Chapter 2

Review of literature
2.1 Ethanol

Ethanol or ethyl alcohol (CH\textsubscript{3}CH\textsubscript{2}OH) is an important organic chemical because of its unique properties and therefore can be used widely for diverse applications. Under ordinary conditions, ethanol is a volatile, flammable, colourless liquid, miscible in both water and non-polar solvents. Ethanol has widespread use as a solvent of substances intended for human contact or consumption, including scents, flavourings, colourings and medicines. In chemistry, it is both an essential solvent and a feedstock for the synthesis of other products. It has a long history as a fuel for heat and light, and more recently as a fuel for internal combustion engines (Hill, 2007).

The production of ethanol has two routes: chemical and biological. The chemical ethanol production is commonly carried out by a catalytic hydration of ethylene in vapour phase and often as a by-product of certain industrial operations. Fermentative ethanol production accounts for 93% of the total ethanol production in the world because its production economical cheaper. Ethanol is produced from fermentation of sugars extracted mostly from crops. \textit{S. cerevisiae} is the most popular micro organism used for ethanol production due to its high ethanol yield and higher tolerance. Brazil is the largest ethanol producer with a capacity of 15.5 Giga-L (in 2004) and uses sugar cane as feedstock, while the USA being in second place (12.9 Giga-L) uses corn as feedstock (Purwadi, 2006). However, these crops are also food for human and animals, thus discouraging the production of ethanol from above mentioned raw materials. In contrast, cheap abundant lignocellulosic materials in the form of agricultural and forestry wastes as well as municipal and industrial wastes are available as alternative feedstock for ethanol production. Therefore, the use of lignocellulosic materials for ethanol production is very promising (Kim and Dale, 2004).

D-xylose, which is present in these lignocellulosic materials, is the second most abundant renewable sugar in nature, forming up to 25% of the total dry weight of some forestry and agricultural residues (Hartley, 1981). The efficient utilization of this pentose sugar, therefore important in the overall bioconversion of plant biomass for the production of chemicals and liquid fuels.
Indian government is also showing interest in such kind of projects because our agronomy based economy and heavy expenditure on the import of petroleum fuel compel us to think about it.

2.2 *Pachysolen tannophilus*

*Pachysolen tannophilus* was the first yeast discovered capable of producing ethanol from xylose and thus has served as a model for studies of other yeasts mediating this conversion (Slininger *et al.*, 1982; Toivola *et al.*, 1984). Xylose is found in the hemicellulose fraction of many cellulosic materials, but is not utilized in commercial fermentation processes. The organism is thus of potential importance to industry; it may prove useful in providing a more efficient method of converting biomass to energy.

2.3 The metabolism of xylose in *Pachysolen tannophilus*

The metabolic steps involved in the fermentation of six carbon sugars have extensively been studied, when compared to five carbon sugars. The first biochemical step in D-xulose fermentation in yeast *P. tannophilus* is the conversion of D-xylose into xylitol in the presence of NADPH linked enzyme D-xylose reductase. In the second step, xylitol is converted to D-xylulose by NAD linked xylitol dehydrogenase. Phosphorylation of D-xylulose is then catalyzed by D-xylulokinase to D-xylulose-5-phosphate, which is a chief driving reaction in the pathway (Stevis *et al.*, 1987). Xylulose-5-phosphate entered in the pentose phosphate pathway for the further processing.

2.4 Ethanol production by the *Pachysolen tannophilus*

The rate of production and its concentrations attained by *P. tannophilus* compare poorly with that of commercial ethanol fermentation by non xylose-fermenting *Saccharomyces cerevisiae* or *Zymomonas mobilis* on glucose-based substrates. There are many major limitations which block this process (Hahn-Hagerdal *et al.*, 1994). The redox imbalance in the initial conversion of xylose to xylulose, sensitivity to high concentrations of ethanol (Jones, 1989), differences in the
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respiratory pathway and sensitivity to microbial inhibitors, particularly those liberated
during pre-treatment and hydrolysis of lignocellulose substrates (Pampulha and
Leureiro, 1989), have been identified as major factors limiting ethanol fermentation
by the xylose-fermenting yeasts (Watson et al., 1984a; 1984b).

2.5 Ethanol stress and yeast

Effects of alcohol can be exerted on the membranes or on several
physiological activities. The effects may also be related to production of stress
proteins or to mitochondrial activity. The complexity of these mechanisms has made
it difficult to establish a suitable model for alcohol action. To understand the effects
of ethanol on various parts of yeast we can simplify the whole process by further
categorization.

2.5.1 Effects of ethanol stress on plasma membrane

Yeast membranes are composed of structured phospholipid assemblies that
form under physiological conditions layer of bimolecular thickness with integrated
proteins. In aqueous solution and in an appropriate environment, the amphipathic
phospholipids self-assemble into bilayer sheets. The dominant force in this process is
the hydrophobic interaction, which favours shielding of the acyl chains from aqueous
contact. In order to avoid hydrophobic exposure, bilayers tend to deform into closed
structures with hydrophilic internal and external faces that are composed of the
phospholipid head groups. Phospholipids in this structure have considerable motional
freedom: for this reason the membrane is described as a fluid mosaic, and the term
membrane fluidity represents the dynamic properties of lipids within the bilayer. This
structure, called the fluid-crystalline lamellar phase, is dominant in functional
membranes. Membranes enclose cells and organelles and have a separating function
related to their hydrophobic core, a barrier for hydrophilic compounds, which allows
maintenance of the difference between the chemical composition of the delimited
internal spaces and their environment (van der Rest et al., 1995).

Membranes are also the matrices into which many functional proteins are
embedded and maintained in an interfacial position. Dynamic and structural
characteristics of membranes can be changed, either by changing the environmental conditions, by changing the molecular composition of the membrane, or by adding foreign molecules that interact with membrane constituents. This explains why changes in membrane fluidity are observed in response to many environmental stresses, and why cells can control their fluidity by the modulation of their membrane composition in order to maintain an optimal level of fluidity within the lipid matrix. Changes in the environment induce variations in the membrane fluidity, and even in the membrane lipid structure e.g. the transition of the fluid-crystalline lamellar phase into the gel lamellar phase. Changes in the membrane’s structural and dynamic characteristics affects the functions of membrane proteins by changing in the lipid environment of enzymes, and may either be involved in the cellular response to stress, e.g. because of the lipid/protein-mediated activation of a signal pathway or in cell injury because of the rupture of the hydrophobic barrier caused by a phase separation of the lipids (Beney and Gervais, 2001).

2.5.1.1 Ethanol stress and membrane fluidity

Yeast membrane is one of the primary targets of ethanol. Alterations in the lipid composition of the cell membrane affects the ethanol tolerance of yeast and increased unsaturated fatty acids result in an increase in membrane fluidity and lead ethanol tolerance (Prashant and Prasad, 1989; Swan and Watson, 1996). The ethanol induces the synthesis of unsaturated fatty acid synthesis, that leads to increased membrane fluidity (Dinh et al., 2008a; 2009b; Alexandre et al., 1994). The compositions of UFAs in *S. cerevisiae* consist mainly of the mono-UFAs palmitoleic acid (C16:1) and oleic acid (C18:1) with the former dominating. Both of them can be catalyzed by a single integral membrane desaturase encoded by OLE1 gene. It is showed that the OLE1 deletion strain conferred the greatest tolerance to the growth inhibiting effects of ethanol when supplied with C18:1, whereas, C16:1 conferred the least tolerance. It is suggested that among the two common types of UFAs, oleic acid is more efficacious in overcoming the toxic effects caused by ethanol in growing yeast cells (You et al. 2003). Similar results that 18:1 fatty acids could rescue ethanol tolerance were also obtained in the sensitive mutants of *Escherichia coli* (Ingram,
However, the mechanism of increasing UFAs under alcohol stress seems relatively more complex. It has been shown that the effect of increasing the content of UFAs can result in over fluidized membrane, which is opposite to regular theory that the factors making cell membranes more rigid are beneficial to ethanol tolerance (Mishra and Prasad, 1989). On the other hand, phospholipids containing 18:1 cis fatty acids have a lower transition temperature than those containing 16:0 fatty acids, and it means that the incorporation of 18:1 cis will further disorder the lipids on membrane. So, the reason for the increase of UFAs in ethanol response has been suggested to antagonize the stereo-chemical effect produced by the hydrophilic ethanol on the head groups of the phospholipids bilayer (Weber and Bont, 1996). Recently, the hypothesis that UFAs are positively correlated to ethanol resistance was experimentally supported again.

Specifically, the supplement of soya flour to yeast growth medium was found to contribute to greater ethanol tolerance than that supplemented with soya flour without fatty acids. When unsaturated fatty acids compositions of cell membrane were analyzed, the concentrations of UFAs increased significantly in all tested strains grown in medium supplemented with soya flour, which also showed increased ethanol concentration by 4–6% (v/v) than the control with defatted soya flour (Xiao et al., 2008). This study further suggested that the composition of UFAs might be used as a criterion in evaluating the potential ethanol tolerance of other microorganisms.

2.5.1.2 Role of ethanol stress on plasma membrane ATPase and glucose uptake

Plasma-membrane ATPase is an electrogenic proton pumps crucial to all fungal and plant cells. It generates the electrochemical $H^+$ gradient which is carrying several cellular functions as the secretion of acids, the uptake of nutrients, the maintenance of $K^+$ levels and the regulation of intracellular pH (Cartwright et al. 1987). Plasma membrane $H^+$-ATPase is the major enzyme activity responsible for maintaining the electrochemical potential gradient across the plasma membrane.
When yeast cells exposed to sub-lethal ethanol levels (Rosa and Sa-Correia, 1991; Meyrial et al., 1997), the activity of this H⁺-ATPase dramatically stimulated. This activation of the H⁺-ATPase by ethanol occurs in vivo being readily detectable as an increased proton extrusion by intact cells and it is not lost during purification of membranes for ATPase assay. It reflects a stress-induced modification of the ATPase. The mere addition of ethanol to the in vitro ATPase assay reaction leading not to activation of the ATPase but instead to a partial inhibition. Remarkably, this ATPase inhibition due to ethanol addition to the in vitro assay is less if the membranes are from ethanol adapted cells, possibly due to the plasma membrane lipid changes that occur with adaptation to ethanol. Although heat shock and ethanol exposure both stimulate plasma membrane H⁺-ATPase, protein levels of this proton-pumping ATPase actually show a rapid initial decline with short periods of either stress (Panaretou and Piper, 1992).

Sudden exposure of cells to ethanol stress probably influences the capacity of the cells to re-establish and sustain homeostasis immediately after imposition of stress. The increased plasma membrane H⁺-ATPase activity in ethanol-stressed cells cause an enhanced catalysed proton efflux. This proton extrusion counteracts the dissipation of proton motive force resulting from stress induced increase in membrane permeability. This is probably one reason that mutations that alter plasma membrane H⁺-ATPase activity can influence cellular tolerance of both ethanol and heat (Coote et al., 1994). Imposition of ethanol stress demands a dramatically increased expenditure of energy as cells struggle to re-establish and subsequently maintain homeostasis.

