and then subjected to oxygen aeration. For induced thermotolerance experiments, cultures were grown aerobically or anaerobically while for intrinsic thermotolerance, cultures were grown aerobically only.



The effect of nitrogen aeration (anaerobiosis) on yeast cell stress survival was also investigated. However, in these experiments the effects of nitrogen were investigated only during and following heat treatments. The procedures were thus similar to the first and third oxygen protocols as above, except substituting oxygen by nitrogen.

The percentage of survivors, expressed as colony forming units, from both oxygen and nitrogen treatments were counted by means of the spread plate method as described previously (section 2.2).

2.10. Effect of inhibition of protein synthesis

These experiments were designed to determine the effect of stress treatments on cells in the absence of protein synthesis. Cyclohemixide is a known inhibitor of eucaryotic cytoplasmic protein synthesis (Schindler & Davies, 1975). According to Whiffen (1948), 10 µg/ml of cycloheximide is enough to inhibit growth of *S. cerevisiae,* although this concentration would be strain dependent. In this study, 50 µg/ml cycloheximide was used to block protein

synthesis, a concentration previously shown to strongly inhibit cytoplasmic protein synthesis in strain K7 (Lewis *et al.* , 1995).

Aerobically grown cells were pre-incubated at 25°C with cycloheximide (50 μg/ml) for 15 min prior to a stress challenge: heat, ethanol or H_2O_2.

2.11. Determination of *de novo* protein synthesis

De novo protein synthesis was determined by radioactive labelling. Thirty ml of culture at 25°C were washed and re-suspended in YNB (yeast nitrogen base without amino acid) plus 2% glucose. Cultures were shocked (37°C) or heat shocked plus oxygen for 5 min before addition of 100μCi of ^{35}S-methionine and incubation continued for 45 min. Cold methionine (3 ml of a stock solution, 10% w/v) was added immediately after incubation to stop incorporation of ^{35}S-methionine. Reaction mixtures were centrifuged (3000 x g for 5 min) and the pellet extracted and analysed for protein as described in 2.12.1.

2.12. Protein electrophoresis

2.12.1. Yeast protein extraction for SDS-PAGE

Mid-to-late exponential phase cells were harvested by centrifuging at 3000 x g for 3 min (Clements centrifuge, model 2000 S) and the pellet transferred to a microfuge tube (1.5 ml). Fifteen μl 0.1M phenyl methyl-sulfonyl fluoride (PMSF), 100μl protein extraction buffer [2 mM ethylenediamine tetraacetic acid (EDTA), 2% w/v ficoll 400, 1% 2-mercaptoethanol, 2% sodium dodecyl sulphate (SDS) and 50 mM tris-HCl, pH 6.8] and an equal volume of glass beads (0.45-0.5 mm diameter, acid washed, Sigma) were added and the pellets extracted by vortexing for 6 x 20 sec with cooling in between on ice for 2 min. Cell debris and glass beads were removed by centrifuging at 13000 x g for 2-3 min in a microcentrifuge. The supernatant containing the protein was transferred to a cold microfuge tube, and an equal volume of SDS sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.25M tris-HCl, pH 6.8)

was added . Samples were boiled for 2 min and stored at - 70°C, until required for electrophoresis.

2.12.2. Protein analysis

Protein analysis was determined by the Pierce Coomassie micro-assay (Pierce, Rockford, Illinois, U.S.A.) which is based on the Bradford procedure (Bradford, 1976). Absorbance was measured at 595 nm (Novaspec 4049 spectrophotometer, 1 cm pathlength cuvettes) against a reagent blank and the protein content of the sample was estimated by reference to a standard curve constructed by using bovine serum albumin (Pierce) as a standard.

2.12.3. Electrophoresis

Proteins were separated using the technique described by Laemmli (1970) and Laemmli and Favre (1973). Electrophoresis was performed on polyacrylamide vertical gels in one dimension (vertical gel with 16 x 20 cm, 1 mm thickness Bio-Rad protein II x i system). The running gel was 10% polyacrylamide [13.32 ml of 30 % (w/v) acrylamide and 0.8 % (w/v) bisacrylamide, 10 ml 1.5 M tris-HCl pH 8.8, 16.10 ml H_2O, 40 µl N,N,N','N' tetramethyl-ethylenediamine (TEMED), 400 µl 10 % (w/v) SDS and 140 µl 10 % (w/v) ammonium peroxydisulphate (AMPS)] and a 4 % stacking gel [1.30 ml 30 % (w/v) acrylamide and 0.8% (w/v) bisacrylamide, 1.25 ml 1 M tris HCl pH 6.8, 7.24 ml H_2O, 10 µl TEMED, 100 µl 10% (w/v) SDS and 100 µl 10 % (w/v) AMPS] was used. Prior to loading, samples were boiled for 2 min, together with low-range molecular mass protein standards (Bio-Rad standards with 97.4, 66.2, 45, 31, 21.5 kDa molecular masses). Samples containing equal amount of protein (10µg) were loaded into each lane and gels run at 10°C and 3 mA. Electrophoresis was stopped when the tracking dye (bromophenol blue) reached the end of the gel (4-5 hr).