Other factors that affect homeostasis, in addition to H⁺-ATPase activity influence cellular tolerances to ethanol (Barbosa and Lee, 1991). Ethanol also affects the glucose transport and it enhances the leakage of membrane, (Pascual et al., 1988; Salmon, 1989; Cardoso and Leao, 1992). Ethanol enhances the passive influx of protons into deenergized cells of S. cerevisiae. It is reported that passive proton influx contributes to the kinetics of acidification in S. cerevisiae and that uncoupling contributes to the overall kinetics of ethanol inhibited secondary active transport across the yeast plasma membrane (Leao and Van Uden, 1984).
The ethanols causes direct enzymatic inhibition, and produce inhibition of alcohol dehydrogenase or cause denaturation. Studies on the inhibitory effects of ethanol on the hexokinase suggest that this is the most important inhibition of ethanol on glycolysis, as hexokinase control, the input of glucose into the metabolism so its inhibition severely affects the metabolism of yeast and uptake of glucose. Similar kind of study on the other enzymes suggests that more than twelve enzymes of the Embden Myerhalf glycolytic pathways are affected by the ethanol (Millar et al., 1982; Osman and Ingram, 1985; Alexandre and Charpentier, 1998; Bisson, 1999).

2.5.2 Ethanol induced water stress

Water activity (a_w) is a measure of water availability and is applicable to both simple solutions (such as those of a single salt) and complex mixtures (such as brewer’s wort). Pure water is designated as a value of 1 a_w, (arbitrary units) at a constant temperature and pressure. Water activity can be determined by measuring the equilibrium relative humidity above a body of water (100%) or substrate (less than 100%) and dividing this value by 100. The majority of yeast species, including S. cerevisiae, grow within a narrow range of a_w, values; between 0.9 and 1 (Jones and Greenfield 1986). Growth of S. cerevisiae is optimal between 0.975 and 0.999 aw, and the growth of most brewing strains is severely inhibited below 0.94. Most strains of S. cerevisiae cannot grow or remain metabolically active below 0.92 a_w. This is a value of general significance, like a temperature of 45°C, beyond which most biological systems cease to function normally. Such values of a_w, and temperature have a physicochemical basis that relates to the instability of the hydrogen bond under extreme conditions. The exclusive use of terms such as ‘osmotic stress’, ‘water potential’ and ‘water availability’ can result in a misinterpretation of cell metabolism. The terms ‘osmotic stress’ and ‘water potential’ imply that water stress involves a net movement of water across a membrane, but this is not always the case. The structure and function of phospholipid bilayers and enzymes are disrupted when hydrogen bonding is disrupted in vitro or in vivo, even when intra- and extra-cellular a_w are equal.
The effect of ethanol on the aₙ of ethanol-water mixtures is shown in Fig. 2.1. As ethanol concentration increases there is a sharp reduction in water availability. A concentration of 20% ethanol (w/v) reduces the aₙ to 0.895 considerably below the growth limit for most yeast species. Of the compounds, that are commonly used to reduce aₙ experimentally (sucrose, sorbitol, glucose, glycerol, NaCl), ethanol is second only to NaCl in its ability to depress water availability when compared on a per cent, weight for weight basis. Furthermore, when a fermentation medium contains 20% ethanol (w/v), the actual aₙ is less than 0.895 because wort sugars and metabolites, such as glycerol or lactic acid reduce aw (Jones and Greenfield 1986). Even ethanol concentrations of less than 5% (w/v) can have an impact, in combination with other medium components, on growth and metabolism. Factors other than medium components can also have synergistic water stress effects (e.g. extremes of pH, temperature or pressure), further weakening hydrogen bonds of structural importance. In addition, ethanol can have effects that are specific to particular metabolic processes as well as indirect metabolic effects and specific effects on hydrophobic regions of cell components, none of which are accounted for by aₙ measurement. Ethanol-induced water stress provides a context in which to consider...
many of the effects of ethanol at concentrations of about 5% (w/v) or above (Jones, 1989; Omi and Kamihara, 1989).

2.5.2.1 Effects of ethanol induced water stress

Ethanol is hydrophilic and is small enough to enter the hydration layer of membranes (Slater et al., 1993). The structure of biological membranes is closely dependent on the maintenance of lipid-lipid interactions; membranes are damaged when lipids either move too far apart or too close together. In the latter case lipid-lipid forces become strong enough to cause crystallization of lipids, and in either case the membrane can rupture. Ethanol adversely affects the bonding of and displaces water molecules in this hydrogen-bonded water network and thereby lowers the transition temperature of bilayers, increases membrane fluidity and sometimes causes cell lysis (Breddam and Beenfeldt 1991; Kollar et al., 1993). The presence of ethanol also reduces the dielectric strength of the intracellular environment, and this enhances its water stress effect.

Proteins are stabilized by many hydrophilic and hydrophobic interactions and are therefore vulnerable to hydrogen bond disruption. When this water is displaced or removed, the 'naked charge' of these hydrogen-bonding sites is revealed and protein configuration changes. It is well-established that ethanol has adverse effects on hydrophilic enzymes (Millar et al., 1982), however, this has not been considered in terms of water stress. The change in the invertase enzyme activity (Dzingeleski and Wolfenden, 1993) and inhibition of glycolytic enzymes results from low water availability. There is typically a 50% reduction of enzyme activity at 10 to 15% ethanol (w/v) which is equivalent to 0.953 to 0.925 a_w (Fig. 2.1.). Enzymes are denatured between 10 and 20% ethanol (w/v), equivalent to a_w values as low as 0.895 (Fig. 2.1.). These ethanol concentrations are approximately equivalent to the a_w values at which growth and metabolic activity cease. As mentioned, at lower concentrations (less than 5% ethanol, w/v) specific effects on cell metabolism may be more important than those relating to water stress (Jones and Greenfield, 1986). Ethanol toxicity results from reactions, in which the primary structure of the reactants is altered, which does not seem to be the case. The response of yeast cells to acute
ethanol stress does, to an extent, resemble a water stress response. Ethanol-induced water stress could be perceived as a physical or physicochemical phenomenon. Ethanol is a disruptive compound like urea and guanidine HCl that bind preferentially to hydrate cell components.

High ethanol concentrations may also have secondary physiological effects. For instance, ethanol can inactivate enzymes in vivo by disrupting vacuolar membranes and releasing proteases into the cytoplasm. Ethanol can have adverse effects on the hydrophobic regions of cell components (Carlsen et al., 1991). However, such effects do not account for the metabolic inhibition that ethanol causes (Jones, 1989). By contrast, the interaction of ethanol with the hydrophilic regions of cell components is probably the predominant cause of metabolic and growth inhibition at concentrations of more than 5% ethanol (w/v). The apparent toxicity of ethanol has sometimes been attributed to the effects of its precursor, acetaldehyde (Stanley and Pamment, 1993). Acetaldehyde has a Mr of 44.1, which is similar to that of ethanol, but the former is more polar (acetaldehyde has a dipole moment of 2.69D, in the vapour phase). As a result, acetaldehyde is even more potent than ethanol as an agent of water stress, and this has not been recognized in toxicological studies. Acetaldehyde is known to be more lethal in its effects on yeast metabolism than ethanol, but the aw of acetaldehyde-water mixtures has not been quantified. Intracellular (endogenous) acetaldehyde concentration was estimated to be less than 0.1% (w/v). The high polarity of acetaldehyde results in the formation of a hydration shell in an aqueous environment. The hydrated molecule is therefore too bulky to move efficiently into the aqueous phase of cell components such as membranes. This is consistent with the slower rate at which acetaldehyde diffuses out of the cell. In consequence, acetaldehyde tends to accumulate more than ethanol, compounding any effects of acetaldehyde-induced water stress. However, acetaldehyde is also a reactive compound that can have considerable adverse effects other than those relating to water stress.
2.5.3 Ethanol induced oxidative stress

2.5.3.1 Reactive Oxygen Species

ROS is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and are easily converted into free radicals (HOCl, HOBr, O₃, ONOO-, H₂O₂). In other words, all oxygen radicals are ROS, but not all ROS are oxygen radicals (Sies, 1986; Ghibelli et al., 1995; Imlay, 2003). ROS are highly reactive due to the presence of unpaired valence shell electrons. ‘Reactive’ is not always an appropriate term, because H₂O₂, NO⁻ and O₂⁻ can react quickly with only a few molecules, whereas OH- reacts quickly with almost everything. In contrast, RO₂·, RO·, HOCl, HOBr, NO₂⁻, ONOO⁻ and O₃ have intermediate reactivity (Halliwell and Whiteman, 2004). Additionally, upon exposure to various environmental stresses (e.g. exposure to UV radiation or heat-shock), ROS levels increase dramatically, resulting in cell structure damage. The accumulation of intracellular ROS produces an oxidative stress, which can trigger death (Hockenbery et al., 1993; Kane et al., 1993; Greenlund et al., 1995). Normally cells are able to protect themselves against ROS damage through the use of detoxifying enzymes, such as superoxide dismutases, catalases, glutathione peroxidases and peroxiredoxins, to diminish ROS levels. Singlet oxygen is the most reactive form of ROS (Scandalios, 1987). Superoxide anion (O₂⁻) is a moderate reactive radical formed by an electron reduction of oxygen. It is mainly generated during respiration in mitochondria. Superoxides are also present abundantly in cells but they are not strongly reactive, although they can directly damage some proteins (Gardner and Fridovich 1991). The toxic effects of both superoxide anion and hydroxyl peroxide result from their complete conversion to the extremely reactive OH species. These reactions are catalyzed by metal ions, through the Haber-Weiss reaction, which generates a reduced form of metal ions from Fe(III) or Cu(II) reacting with superoxide; reduced forms of active metals [e.g. Fe(II), Cu(I) and Ti(III)] can participate in these reactions (Kehrer, 2000).

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\begin{align*}
\text{Fe}^{3+} + \text{O}_2 & \rightarrow \text{Fe}^{2+} + \text{O}_3 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\end{align*}
\]

(Fenton reaction)
The hydroxyl radical indiscriminately reacts, in a diffusion-limited manner, with sugars, amino acids, phospholipids, nucleotides and organic acids.

2.5.3.2 Sources of oxidative stress in yeast

Sources of oxidative stress can be related to a decrease in antioxidants or an increase in oxidizing species (Davies et al., 1995). Diminished antioxidant levels can promote oxidative stress. For instance, mutations affecting the activity of enzymes, such as superoxide dismutases or glutathione peroxidase reduce antioxidant defenses. Deficiencies in some minerals (e.g. Zn$^{2+}$, Mg$^{2+}$, Fe$^{2+}$, Cu$^{2+}$) can alter the function of some of these enzymes due to their role as cofactors (Halliwell and Whiteman, 2004). Furthermore, exposure to a number of toxins can decrease antioxidant levels. For example, many xenobiotics are metabolized by conjugation with glutathione (GSH) causing oxidative stress even if the xenobiotic is not itself a generator of ROS. Furthermore, increased oxidants can be caused by the exposure of cells to elevated oxygen levels or toxins that are themselves reactive species (e.g. NO$_2^-$) or are metabolized to generate reactive species.

The types of cell damage resulting from ROS during oxidative stress are:

1. Cell adaptation by up-regulation of defence systems, which may completely protect against damage or under/over-protect.

2. Cellular injury through several molecular targets (lipids, DNA, protein, or carbohydrate). Not all damage caused by oxidative stress is oxidative damage.

3. Cell death as a consequence of oxidative injury. Cells may recover from oxidative damage by repairing or replacing damaged molecules, survive with persistent oxidative damage or they may die or experience overall DNA damage through apoptosis or necrosis.
2.5.3.3 The relationship between ethanol and oxidative stress

Dissolved oxygen and mitochondrial activity are important for the biosynthesis of unsaturated fatty acids and ergosterol (Casey et al., 1984; O'Connor-Cox et al., 1996; Higgins et al., 2003). In brewing, for example, oxygenation of wort at pitching is important for sterol, unsaturated fatty acid and lipid metabolism, and this impact on yeast performance and beer flavour. However, growth under aerobic conditions exposes cells to oxidative stress due to the production of partially reduced forms of molecular oxygen, known as reactive oxygen species (ROS). These highly reactive forms of ‘oxygen’, including the hydroxyl radical (OH'), the superoxide anion (O2·) and peroxide (H2O2), are highly damaging to cellular components causing DNA lesions, lipid peroxidation, oxidation of proteins and perturbations to the cellular redox balance. ROS are formed during respiration, β-oxidation of fatty acids, and a range of other reactions. They are also produced by yeast cells exposed to ethanol or chemical stresses (Costa et al., 1993; Steels et al., 1994; Jamieson, 1998; Georgiou and Masip, 2003). Cellular defenses that can inactivate ROS include the activities of a number of enzymes, such as the cytoplasmic superoxide dismutase (Cu, ZnSOD) encoded by the SOD1 gene, the mitochondrial superoxide dismutase (MnSOD) encoded by the SOD2 gene, cytochrome c peroxidase (CCP) and cytoplasmic catalase T (CTT1). Using respiratory deficient mutants as controls, Costa et al. (1997) showed that ethanol toxicity correlates with the production of ROS in the mitochondria and mitochondrial superoxide dismutase. MnSOD, is essential for ethanol tolerance in diauxic and post-diauxic-phase cells. Consistent with this, yeast lacking Sod1p (SOD1Δ) was found to have lower tolerance not only to oxidative stress but also to heat and ethanol stresses (Pereira et al., 2003). Many anti-oxidant genes are glucose-repressed; both intracellular and extracellular catalase activities in an aerated S. cerevisiae distillery strain are greater with ethanol as substrate than with glucose (Gille et al., 1993). Earlier research shows that extracellular catalase acts as a protectant against the damaging effects of ethanol by oxidizing ethanol outside the cell. Cytochrome P-450, an enzyme catalysing the oxidation of endogenous and exogenous substrates in S. cerevisiae, accumulates to a high level when yeast grows fermentatively on glucose and is also present when ethanol is added to cultures grown
Review of literature on low levels of glucose. Encoded by *ERG11*, this cytochrome oxidatively detoxifies ethanol. Furthermore, studies by Gupta *et al.* (1994) indicate that ethanol might interfere with the antioxidant defence system of yeast cells and, as a result, catalase was unable to counter the toxic effects of ethanol. When *S. cerevisiae* cells treated with ethanol, lipid peroxidation increases. Ethanol-induced lipid peroxidation is associated with a decline in plasma membrane lipid order and interfered with catalase defensive activity, resulting in the deterioration of membrane integrity and loss of membrane impermeability (Gupta *et al.*, 1994).

### 2.5.3.4 Effects of oxidative stress in yeast

Yeast cells can be cultured in aerobic conditions and therefore are continuously exposed to reactive oxygen species generated as by-products of cellular metabolism. The major source of reactive oxygen species is the mitochondrial respiratory chain, which accounts for 85-90% of the oxygen consumed in the cells. These reactive oxygen species, namely superoxide radical, hydroxyl radical and hydrogen peroxide, oxidatively damage lipids, proteins, and nucleic acids (Jamieson, 1998).

Under normal physiological conditions, cellular damages are prevented by antioxidant defences that neutralise the reactive oxygen species (primary defences) and repair molecular damage or degrade oxidised molecules (secondary defences). However, under specific stress conditions, the levels of reactive oxygen species exceed the antioxidant capacity of the cells, and the cells face an oxidative stress. This unbalanced situation can result from: (i) a decrease in antioxidants, due to depletion of such defences (e.g. by xenobiotics that are metabolised by conjugation to glutathione, or to mutations that weaken antioxidant defences); (ii) an increased production of reactive oxygen species (e.g. by exposure to hyperoxia, compounds that generate reactive oxygen species like ethanol or to excessive activation of systems that produce reactive oxygen species), or both (Flattery-O’Brien *et al.*, 1993; Ng *et al.*, 2008). The presence of reactive oxygen species can produce specific cellular damages; however the hydroxyl radicals are the key causative agents in oxidative stress. Any increase in the levels of superoxide radicals, hydrogen peroxide, or redox active metal ions (e.g.
copper and iron), is likely to promote the production of the highly reactive hydroxyl radicals. These reactive oxygen species, together with other molecules, can generate other oxygen radicals, such as alkoxyl or peroxyl radicals in lipids that further enhance the molecular damages. The accumulation of oxidised molecules is ultimately associated with cell death (Costa and Moradas-Ferreira, 2001; Grzelak et al., 2006).

2.5.3.5 Oxidative stress and Protein oxidation in yeast

This response is triggered by mechanisms sensing ROS and changes in the redox state, including the Yap1 transcription factor, that increase oxidative stress resistance (Lee et al., 1999; Thorpe et al., 2004; Mager and Winderickx, 2005; Costa et al., 2007). Proteins are directly oxidised by all reactive oxygen species, and can be oxidatively modified by reaction with lipid peroxidation products (Fig. 2.2). Superoxide radicals specifically oxidise 4Fe±4S clusters in enzymes, such as the mitochondrial aconitase (citric acid cycle), homoaconitase (lysine biosynthesis) and isopropyl malate isomerise (leucine biosynthesis), releasing iron from the cluster and inactivating the enzyme. Elevated levels of "free" iron are detected in yeast cells exposed to superoxide generating drugs, as well as in SOD mutants, deficient in superoxide dismutases (either the cytosolic Cu ZnSOD or the mitochondrial MnSOD, or both) (Srinivasan et al., 2000; De Freitas et al., 2000). The inactivation of aconitase results in an impaired respiratory activity that limits the capacity of SOD mutants to grow on non-fermentable carbon sources, and accelerates ageing of yeast cells. Despite having high levels of iron, SOD mutants are iron-deficient, suggesting that the iron released from the 4Fe±4S clusters accumulate in a form that cannot be used for the biosynthesis of the clusters. This pool of "free" iron has been implicated in oxidative damage to vacuoles, the organelle where iron is stored. As a consequence, the vacuoles are fragmented, and cells become sensitive to pleiotropic stress conditions (pH, nutrient limitation and metals) (Corson et al., 1999; Srinivasan et al., 2000).
Fig. 2.2 Protein oxidation and proteolysis of oxidized proteins. The increased production of reactive oxygen species (ROS) due to stress or associated with ageing and diseases leads to the accumulation of oxidized proteins. Irreversibly damaged proteins can be degraded by the 20S proteasome or by vacuolar proteases. Extensively oxidized proteins cannot be degraded and tend to cross-link forming aggregates that impair the 20S proteasome and mitochondrial function, thereby increasing ROS production (Courtesy of Costa et al. 2007).

Studies with the Hydrogen peroxide suggest that increased oxidative stress is responsible of inactivating a few enzymes by oxidation of labile essential thiol groups of cysteine residues at the active site. The oxidation of thiol groups leads to the generation of mixed disulphides between cysteine residues in the protein and the thiol group of glutathione, cysteine or $\gamma$-glutamyl-cysteine. It has been suggested that protein S-thiolation plays a protective role by preventing the irreversible oxidation of cysteine residues. Proteins containing oxidised cysteine residues are reactivated by dethiolation, probably via reduction by glutathione, glutaredoxins, thioredoxins, or protein disulphide isomerase. Consistent with this hypothesis, all these antioxidant defences are induced by hydrogen peroxide (Lee et al., 1999). The importance of this protective mechanism is illustrated by Tdh3p, a glyceraldehyde-3-phosphate dehydrogenase isoform. Tdh3p is S-thiolated and the activity is fully restored within 2 h after removal of hydrogen peroxide. In contrast, the Tdh2p isoform is not S-thiolated and the activity is only partially restored (45%). The recovery of Tdh3p activity is important for hydrogen peroxide resistance (Grant et al., 1999).
Hydrogen peroxide also oxidises methionine residues to methionine sulfoxide or sulfone. Methionine sulfoxide reductase is able to repair mildly oxidised proteins, by reducing methionine sulfoxide to methionine. In agreement, mutants deficient in this reductase accumulate high levels of methionine sulfoxide derivatives when exposed to hydrogen peroxide (Moskovitz et al., 1997).

The hydroxyl radicals, generated by reduction of hydrogen peroxide catalysed by the metal ions Fe$^{2+}$ or Cu$^+$ (Fenton reaction), account for most of the oxidative damages induced by hydrogen peroxide. The oxidation of amino acid residues to carbonyl derivatives is thought to be a major cause of oxidative stress toxicity. The formation of carbonyls is due to the oxidation of specific amino acids (arginine, proline, lysine and histidine) and to scission of the polypeptide chain (at proline, glutamate or aspartate residues) (Stadtman, 1993; Levine et al., 1994). It is also reported that hydrogen peroxide induced protein carbonylation is specific, and glyceraldehyde-3-phosphate dehydrogenase and mitochondrial enzymes are the major targets inactivated (Cabiscol et al., 2000). The inactivation of glyceraldehyde-3-phosphate dehydrogenase may contribute to cellular protection as it increases the levels of glucose-6-phosphate to be used in the pentose phosphate pathway in order to increase the production of NADPH.

Proteins irreversibly inactivated by formation of methionine sulfone and carbonyl derivatives cannot be repaired. Therefore, these proteins have to be targeted to proteolytic pathways. Indeed, it is known that oxidised proteins exhibit enhanced rates of proteolytic degradation. In S. cerevisiae, several components of the ubiquitin-26S proteasome pathway and vacuolar proteases are induced by hydrogen peroxide (Godon et al., 1998; Lee et al., 1999), which is consistent with the involvement of these proteolytic pathways in the degradation of irreversibly oxidised proteins.

2.5.3.6 Oxidative stress and DNA damage in yeast

The oxidation of nucleic acids leads to bases and sugar damages, single strand breaks, abasic sites and DNA-protein cross-links. Amongst DNA oxidative damages, base modification is an important class of lesions due to its lethal or mutagenic effect.
Indeed, failure to maintain the genomic integrity has been associated with ageing and degenerative diseases. In *S. cerevisiae*, hydrogen peroxide and superoxide generating compounds induce base oxidation, generate strand breaks and increase the frequency of intra chromosomal recombination (Frankenberg *et al*., 1993; Brennan *et al*., 1994; Lee and Park, 1998). Oxidised bases removed and replaced by a system of secondary antioxidant defences. The mitochondrial DNA is particularly susceptible to oxidation, due to the lack of protective histones, and the repair of oxidatively damaged mtDNA is essential for hydrogen peroxide resistance. Yeast mitochondria contain enzymes for the protection of mtDNA. Other factors are also important for the protection of the mtDNA. For example, glutathione has a key role in the maintenance of the genetic integrity of the mitochondrial genome. The disruption of GSH1 gene, encoding the rate-limiting enzyme of glutathione biosynthesis, increases hydrogen peroxide sensitivity but also leads to a high frequency of petite (respiration deficient cell) generation. The function of glutathione in the protection of the mtDNA is independent of its role in oxidative stress resistance, as suppressors of the gsh1 mutation that decrease the rate of generation of petites does not increase hydrogen peroxide resistance of gsh1D cells (Lee *et al*., 2001). Atm1p, an ATP-binding cassette transporter that controls iron homeostasis within the yeast mitochondria, is also important for mtDNA integrity. Loss of Atm1p increases free iron levels and leads to the accumulation of dsDNA breaks and generation of petite mutants (Senbongi *et al*., 1999).