2.13. Gel staining

Following electrophoresis, the next step was gel staining, using a silver stain kit consisting of oxidiser, silver reagent and silver-stain developer (Bio-Rad Laboratories). Prior to staining, gels were fixed by soaking in 40 % methanol and 10 % acetic acid for at least 30 min, and continued with 10 % ethanol and 5% acetic acid for 20 min. Staining was stopped by immersion of gels in 5 % acetic acid.

2.14. Gel drying

Gels were dried in a Bio-Rad model 543 gel drier at 70°C for 70 min as outlined in the manufacturer's guide. Gels were placed between two pre-wetted cellophane sheets.

2.15. Autoradiography

Labelled protein separated by electrophoresis was detected by exposing the dried gel (cassette holder 18 x 24 cm) to hyperfilm-MP (Amersham) for 2-3 weeks in a -70°C freezer. Films were developed by immersion in Kodak liquid x-ray developer for 1 min, rinsed with water, immersed in Kodak liquid x-ray fixer for 1 min and finally rinsed with water.

2.16. Trehalose assay

Trehalose was extracted by trichloroacetic acid (TCA) and determined by the anthrone procedure (Lillie & Pringle,1980) with modification. Twenty to forty ml of mid-to-late exponential phase cells were centrifuged at 3000 x g for 5 min. Cells were washed twice with 5 ml of chilled distilled water and spun down at 3000 x g for 5 min. Extraction was continued by resuspending the pellet in 0.5 M-TCA, twice for 90 min each.

All samples were boiled for 15 min after addition of anthrone reagent and absorbance at 620 nm read. A standard curve was prepared using glucose (500 μg/ml) as a standard. The final concentration of trehalose (μg/mg dry weight) was calculated from a standard curve.

2.17. Lipid analysis

2.17.1. Lipid extraction

Cells (100 - 200 ml) were harvested by centrifugation. The cells were then washed twice with H_2O. Lipids were extracted using a modification of the Letters (1968) and Watson (personal communication) procedures.

Washed cells were resuspended in 10 ml of 80 % (v/v) ethanol and placed in a 80°C waterbath for 20 min. The sample was cooled and centrifuged at 3000 x g for 5 min. The supernatant was removed into a clean, dry 15 ml Corex tube. Ten ml of chloroform/methanol [2:1 (v/v) ratio] was added to the pellet and the mixture left (with occasional shaking) at room temperature for 30 - 45 min. The solutions were centrifuged to obtain a second supernatant. The supernatants were combined and transferred to a separating funnel. The supernatant was washed with 0.25 volume of 0.88 % (w/v) KCl and the mixture allowed to separate (5-10 min).

The lower phase was removed into a 50 ml round-bottom flask, and the mixture dried at 50°C in a rotary evaporator. The residue was immediately dissolved in 0.5 ml of chloroform/methanol (2:1, v/v). Samples were stored at - 20°C until required for phospholipid analysis.

2.17.2. Total phospholipid

Total phospholipids were separated from neutral lipids by thin-layer chromatography using silica gel G-60 plates (Merck, 0.25 mm thick) using a solvent system of petroleum ether (bp = 40-60 °C)/diethyl ether/acetic acid (70:30:2, by volume) for 30 - 45 min. The phospholipids, which remained at the origin, were scrapped from the plates into glass tubes and methylated with BF_3 / methanol at 80°C for 30 min.

The fatty acid methyl esters were extracted into hexane and concentrated to a small volume prior to analysis. Samples were analysed

using a Hewlett Packard 5890 model gas chromatograph. The conditions were: carrier gas, helium flow rate 10ml min-1, detector and injector temperature 220°C and oven temperature 180°C. Fatty acid methyl esters were identified from their retention times relative to appropriate standards (Sigma) and percentage fatty acid composition determined by using the Delta chromatography data system (Digital Systems, Brisbane).

2.18. Statistical analysis

Repetition is very important in any experimental procedure. In this study, all experiments were carried out a minimum of two times and generally 3-4 times. However, in some experiments a satisfactory statistical analysis could not be performed, particularly with respect to percentage survivors following a stress. Whenever possible, the results reported in this study were average values. In some experiments, only the results from a typical experiment were presented, reflecting the trends of a particular experimental procedure.