2.5.3.7 Oxidative stress and lipid peroxidation in yeast

Damages to lipids involves the oxidation of polyunsaturated fatty acids by an autocatalytic process (Fig. 2.3), leading to the production of fatty acid hydroperoxides, which undergo fragmentation, generating a variety of highly reactive products, such as epoxides, aldehydes and alkanes. Some of these products are highly reactive and disseminate and increase initial free radical events by damaging DNA and proteins. Yeast cells are unable to synthesise polyunsaturated fatty acids and exogenous fatty acids are preferentially internalised and incorporated into membranes when present in the growth media. Lipid peroxidation depends, therefore, on the amount of polyunsaturated fatty acids present in membranes (Marnet, 2002).
Lipid peroxidation generates a constellation of products among which are reactive electrophiles, such as epoxides and aldehydes (Janero, 1990). Aldehyde induced lipid peroxidation shows inhibition in cell proliferation of yeast (Wonisch et al., 1995). Malondialdehyde (MDA) is a major product of lipid peroxidation; its $pK_a$ is 4.6 and as the neutral species it is highly electrophilic as well as nucleophilic. It not only allows reaction with cellular nucleophiles but leads to self-condensation to form MDA oligomers. Many oligomers of MDA are formed but, to date, only dimers and trimers have been isolated. MDA acts as a mutagen in *S. typhimurium hisD3052* (Riggins and Marnett, 2001). Subsequently, MDA was shown to be mutagenic in eukaryotic cells (Esterbauer et al. 1993). MDA reacts with nucleic acid bases at physiological pH to form adducts to dG, dA, and dC (Stone et al., 1990a; 1990b). The toxicity of lipid peroxidation has been associated to a decrease of coenzyme Q and glutathione levels. Coenzyme Q protects cells by reducing lipid peroxyl radicals and therefore inhibiting the propagation of lipid peroxidation (Do et al., 1996).

### 2.5.4 Ethanol stress and Heat Shock Proteins

Heat shock proteins (HSPs) are a group of proteins whose expression is increased when the cells are exposed to elevated temperatures or other stress. This
increase in expression is transcriptionally regulated. This dramatic upregulation of the heat shock proteins induced mostly by Heat Shock Factor (HSF) is a key part of the heat shock response. The HSPs are named according to their molecular weights. For example, HSP 60, HSP 70 and HSP 90 (the most widely-studied HSPs) refer to families of HSPs on the order of 60, 70 and 90 kilodaltons in size, respectively. The small 8 kilodalton protein ubiquitin, which marks proteins for degradation, also has features of a HSP (Feder and Hofmann, 1999). Exposure of cells or organisms to elevated temperatures triggers the synthesis of heat shock proteins (hsps), which help protect cells against high temperatures and a variety of other potentially toxic agents (Plesset et al., 1983; Welch, 1992; Hendrick and Hartl, 1993). Many of these hsps function as molecular chaperones that prevent the accumulation of unfolded or aggregated polypeptides (Elliott et al., 1996). In growing cells, the HSPs catalyze the proper folding of nascent polypeptide chains, and upon heat shock, these chaperones prevent protein aggregation and promote the refolding of damaged polypeptides (Georgopoulos and Welch 1993). Another important function of certain hsps is to promote the rapid degradation of such abnormal proteins (Kandror et al., 1994; Lee et al., 1996).

The induction of heat shock response can lead to increased tolerance of cells to otherwise lethal, high temperatures. Several of the changes induced in yeast by exposure to stressful ethanol levels are identical to those caused by a heat stress. Ethanol acts in a synergistic way to increase the damage caused by heat. Both heat and ethanol cause membrane disordering and protein denaturation (Piper, 1993). Heat and ethanol also induce HSPs in yeast and other organisms (Plesset et al., 1983). The trigger for the induction of HSPs is probably the cytoplasmic accumulation of aberrant or partially denatured protein (Ananthan et al., 1985). Thus ethanol may be an inducer of HSPs through its destabilization of the hydrophobic interactions of protein structure which leads to the association of Hsp chaperones with these destabilized proteins and the concomitant activation of heat shock genes (Mager and Moradas-Ferreira, 1993).
2.5.4.1 Ethanol and HSP30

Several stresses lead to induction of HSP30, a highly hydrophobic integral membrane protein and the only heat shock protein tightly associated with the yeast plasma membrane (Piper, 1993; Panaretou and Piper, 1992). HSP30 is induced to similar levels by heat shock and by treatment with 6% ethanol, although induction appears to be appreciably less with 8% ethanol. Cells lacking HSP30 give lower final biomass yields in batch fermentations. In addition, they take longer to adapt to enable subsequent growth when exposed to non-growth-inhibitory levels of several energy-demanding stresses including low pH, osmo-stress, the presence of weak organic acids and 10% ethanol.

2.5.4.2 Ethanol and HSP70

Members of the HSP70 family of proteins are involved in a variety of cellular processes, including the targeting of polypeptides to different organelles, such as the nucleus (Shi and Thomas, 1992; Piper et al. 1994; Shulga et al., 1996). In addition, HSP70s are implicated in repair of cell and tissue damage in response to heat shock and other forms of insults. The yeast *S. cerevisiae* contains six cytoplasmic HSP70s, which are members of two families. The first family consists of Ssa1p, Ssa2p, Ssa3p, and Ssa4p; proteins Ssb1p and Ssb2p are members of the second family. Gene products of the SSA family are highly homologous, however, their expression is regulated differently. Proteins Ssa1p and Ssa2p are produced under normal growth conditions; upon heat shock, SSA1 gene expression increases, and the synthesis of Ssa3p and Ssa4p is highly induced. Like heat stress, ethanol concentrations above 4–6% lead to a strong induction of HSP synthesis (Piper, 1995). In response to stress, some cytoplasmic hsp70s accumulate in nuclei. Quan et al. (2004) demonstrated that ethanol, but not other environmental stresses like oxidants or osmotic stress, promotes nuclear import of Ssa4p. This indicates that a specific response is triggered in cells that have been treated with ethanol, distinct from the signalling events activated upon treatment with hydrogen peroxide or salt.
2.5.4.3 Ethanol and HSP104

One of the proteins induced most strongly by ethanol is HSP104, an important determinant of ethanol tolerance (Sanchez et al., 1992). The form of the HSP90 protein which is most strongly heat-inducible (HSP82) was observed to be induced by ethanol to a lesser degree than by heat shock. *S. cerevisiae* has two functionally equivalent HSP90 genes (HSP82 and HSP82) (Borkovich et al., 1989). HSC82 displays a high constitutive expression that increases only slightly after heat shock, whereas HSP82 displays much lower basal expression yet is strongly activated by heat shock. HSP82 therefore contributes most of the Hsp90 present during normal vegetative growth, whereas HSP82 expression is mainly responsible for the increase in HSP90 protein with heat shock (Borkovich et al., 1989; Mager and Ferreira 1993; Piper et al., 1994). Loss of HSP104 has been shown to reduce both ethanol tolerance, tolerance to heat, and heat induced tolerance to ethanol (Parsell & Lindquist, 1993; Piper, 1995).

2.5.4.4 Ethanol and small HSPs

Several small Hsps are induced by ethanol (Gropper and Rensing, 1993) although loss of at least one of these proteins (HSP26) seems to affect ethanol tolerance (Petko and Lindquist, 1986; Susek and Lindquist, 1989). Northern analysis of the mRNA of strain BJ2168 probed for HSP26 gene transcripts indicated that Hsp26 mRNA, undetectable with addition of 2% ethanol, is barely detectable after addition of 4% ethanol. However, it is much more strongly induced with further increases in ethanol concentration eventually (at 10% ethanol) becoming as strongly induced by ethanol as by heat shock. HSP12 also induced by the ethanol (Praekelt and Meacock, 1990). Odumeru et al. (1992) also reported the induction of HSP70, HSP38, HSP26 and HSP23 in two laboratory strains of *S. cerevisiae*. 
2.5.5 Ethanol stress and changes in the gene expression

The multiple effects of ethanol suggest that a large number of genes involved the specific stress response.

![Distribution of ethanol induced genes in the most representative classes. A: Induced genes, B: down-regulated genes (Courtesy of Alexandre et al., 2001)](image)

The distribution of genes, either up or down regulated upon ethanol exposure, in functional classes provides interesting information on the molecular mechanisms that allow the cell to survive ethanol stress (Fig. 2.4.). However, it should be emphasized that a large number of the genes, whose expression altered, encoded proteins of unknown functions. Most of the down-regulated genes are involved in protein biosynthesis (34%), cell growth (4%), RNA metabolism (13%) and cellular biogenesis (3%) (Fig. 2.4). The down-regulation of these genes is thought to reflect growth arrest which occurs during different stress treatments and allows the cell to save energy and to adapt to new conditions (Gasch et al., 2000). Conversely, the genes that are up-regulated by ethanol are mainly involved in energetic metabolism, protein destination, ionic homeostasis, and the stress response (Fig. 4). Gasch et al. (2000) depicted the environmental stress response (ESR) family genes. ESR corresponds to the cluster of all the genes that have similar expression profiles under various stress conditions. Alexander et al. (2001) found that many of the genes whom expression increased after ethanol stress are from ESR family. One of the first studies in this area compared gene expression in a sake yeast and an ethanol-tolerant sake mutant to determine the mechanisms of ethanol tolerance acquired by the mutant
The following genes are found to be highly expressed only in the mutant in the absence of ethanol stress, with their level of expression increasing following exposure to ethanol; CTT1 (encodes cytosolic catalase T; important for resistance to oxidative stress), GPD1 (encodes glycerol-3-phosphate dehydrogenase; adjusts intracellular osmolarity during osmotic stress), SPI1 (encodes a putative cell wall protein; known to be induced during stationary phase), HSP12 (encodes a membrane-associated HSP that protects liposomal membrane integrity against desiccation and ethanol) and HOR7 (a hyper osmolarity responsive gene encoding a small type I membrane protein that localizes at the plasma membrane).

The Ogawa et al. (2000) also found that catalase, glycerol and trehalose accumulated to a greater extent in the mutant compared to the parent, and the mutant exhibited higher resistance to other stressors such as heat, high osmolarity and oxidative stress. Only a few genes in the mutant are reported to have higher expression levels compared to the parent, which may be attributed to a number of factors. Global transcription differences between the parent and mutant strains are determined for cells grown in the absence of stress, suggesting that the reported stress response genes are constitutively expresses in the mutant; ethanol stress conditions are used only to confirm the increased expression of six genes in the mutant when exposed to inhibitory ethanol concentrations. Other studies directly compare the transcriptomes of stressed and non-stressed S. cerevisiae during short-term sub-lethal ethanol exposure (Alexandre et al., 2001; Chandler et al., 2004; Fujita et al., 2004).

As shown in table 2.1, there is considerable overlap in the findings of these gene expression studies, with a large number of genes similarly affected by ethanol exposure. Various studies proves that enhanced gene expression are associated with cell energetics, transport mechanisms, cell surface interactions, lipid metabolism, general stress response, trehalose metabolism, protein destination, ionic homoeostasis and an increase in the expression of many glycolysis and TCA cycle-associated genes, despite the presence of surplus glucose in the medium (Alexandre et al., 2001; Chandler et al., 2004; Fujita et al., 2004).
Table 2.1 Genes reported as more highly expressed in *Saccharomyces cerevisiae* during ethanol stress (Courtesy of Stanley et al., 2010)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP12, 26, 30, 42, 78, 82, 104</td>
<td>Heat shock proteins (HSP)</td>
</tr>
<tr>
<td>CTT1</td>
<td>Cytosolic catalase T, has a role in protection from oxidative damage</td>
</tr>
<tr>
<td>DDR2</td>
<td>Multi-stress response protein</td>
</tr>
<tr>
<td>SSA4</td>
<td>Member of the HSP70 family</td>
</tr>
<tr>
<td>YRO2</td>
<td>Putative protein of unknown function</td>
</tr>
<tr>
<td>TDH1</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>TSL1</td>
<td>Large subunit of trehalose 6-phosphate synthase</td>
</tr>
<tr>
<td>TPS1</td>
<td>Synthase subunit of trehalose-6-phosphate synthase</td>
</tr>
<tr>
<td>ALD4</td>
<td>Mitochondrial aldehyde dehydrogenase</td>
</tr>
<tr>
<td>GLK1</td>
<td>Glucokinase, catalyses the phosphorylation of glucose</td>
</tr>
<tr>
<td>YGP1</td>
<td>Cell wall-related secretory glycoprotein</td>
</tr>
<tr>
<td>HOR7</td>
<td>Protein of unknown function; induced under hyperosmotic stress</td>
</tr>
<tr>
<td>PYC1</td>
<td>Pyruvate carboxylase isoform</td>
</tr>
<tr>
<td>DAK1</td>
<td>Dihydroxyacetone kinase, required for detoxification of dihydroxyacetone (DHA); involved in stress adaptation</td>
</tr>
<tr>
<td>YER053C,</td>
<td>Products have unknown function</td>
</tr>
<tr>
<td>YDR516C</td>
<td></td>
</tr>
<tr>
<td>YBR139W</td>
<td></td>
</tr>
<tr>
<td>HXX1</td>
<td>Hexokinase isoenzyme 1, a cytosolic protein that catalyses phosphorylation of glucose during glucose metabolism</td>
</tr>
<tr>
<td>PGK1</td>
<td>3-phosphoglycerate kinase, enzyme in glycolysis and gluconeogenesis</td>
</tr>
<tr>
<td>SPI1</td>
<td>GPI-anchored cell wall protein involved in weak acid resistance</td>
</tr>
<tr>
<td>CYC7</td>
<td>Cytochrome c isoform 2, expressed under hypoxic conditions</td>
</tr>
</tbody>
</table>

The predominance of hexose transport and glycolysis genes with higher expression levels led to the proposal that the cell enters a pseudo-starvation state during ethanol stress (Chandler et al., 2004). The reason for the pseudo-starvation state during stress is reported due to the loss of intracellular acetaldehyde in ethanol-stressed yeast, leading to cellular redox imbalance and a NAD⁺ shortage; NAD⁺ is a...
cofactor for glycolysis enzyme, glyceraldehyde 3-phosphate dehydrogenase, for which activity is affected by NAD\(^+\) supply (Stanley et al., 1997; Chandler et al., 2004; Valadi et al., 2004). Chandler et al. (2004) found the gene expression profiles of ethanol-stressed cells to be quite different in the later stages of ethanol stress, noting that the cell population was >99% viable after exposure to 5% (v/v) ethanol for 3 h. The total number of highly expressed genes decreases from 100 after 1 h of stress exposure to 14 (YRO2, ALD4, ARG4, CPS1, LAP4, PCL5, CUP1, DLD3, SSU1, FET3, SNZ1, FRT2, YLR089C, YGL117W) after 3 h of stress exposure, 7 of which (YRO2, ALD4, ARG4, LAP4, PCL5, SSU1, YGL117W) are also induced during the early stress response; these latter genes are associated with energy utilization, general stress response and vacuole function. The number of genes with decreased expression rates changed from 274 (1 h of ethanol stress) to 99 (3 h of ethanol stress), most of these being associated with ribosomal function. Tryptophan biosynthesis in particular has been implicated in the ethanol stress response of \textit{S. cerevisiae}. Microarray analysis and two-dimensional clustering was used to identify a cluster of tryptophan-related genes that are induced by ethanol stress (Hirasawa et al., 2007). Strains overexpressing tryptophan biosynthesis genes show improved tolerance to 5% (v/v) ethanol, as did the addition of tryptophan to the culture medium. The role of tryptophan biosynthesis in improving ethanol stress tolerance is unclear although a number of studies have implicated amino acid biosynthesis and transport to ethanol stress tolerance, suggesting that ethanol disruption of membrane function may affect the delivery of amino acids into the cell (Pham and Wright, 2008; Yoshikawa et al., 2009).

Hallsworth (1998) suggested that water stress can be responsible for the alteration in the coiling of the DNA that resulted in the altered gene expression. Water stress related control of gene expression involves a disruption of the hydrogen bonds that stabilize super coiled DNA. This structural transition has a direct effect on replication and transcription that has been best characterized in bacteria (Dorman et al., 1988; Higgins et al., 1988). Hydrogen bonds provide the structural specificity of the DNA double helix and the secondary structure of DNA helices depends on the energetic status of the aqueous environment. Water stress mediated
control operates both at a DNA-structural level and by direct effects on proteins can play a role in gene expression. Glycerol production is invariably increased at high ethanol concentration suggests that the HOG pathway can be induced by ethanol (D'Amore et al., 1988; Julian et al., 1990). Schiiller et al. (1994) showed that 6% ethanol (w/v), as well as high salt concentration induces stress response element sequences that are also involved in heat shock response. Furthermore, Sugiura et al. (1994) found that expression of the gene for a putative osmosensor can be controlled by both salt and ethanol stress. In cells of Saccharomyces, mitochondrial DNA is also particularly sensitive to the effects of ethanol (Ibeas and Jimenez, 1997).

2.5.6 Ethanol stress and cell death in yeast

Cell death can be divided into two main categories, necrosis and apoptosis (Longo et al., 2005). Whereas necrosis is a catastrophic "death by assault", apoptosis can be thought of as an altruistic death, in which a cell positively executes death. Multicellular organisms utilize apoptosis for a variety of biological programs, including in development and disease prevention, and defects in apoptotic programs in multicellular organisms can lead to diseases like cancer. Although the yeast S. cerevisiae is an unicellular organism, there are compelling evidences that yeast can undergo altruistic programmed cell death similar to apoptosis (Madeo et al., 2004; Buttner et al., 2006; Gourlay et al., 2006; Leadsham and Gourlay, 2008; Eisenberg et al., 2010; Carmona-Gutierrez et al., 2010). The stimuli reported to induce this type of cell death in yeast include hydrogen peroxide, acetic acid (Madeo et al., 1999, Ludovico et al., 2002), high osmotic shock (Silva et al., 2005), viruses (Ivanovska et al., 2005), pheromone (Severin and Hyman, 2002; Zhang et al., 2006) and defects in N-glycosylation (Hauptmann et al., 2006). In yeast, ethanol has an effect on many different cellular behaviours and processes related to cell death, including the stress responses (van Voorst et al. 2006; Teixeira et al. 2009), changes in membrane fluidity (You et al., 2003), protein structure (Sanchez et al., 1993) and mRNA export from the nucleus (Takemura et al., 2004).
2.5.6.1 Effects of ethanol on the DNA of yeast

Altered morphology of the chromatin is always associated with the apoptosis after treatment with ethanol. Ethanol-treated cells have chromatin condensation and fragmentation. Apoptotic stimuli have also been reported to cause DNA cleavage in the nucleus. TUNEL staining in ethanol treated cells proves the DNA cleavage (Kitagaki et al., 2007). Few ethanol treated cells also positive for PI staining, which stands for necrotic cells, indicating that apoptosis rather than necrosis is the major event in this condition. Moreover, DNA breakdown was observed in ethanol-treated cells as visualized by PFGE, similar to hydrogen peroxide, acetic acid and glucose-induced apoptosis (Kitagaki et al., 2007, Ribeiro et al., 2006). Increase viability in the ethanol with cyclohexamide treated yeast cells suggests the role of apoptosis in yeast.

2.5.6.2 Role of mitochondria and ROS in ethanol induce apoptosis in yeast

Mitochondrial pathway plays the central pathway of apoptosis in mammalian cells (Skulachev, 2000; 2002; Longo et al., 2005) and in pheromone (Pozniakovsky et al., 2005; Zhang et al., 2006), virus (Reiter et al., 2005) and acetic acid (Ludovico et al., 2002) induced apoptosis in yeast. Kitagaki et al. (2007) reported the role of mitochondria in ethanol induced apoptosis in yeast. Mitochondrial fragmentation is an early maker of apoptosis in mammalian cells (Cereghetti and Scorrano, 2006). The studies of mitochondria visualization by using a mitochondria-localized GFP marker (mito-GFP) (Westermann and Neupert, 2000; Kondo-Okamoto et al., 2006; Kitagaki et al., 2007) clearly indicate that ethanol is a potent inducer of mitochondrial fragmentation.

The increased level of ROS have been reported to be involved in many forms of apoptosis in yeast (Ludovico et al., 2002; Gourlay and Ayscough, 2005; Pozniakovsky et al., 2005; Silva et al., 2005). Study reported from microarray analysis suggests that the genes involves in antioxidant defense are upregulated in response to ethanol (Alexandre 2001). Kitagaki et al. (2007) reported the elevated level of ROS in ethanol induced apoptotic cells.
2.5.7 Ethanol stress and morphology of yeast cells

The atomic force microscopic study showed that the ethanol affects morphology and physiology of the yeasts. The dramatic changes in cell surface morphology observed for *Sc. pombe* indicate a higher loss of cell membrane integrity due to ethanol-imposed stress. (Canetta *et al.*, 2006) In fact, cell membrane integrity is needed for ethanol tolerance in yeasts (Takahashi *et al.*, 2001). Canetta *et al.* (2006) reported that the stronger the stress, the higher the alterations in cell membrane integrity and fluidity will occur. Birch and Walker (2000) also reported the altered cell volume after ethanol stress in yeast.

2.6 Protective mechanism of yeast cell from ethanol and other stress

The yeast stress response is a transient reprogramming of cellular activities to ensure survival in challenging conditions, protect essential cell components and enable resumption of ‘normal’ cellular activities during recovery. The response of yeast to environmental stress is complex, involving various aspects of cell sensing, signal transduction, transcriptional and posttranscriptional control, protein-targeting, accumulation of protectants, and increased activity of repair functions (Mager and Ferreira, 1993). The efficiency of these processes in yeast strain determines its robustness and, to a large extent, ability of yeast to perform well in industrial processes. A better understanding of the cellular consequences of microbial ethanol stress and of the underlying ethanol stress defence mechanisms is crucial for improving the performance of yeast strains during stress (Stanley *et al.*, 2010). We can divide the protective mechanisms of yeast against ethanol stress in two main categories non enzymatic and enzymatic defence.

2.6.1 Non enzymatic defences

There are non enzymatic defences against ethanol and oxidative stresses in the form of several metabolites that are important in maintain the normal homeostasis of yeast cells. The most common of these metabolites include and trehalose, glycogen, glycerol, mannitol etc. (Missall *et al.*, 2004).
2.6.1.1 Trehalose

Trehalose is a disaccharide that is ubiquitous in the biosphere. It consists of two subunits of glucose bound by an α:1→1 linkage (α-D-glucopyranosyl α-D-glucopyranoside) and is thus non-reducing. Trehalose has been isolated and characterized from a large variety of both prokaryotic and eukaryotic organisms, ranging from bacteria to plants and mammals (Thevelein, 1984; 1996, Strom and Kaasen, 1993). The original term “trehalose” was introduced to describe the external cuticula “trehalamana” of an insect parasite of plants (Elbein 1974). The success of trehalose in nature compared to other sugars can be explained by its peculiar structure. In addition to being nonreducing, it possesses several unique physical properties, which include high hydrophilicity and chemical stability, nonhygroscopic glass formation and the absence of internal hydrogen bond formation. These features account for the principal role of trehalose as a stress metabolite. A variety of functions have been proposed for trehalose, which depend on the specific biological system analyzed. Hence, in prokaryotic organisms, the carbohydrate can be used as external carbon source, stored as compatible solute by photosynthetic bacteria, or serve as a structural component of the cord factor in mycobacteria (Galinski and Herzog, 1990; Spargo et al., 1991; Strom and Kaasen 1993). In yeast and filamentous fungi, large amounts of trehalose are stored both as a reserve carbohydrate and as protector against stress challenges to cells. Several species of insects contain trehalose in the “fat body” and the hemolymph, which is quickly mobilized during flight. The enzymes involved in trehalose metabolism are even present in higher animals, although their precise role is not well understood. In humans, trehalase has been located both in the brush border membranes of epithelial cells of the small intestine and in the kidney proximal tubule. It may be involved in sugar transport across the membrane and in the hydrolysis of ingested trehalose. The physiological role of kidney trehalase remains a mystery, because trehalose is not found in blood (Ishihara et al., 1997). In addition, genes coding for trehalose metabolism in higher plants have recently been cloned and characterized (Müller et al., 1999; Avonce et al., 2005; Gupta and Kaur, 2005). Finally, trehalose increases the stability of several unstable products, including enzymes, foods, pharmaceuticals and cosmetics, during dry
storage or freezing and become a widely valued preservative (Colaco et al., 1992; Argüelles, 2000).

2.6.1.1.1 Chemical synthesis of trehalose

\( \alpha, \alpha \)-Trehalose has been synthesized chemically using the ethylene oxide addition reaction between 2,3,4,5-tetra-O-acetyl-D-glucose and 3,4,6-tri-O-acetyl-1,2-anhydro-D-glucose. This same series of reactions also gives rise to one of the other trehalose anomers, specifically, \( \alpha,\beta \)-trehalose, also referred to as neotrehalose. Neotrehalose has also been synthesized using the Koenigs-Knorr reaction. This anomer has not been isolated from any living organisms. The other anomer of trehalose, \( \beta, \beta \)-trehalose, or isotrehalose, also has not been isolated from any living organisms, but it was found in starch hydrolysates (Sato and Aso, 1957); it also has been synthesized chemically using the Koenigs Knorr reaction, as well as by a dehydration reaction. Trehalose also can be produced chemically by an acid reversion of glucose (Thompson et al., 1954). On the other hand, \( \alpha, \alpha \)-trehalose is the only anomer of trehalose that has been shown to be biosynthesized in many different types of organisms (Elbein et al., 2003).

2.6.1.1.2 Biological synthesis of trehalose by trehalose synthase complex

The most widely reported and best studied pathway for the biosynthesis of \( \alpha,\alpha 1,1 \)-trehalose is that involving the enzyme trehalose-phosphate synthase (TPS) (Reinders et al., 1997; Bell et al., 1998) that catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate to produce trehalose 6-P plus UDP (see Fig. 2.5, Table. 2.2). As indicated, the TPSs purified from yeast, insects, or streptomycetes are found to be specific for either UDP glucose or GDP-glucose as the glucosyl donor, but none of these enzymes can use both donors. TPS is present as part of a complex that is made up of four subunits. One of these subunits is the synthase (TPS1), another is a specific trehalose-P phosphatase (TPS2), and the other two subunits are thought to be regulatory proteins (TPS3 and TSL1) (Bell et al., 1998).
Yeast use this enzyme complex to synthesize trehalose, whereas other organisms apparently do not, is not understood, but it may have to do with a regulatory function on trehalose metabolism, and the interaction between trehalose metabolism and glycolysis and fermentation (Noubhani et al., 2000). TPS, in addition to synthesizing trehalose-P, may have a regulatory function that restricts glucose influx by its interaction with the glucose transport and sugar kinase activities (Thevelein, 1992).

Trehalose metabolism may prevent the overflow of glycolysis by utilizing or diverting sugar-Ps into trehalose synthesis, and this pathway then produces inorganic phosphate which is required by glyceraldehyde-3-P dehydrogenase for activity (Hohmann et al., 1993). Trehalose-P may restrict sugar influx into glycolysis by inhibiting hexokinase activity (Blazquez et al., 1993). This study demonstrates that
trehalose-6-phosphate competitively inhibits the hexokinases of *S. cerevisiae*. There is also some evidence for a second pool of TPS in yeast that is present as the free enzyme and is not associated with the complex (Bell et al., 1998).

**Table 2.2 Enzymes of trehalose metabolism in *Saccharomyces cerevisiae***

<table>
<thead>
<tr>
<th>Trehalose synthesis Protein</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tps1p</td>
<td>Catalyses the synthesis of trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate</td>
</tr>
<tr>
<td></td>
<td>Tps1p is expressed constitutively and is further induced by heat</td>
</tr>
<tr>
<td></td>
<td>tps1 mutants cannot grow on glucose-containing media and have impaired thermotolerance</td>
</tr>
<tr>
<td>Tps2p</td>
<td>Removes phosphate from trehalose-6-phosphate to yield trehalose</td>
</tr>
<tr>
<td></td>
<td>Tps2p is expressed constitutively and is further induced by heat</td>
</tr>
<tr>
<td></td>
<td>tps2 mutants have decreased survival of extreme heat</td>
</tr>
<tr>
<td>Tsl1p</td>
<td>Regulator of trehalose synthesis</td>
</tr>
<tr>
<td></td>
<td>Expression of Tsl1p increases during stationary phase</td>
</tr>
<tr>
<td>Tps3p</td>
<td>Regulator of trehalose synthesis</td>
</tr>
<tr>
<td></td>
<td>Expression of Tps3p is constant during exponential and stationary phase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trehalose degradation Protein</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nth1p</td>
<td>Primary enzyme for trehalose degradation</td>
</tr>
<tr>
<td></td>
<td>Located in cytoplasm</td>
</tr>
<tr>
<td></td>
<td>Nth1p is expressed constitutively and is further induced by heat</td>
</tr>
<tr>
<td></td>
<td>nth1 mutants have diminished thermotolerance and diminished recovery from heat shock</td>
</tr>
<tr>
<td>Nth2p</td>
<td>Highly homologous to Nth2p</td>
</tr>
<tr>
<td></td>
<td>Located to cytoplasm</td>
</tr>
<tr>
<td></td>
<td>In vivo role in trehalose degradation is unclear</td>
</tr>
<tr>
<td></td>
<td>Nth2 is expressed constitutively and is further induced by heat</td>
</tr>
<tr>
<td></td>
<td>nth2 mutants are impaired in thermotolerance and in recovery from heat shock</td>
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<tr>
<td>Ath1p</td>
<td>Located in vacuole</td>
</tr>
<tr>
<td></td>
<td>nth1 mutants are unable to utilize trehalose as a carbon source; mutants are reported to show enhanced survival of dehydration, freezing and ethanol stress</td>
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This TPS does not appear to be free as a result of insufficient amounts of the other proteins that make up the complex; in fact, it may have another and distinct role in trehalose metabolism and in glycolysis. Some recent studies indicate that TPS1-mediated protein-protein interactions are involved in control of glucose influx into yeast glycolysis, that trehalose-P inhibition of hexokinase might not be competitive with respect to glucose in vivo, and that TPS2 also may play a role in the control of hexokinase activity.
2.6.1.1.3 Hydrolysis of trehalose

Most yeast and fungi species exhibit two types of trehalose hydrolyzing activities, one termed acid trehalase which is optimally active at pH 4.5-5.0, and the other one, designated neutral trehalase with an optimum of activity at pH 6.8-7.0 (Thevelein, 1984; Londesborough and Varimo, 1984; App and Holzer, 1989; Yarar et al., 2000; Vicente-Soler et al., 2009). It is also considered that the acidic trehalase is vacuolar, while the neutral enzyme is Cytosolic (Keller et al., 1982; Thevelein, 1984; Mittenbuhler and Holzer, 1988; Harris and Cotter 1988). NTH1 is cloned by complementation of a neutral trehalase-deficient yeast mutant that is isolated by a sophisticated enzymatic overlay assay (Kopp et al., 1993). The gene is localized on chromosome IV and encodes a 80-kDa protein which is 77% identical with the product of YBR0106 (NTH2), another gene identified by systematic sequencing of the yeast chromosome II (Wolfe and Lohan, 1994). Amino acid sequence comparison with the trehalases from S. cerevisiae, Kluyveromyces lactis, Schizosaccharomyces pombe, insect, rabbit small intestine and Escherichia coli reveals that regions of homology (about 25-28% identity) are restricted to two domains corresponding to the central (likely catalytic) core and C-terminal parts, and that only neutral trehalases from yeasts exhibit a N-terminal extension that contains the phosphorylation regulatory domain. Deletion of NTH1 leads to inability of yeast cells to mobilize endogenous trehalose and to an apparent complete loss of measurable trehalase activity. In contrast, no change in trehalose and trehalase activity was observed in a nth2 mutant. The only phenotypic feature found for nth2 mutant is its lower thermotolerance also found for nth1 mutants (Nwaka et al., 1995). The purified neutral trehalase is a homodimer of two 80-86-kDa subunits which displays a Km for trehalose between 5-35 mM (Londesborough, 1984; Dellamora-Ortiz, 1986; App and Holzer, 1989). In order to clone ATH1 which encodes the acidic trehalase, it was made use of the property that vacuolar proteins, when they are overproduced, mislocalize at the cell surface (Rothman, 1994). Therefore, colonies that expressed higher acidic trehalase activity after transformation with a multicopy yeast DNA library can be screened on plates for their capacity to develop a dark green colour after permeabilization and incubation with glucose oxidase reagents (Destruelle,
1995). A surprising phenotype associated with the loss of acid trehalase function is the apparent inability of athl mutants to growth on trehalose, since the vacuolar localization of Athlp is in contrast with this property (Nwaka et al., 1996). In addition, the transport of Athlp to vacuoles via the secretory pathway as proposed from previous works (Mittenbuhler and Holzer, 1988; Harris and Cotter, 1988) is doubtful since the amino acid sequence of this protein does not present any signal sequence and consensus cleavage sites characteristic of proteins following this pathway (Nwaka and Holzer, 1998). It is therefore suggested that the acid trehalase can be secreted to the periplasm similarly to the externalization of some permeases and H⁺-ATPase (Tschopp et al., 1984; Holcomb et al., 1988). The acid trehalase and extracellular trehalose will then be internalized from the surface and delivered to the vacuoles by endocytosis. The fact that mutants in the endocytosis pathway (end1 mutants) cannot grow on trehalose like athl mutant is consistent with this scenario. The ability of acid trehalase to sustain growth on low trehalose concentration also fits with its high affinity for this substrate (Londesborough, 1984; Mittenbuhler and Holzer, 1988).

2.6.1.1.4 Trehalose transport

Growth of certain yeast species is possible on trehalose. In S. cerevisiae it is preceded by a long lag phase due to the 'induction' of the trehalose uptake. Prior incubation of yeast cells with K-methylglucoside or maltose favours the subsequent uptake and growth on trehalose whereas glucose galactose or ethanol is strongly inhibitory (Kotyk and Michaljanicova, 1979). Kinetic studies showed the existence of at least two different trehalose transporter activities: a high affinity H⁺-trehalose symporter (K_m = 2-4 mM) and a low affinity no concentrative trehalose transporter (K_m >50 mM). The high affinity transporter is repressed in glucose and highly expressed in maltose-containing medium, whereas the low affinity transporter appears insensitive to the carbon source (Stambuk et al., 1996; 1998). Michels' group isolated AGT1 as encoding a permease that restores growth on maltose of a mall yeast strain (Han et al. 1995). The protein sequence is 57% identical to the maltose permease encoded by MAL61, and exhibits structural homology with members of the 12 transmembrane domain superfamily of sugar transporters (Boles and Hollenberg,
Analysis of the AGT1 promoter revealed the presence of a 489-bp region identical to that of the maltose-inducible MAL61 gene, and the presence of the maltose specific enhancer (UASma), which could explain the tight control of AGT1 expression by maltose (Hong and Marmur, 1987). Unlike Mal61p which is specific for maltose uptake, Agt1p can also actively transport other K-glucosides including trehalose, turanose, isomaltose, K-methylglucoside and maltotriose (Han et al., 1995). The proposed function of Agt1p to actively transport trehalose from the medium (Stambuk et al., 1998) is genetically conformed by showing that a maltose positive yeast strain defective in trehalose synthase subunit (tps1 mutant) no longer accumulates trehalose upon deletion of AGT1 in the presence of a low concentration of the disaccharide (<4 mg ml⁻¹) in the medium (Plourde-Owobi et al., 1999).

2.6.1.1.5 Trehalose as a protein stabilizer

Trehalose is such an important bioprotectant is due to the existence of a number of polymorphs, both in the crystalline as well as amorphous states. At least two fully crystalline forms and several more transitory intermediates between crystalline and amorphous forms are known (Sussich et al., 2001; Nagase et al., 2002). The most commonly immobile and hence stable when exposed to stress conditions. Trehalose can stabilize proteins as well as lipid bilayer. The stabilization induced by trehalose is nonspecific and is not localized to any particular secondary structure motif. This also agrees well with the change in transfer of free energy on unfolding and the stabilization inferred from thermal transitions.

Trehalose favours the collapse of the unfolded state coupled with an increase in the rate of folding and a decrease in the rate of unfolding of the protein (Chen et al., 2005). In case of unfolding of cutinase by heat, trehalose increases the melting temperature of the enzyme by delaying thermal unfolding of an intermediate (Baptista et al., 2008). Trehalose compensates for the altered entropy of the process by increasing the contribution of enthalpy to the process, making unfolding a thermodynamically unfavourable process. On exposure to heat, yeast produces trehalose, along with heat shock proteins, on a large scale (Ellis, 1988). It is also reported in yeast that trehalose stabilizes partially folded proteins during heat shock.
and is rapidly hydrolyzed on activation of the partially folded form by molecular chaperones (Singer and Lindquist, 1998). The studies of Kaushik and Bhat (2003) demonstrated that trehalose-induced thermostabilization of the protein structure is also helpful in the retention of biological activity of proteins at high temperatures. Trehalose offers protection to yeast glucose-6-phosphate dehydrogenase and phosphoglucoisomerase against thermo inactivation (Hottiger et al., 1994). Induction of thermotolerance by trehalose is also inferred from the fact that the level of trehalose is correlated with thermotolerance (Hottiger et al., 1994). Trehalose has also been found to inhibit aggregation of denatured proteins after heat shock. In some cases, trehalose is thought to influence chaperone activity of heat shock proteins.

Fig. 2.6 Role of trehalose as a protein stabilizer during heat stress in yeast
There is evidence that heat shock leads to increased synthesis of components of the trehalose-6-phosphate synthase complex in *S. cerevisiae* (De Virgilio *et al.*, 1993). Yeast mutants defective in genes for trehalose anabolism display much lower survival rates than the native species (Sun and Leopold, 1997). Trehalose also tries to minimise the inactivation of enzymes caused by the heat stress. Trehalose has been found to protect yeast pyrophosphatase against exposure at 50°C much better than other sugars like sucrose, fructose, or glucose (Soía-Penna and Meyer-Femandes, 1998). The hydrated volume not the molar concentration of trehalose determines the amount of stabilization of proteins. Fluorescence emission studies have confirmed that trehalose prevent unfolding of the enzyme when used at the same volumetric concentration.

It is possible that trehalose and other sugars solubilise in the bulk water and are excluded from the solvation layer of the protein (preferential exclusion theory). This leads to a decrease in the solvation layer around the protein, restricting its mobility, and stabilizing it against stress conditions. As the hydrated radius of a trehalose molecule is almost 2.5 times higher than that of sucrose or maltose, the size-exclusion effects become more pronounced and the stabilization effect is high (Soía-Penna and Meyer-Femandes, 1998). The presence of trehalose also tries to overcome damage caused by the H$_2$O$_2$ stress, presumably by acting as a free radical scavenger. Trehalose-deficient mutants of *Candida albicans* are extremely sensitive to exposure to H$_2$O$_2$ while the same condition induces trehalose accumulation in wild-type cells, with improved life spans (Gonzalez-Parraga *et al.*, 2003). In mammalian cells, O$_2$ deprivation or hypoxia for 5–10 min results in irreversible tissue damage (Haddad and Ma, 2001). Drosophila however can survive exposure to complete N$_2$ atmosphere for up to 4 h without any damage. In this case, over expression of TPS1 gene leads to accumulation of trehalose with concomitant increase in tolerance to O$_2$ deprivation (Chen and Haddad, 2004). Trehalose is also believed to reduce anoxia-induced protein aggregation in vitro (Chen and Haddad, 2004). Transfection of Drosophila TPS1 gene into HEK-293 cells has given rise to populations that show decreased trehalose metabolism when grown in glucose-deficient media. These transfected cells shows increased trehalose accumulation when exposed to hypoxic
stress and shows much higher survival rates as compared to control cells. The amount of aggregated as well as ubiquitinated proteins in the transfected cells is much less when exposed to O₂-depleted atmosphere (Chen and Haddad, 2004). Thus stabilization of proteins by trehalose is probably responsible for survival of lower organisms when exposed to hypoxic stress. Trehalose also has shown the reduction in the formation of protein aggregation (Tanaka et al., 2004). Trehalose also shown positive results in the therapeutics in the huntingtin and other neuronal diseases (Tanaka et al., 2004). Davies et al. (2008) shown the therapeutic role of trehalose in another codon reiteration disease is oculopharyngeal muscular dystrophy (OPMD). Trehalose can also be useful in the alzheimer and osteoporosis diseases (Liu et al., 2005).

2.6.1.1.6 Trehalose in oxidative stress

Trehalose has already proved its useful as a protectant against oxidative stress. Trehalose accumulation can be induced in S. cerevisiae following exposure to gentle stress conditions such as mild heat shock (Benaroudj et al., 2001). These cells show increased tolerance when exposed to a free radical generating system. Mutants unable to synthesize trehalose show exceedingly low viability when exposed to oxidative stress. This effect can be reversed if trehalose is made available to the mutants. The reaction of oxygen radicals with proteins gives rise to free carbonyl groups in the oxidative stress conditions and it seems that trehalose protect damaging proteins by working as a free radical scavenger or by protecting protein denaturation (Benaroudj et al., 2001; Alvarez-Peral et al., 2002; Zaragoza et al., 2003; Herdeiro et al., 2006; Li et al., 2010)

2.6.1.1.7 Trehalose in thermal and ethanol stress

Trehalose has been shown to be crucial for yeast survival during heat stress. During this condition, dramatic changes in cell viability are accompanied by aggregation of macromolecules and denaturarion of proteins. Trehalose can reduce protein aggregation and maintains polypeptide chains in partially folded state, thus increasing tolerance against thermal stress (Singer and Lindquist, 1998). Level of
trehalose increase up to 15 times when yeast cells are submitted to heat shock (De Virgilio et al., 1991). Trehalose plays an important role on the stress-survival of yeasts when submitted to a heat shock, 1.5 M trehalose increases 13-fold the half-time for thermal inactivation of yeast cytosolic pyrophosphatase at 50°C. This thermal protection conferred by trehalose is dose-dependent, after 10 min at 50°C, a condition which inactivated pyrophosphatase, the presence of 2 M trehalose preserves 95% of total activity (Panek and Panek, 1990). In addition, during heat stress the production of ROS also increases and cause oxidation injuries. ROS not only damage protein by modifications of certain residues but also damage membranes and in some circumstances, to a greater extent than they do to protein. Luo et al. (2008) demonstrated, in vitro, that trehalose can scavenge ROS directly. Herdeiro et al. (2006) also demonstrated that trehalose can improve the resistance of yeast cells to menadion stress by reducing intracellular ROS concentration and decreasing in vivo lipid oxidation. So in this way the mechanism of the protection of trehalose under heat stress is also related to its antioxidant role. It is proved by various studies that ethanol; heat stress and osmotic cause similar kind of effects on the yeast as it is observed that ethanol stressed yeast cell responses are similar to the heat stressed cells (Piper, 1995; Sharma, 1997). Mansure et al. (1994) noted a positive correlation between trehalose levels of yeast strains and their ability to withstand 10% ethanol. They also obtained evidence that trehalose reverses electrolyte leakage from ethanol-treated yeast cells and carboxy fluorescein leakage from liposomes. The strong trehalose induction in cells subjected to heat shock or ethanol (Plesset et al., 1983) may therefore partially counteract the deleterious effects of the increased membrane permeabilization associated with these stresses.

2.6.1.8 Trehalose in drought stress

Trehalose has been classified as a water-structure maker that is the interaction between trehalose/water is much stronger than water/water interaction and may be involved in its bio protective action (Branca et al., 2005). Raman scattering experiments reveal the disruption of the tetrahedral network of water molecules on addition of trehalose (Lerbret et al., 2005). This destrucuring of the water network by trehalose and ordering the water molecules around itself (as a kosmotrope) does not
allow the formation of ice and makes trehalose one of the best cryoprotectants known (Wiemken, 1990; Eroglu et al., 2009; Uyar et al., 2010).

2.6.1.1.9 Trehalose as a water stress/osmotic stress protectant

Trehalose performs a protective role in circumstances that involve absolute water stress such as freezing and dehydration (Attfield, 1987; Eleutherio et al., 1993; Attfield et al., 1994; Leslie et al., 1994). Trehalose also protects against the water stress caused by high ethanol concentrations (Odumeru et al., 1993; Mansure et al., 1994). Yeast cells are more sensitive to ethanol when it is added exogenously than under steady-state conditions of ethanol stress (Attfield, 1987; Odumeru et al., 1992; Mansure et al., 1994). When ethanol is produced endogenously, there is a gradual increase in trehalose and glycerol production (D’Amore et al., 1988; Julian et al., 1990; Schulze et al., 1996) that maintains the function and integrity of enzymes and lipid bilayers. Both the synthesis of compatible solutes and changes in membrane composition must take place before hydrated cell components are protected from ethanol. When ethanol is suddenly added to a culture, the damage to the cell (due to a shock water stress) can be irreversible, if lysis occurs for example., On the other hand when cells that are subjected to sudden ethanol stress contained high concentrations of trehalose, therefore cell metabolism and integrity were not severely affected (Millar et al., 1982). Trehalose is also an important compound involved in the survival of *S. cerevisiae* when exposed to severe osmotic stress conditions. The trehalose also showed to increase the viability of yeast cells after osmotic stress (Hounsa et al., 1998).

2.6.1.2 Glycogen

Glycogen, a polymer of glucose, is a major storage form of carbohydrate in many cell types (Mu et al., 1996). Glycogen plays a major role in providing energy to the yeast for its metabolic activities and in times of starvation.

2.6.1.2.1 Glycogen synthesis in yeast

Glycogen synthesis starts with an initiator protein encoded by *GLG1* and *GLG2* (Cheng et al., 1995), which catalyses the formation of an α (1, 4) glucosyl
primer from UDP-glucose. Elongation of this primer proceeds with glycogen synthase, which exists in two isoforms, encoded by GSY1 and GSY2 (Farkas et al., 1991), and a branching enzyme encoded by GLC3 (Thon et al., 1992; Rowen et al., 1992), which introduces the α (1, 6) branches on the mature glycogen. Gac1p has been identified as a component of the protein-phosphatase complex which specifically activates glycogen synthase by dephosphorylation (François et al., 1992). The breakdown of glycogen into Glc1P and free glucose occurs by the combined action of a glycogen phosphorylase encoded by GPH1 (Hwang et al., 1989), and a debranching enzyme carrying transferase/glucosidase activities (Tabata and Hizukuri, 1992).

2.6.1.2.2 Glycogen and stress tolerance in yeast

Heat and osmotic stress cause a sizeable rise in glycogen content (Lewis et al. 1995). In response to various stresses glycogen and trehalose genes displayed the same induction pattern. The induction factor ranged from five to seven fold for the temperature shift to 37 °C, to two to three fold for the osmotic shock with 0.3 M NaCl. In contrast, treatment with 0.4 M sorbitol, and oxidative stresses carried out with 0.4 mM H₂O₂ or 5 mM benzoate, caused a weak but definite induction of genes (Parrou et al., 1997). Parrou et al. (1997) showed that all the genes encoding enzymes of glycogen and trehalose metabolism are induce in a similar fashion and to the same extent in response to stress (Hottiger et al., 1987).

2.6.1.3 Glycerol

In wine fermentation, next to ethanol and carbon dioxide, glycerol is quantitatively the most important fermentation product. Because of to its non volatile nature, glycerol does not contribute to the aroma of wine, but it promotes the smoothness of wine (Berovic et al., 2007).

2.6.1.3.1 Glycerol synthesis in yeast

The biosynthesis of glycerol in a cell is closely associated with osmotic regulation. The anaerobic conversion of glucose into ethanol by S. cerevisiae is redox neutral, that is, NAD⁺, which is consumed initially in the Embden-Meyerhof-Parnas
(EMP) pathway, is regenerated when ethanol is produced. However, when intermediates in the EMP pathway are withdrawn as precursors for the synthesis of the cellular material, this balance is disturbed, because the surplus of NADH produced is not converted back into NAD⁺. This eventually causes metabolism to stop, unless other processes are employed for the regeneration of NAD⁺. During the anaerobic growth of *S. cerevisiae*, NADH cannot be oxidized by oxygen, but must be disposed by the formation of reduced by-products such as glycerol. The accumulation of glycerol is caused by the need to maintain a favourable redox balance (Van Dijken and Alexander Scheffers, 1986) by converting the excess NADH, which is generated during the biomass formation, to NAD⁺. Glycerol formation requires the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (G-3-P) by dihydroxyacetone phosphate reductase, converting one molecule of NADH, which is generated in the oxidation of glyceraldehyde 3-phosphate, to NAD⁺. The reaction is catalyzed by G-3-P dehydrogenase (GPDH) and followed by the dephosphorylation of G-3-P to glycerol by glycerol-3-phosphatase. G-3-P is then dephosphorylated by glycerol phosphate phosphatase yielding glycerol. Glycerol formation may serve as the route for the generation of NAD⁺ from NADH. However, the coupling of these two reactions does not generate net ATP (Albers et al., 1996), if all triose units are shunted to glycerol. It is therefore energetically unfeasible for the overproduction of glycerol to be an end product of glycolysis.

2.6.1.3.2 Glycerol in stress tolerance in yeast

Glycerol formation in yeast is well-known as a response to aeration conditions, due to its role as a redox sink, and to the medium osmotic pressure (Wang et al., 2001; Hohmann, 2002). Increasing glycerol production was also reported after submitting yeast cells to heat shock due to overexpression of the glyceraldehyde-3-phosphate dehydrogenase (Omori et al., 1996, Kajiwara et al., 2000). Compatible solutes are general protective agents against different forms of water stress. The studies of Hallswort et al. (2003) suggest that osmoprotectants glycerol protect against ethanol-induced water stress in fungi *A. nidulans*. Glycerol provides the stability to protein by reducing surface tension of water. Jarabak et al. (1966) and Ruwart and Suelter (1971) explained the stabilization of enzymes by glycerol in terms
of the formation of water-glycerol structure around the protein molecules. Gerlsma (1968, 1970) and Gerlsma and Sturr (1972, 1974) have proposed that the stabilizing effect of polyhydric alcohols is the result of a decrease in the hydrogen-bondrupturing capacity of the medium. It is also reported that glycerol provide thermodynamically stability to the proteins during water stress conditions (Gekko and Timasheff, 1981; Tiwari and Bhat, 2006).

2.6.1.4 Glutathione

Glutathione (GSH), L-\(\gamma\) glutamyl-L-cysteinyl-glycine, is the most abundant non protein thiol present in almost all eukaryotes, with the exception of a few parasitic protozoans such as *Entamoeba histolytica* and *Giardia duodenalis* that lack mitochondria. Glutathione is also present in some bacteria such as the cyanobacteria and the proteobacteria, and a few gram-positive bacteria. It appears to be absent in the archaeabacteria (Fahey and Sundquist, 1991).

2.6.1.4.1 Properties of glutathione

GSH is present in high concentrations up to 10mM in most living cells from prokaryotes to eukaryotes. It's very low redox potential (E’o = 3240mV for thiol disulfide exchange) and the fact that its reduced state is maintained by NADPH-dependent glutathione reductase granting the tripeptide the status of a cellular redox buffer (Meister and Anderson, 1983). This property was already anticipated in the original papers of de Rey-Pahlade, the French discoverer of GSH (Meister, 1988). In bacteria GSH occurs primarily in Gram negative aerobic species but very rarely in anaerobes and Gram-positive strains (Fahey, 2001). GSH can occur under the reduced GSH form, the oxidized form GS-SG, and different mixed disulfides, for example GS-S-CoA and GS-S-Cys (Penninckx and Elskens, 1993; Hell, 1997; Gasch et al., 2000; Cereghino et al., 2000). Mycothiol (2-(N-acetylcysteinyl) amido-2-deoxy-\(\alpha\)-D-glucopyranosyl-(1-1)-myo-inositol) is an alternative thiol produced by Gram positive bacteria of the Actinomycetes lineage (Newton et al., 1996). The thiol moiety of GSH also has strong nucleophilic properties permitting its conjugation with electrophilic compounds, in particular xenobiotics (Vuilleumier, 1997). The \(\gamma\)-glutamyl linkage
present in GSH may confer on the tripeptide a privileged resistance to proteolytic action.

There seems to be a connection between GSH and the aerobic style of life. High levels of GSH present in cyanobacteria and in purple photosynthetic bacteria point in favour of a bacterial emergence of GSH at a time when aerobic conditions became widespread on earth. GSH could thus have appeared in the evolution as a protective agent against oxidative damages.

### 2.6.1.4.2 Glutathione biosynthesis

GSH is synthesized by the consecutive action of $\gamma$-GCS (L-glutamate+L-cysteine+ATP $\rightarrow$ L-$\gamma$-glutamy-L-cysteine+ADP) and L-$\gamma$-glutamylcysteine-glycine $\gamma$-ligase (L-$\gamma$-glutamyl-L-cysteine+glycine+ATP $\rightarrow$ GSH+ ADP: GSH synthetase) encoded respectively by the GSH1 and GSH2 genes (Fig. 2.7.) (Grant and Dawes, 1996).

In *S. cerevisiae* GSH1 comprises a segment of 2034 bp that encodes a protein Gsh1p ($\gamma$-GCS) of 678 amino acid residues. Blast search revealed that *S. cerevisiae* Q-GCS shares sequence homology with the $\gamma$-GCS of the NCY *S. pombe* (41% of...
identity over 669 residues). \( \gamma \)-GCS appears to be a highly regulated enzyme transcriptionally responsive to the Yap1 (Lee et al., 1999) protein, a central modulator in mechanisms for drug and metal resistance (Carmel-Harel and Storz, 2000). Skn7p, another yeast transcriptional regulator, apparently exerts a negative effect upon the response to cadmium. \( \gamma \)-GCS activity was also shown to be feedback-inhibited by GSH, preventing over-accumulation of the tripeptide. Unlike \( \gamma \)-GCS, GSH synthetase appeared to be a constitutive unregulated enzyme (Inoue et al., 1998).

2.6.1.4.3 Glutathione degradation

\( \gamma \)-Glutamyltranspeptidase [\( \gamma \)-GT: GSH + amino acid (H\(_2\)O)\( \rightarrow \)L- \( \gamma \)-glutamyl amino acid (L-glutamate)], is the only GSH-degrading enzyme currently characterized in yeast (Penninckx et al., 1980; Mehdi et al., 2001). It catalyzes transfer of the \( \gamma \)-glutamyl moiety of GSH and other \( \gamma \)-glutamyl compounds to amino acids and also the hydrolytic release of L-glutamate from GSH, various \( \gamma \)-glutamyl compounds, and substituted derivatives (Penninckx et al., 1980). L-Cysteinyl glycine dipeptidase (CGase: L-cysteinyl glycine + H\(_2\)O\( \rightarrow \)L-cysteine + glycine) is assumed to catalyze the last step in GSH hydrolysis (Fig. 2.7). This activity is reported to be present in \textit{S. cerevisiae} and associated with the vacuolar membrane (Jaspers and Penninckx, 1984). A variety of L-Cys-Gly-hydrolyzing peptidases are produced by microorganisms and mammals (Rankin et al., 1980; McIntyre and Curthoys, 1982; Van Den Hazel et al., 1996). \( \gamma \)-GT biosynthesis is found to be regulated by at least two distinct pathways. Ammonium ions as a nitrogen source repressed the transpeptidase, whereas the cellular enzyme level is higher in the presence of a variety of other nitrogen sources, including amino acids or urea (Penninckx et al., 1980). The enzyme production is strongly derepressed by nitrogen starvation as shown previously (Mehdi and Penninckx, 1997) and recently conformed in an exploration of genomic expression patterns in \textit{S. cerevisiae} responding to environmental changes (Gasch et al., 2000). It has been suggested that \( \gamma \)-GT might play a role in amino acid transport in yeast, but this has been ruled out as far as bulk transport of amino acids in \textit{S. cerevisiae} is concerned (Payne and Payne, 1984; J. Jaspers et al., 1985). It is reported that it play a role in the mobilization of GSH as alternative sulfur and nitrogen source during starvation (Penninckx, 2002).
2.6.1.4.4 Transport of glutathione

GSH may also be taken up from the extracellular environment. Specific transporters mediating GSH uptake in microorganisms (Sherrill and Fahey, 1998), plants (Hell, 1997) and animal tissues (Iantomasi et al., 1997) have been described.

Two kinetically distinguishable GSH transport systems are shown in *S. cerevisiae*. GSH-P1 is a high affinity ATP-driven and regulated system, responding to sulfur starvation, whereas GSH-P2 has a lower affinity and is not regulated (Miyake et al., 1998). Hgt1p, a high affinity plasma membrane GSH transporter is cloned and characterized in *S. cerevisiae* (Bourbouloux et al. 2000; Kaur and Bachhawat, 2009; Thakur and Bachhawat, 2010). Apparently nothing is known about the possible relationship between GSH P-1 and Hgt1p. Sequence analysis of Hgt1p revealed virtually no homology with the yeast YCF1 vacuolar pump mediating low affinity vacuolar transport of GSH and different GSH conjugates (GS-X) (Li et al., 1996; Rebbeor et al., 1998; Mehdi et al., 2001). The YCF1 gene was initially isolated according to its ability to confer cadmium resistance to *S. cerevisiae*. It encodes a 1515-amino acid ATP binding cassette (ABC) protein with extensive sequence homology to the human multidrug resistance-associated protein (MRP1).

2.6.1.4.5 Glutathione and stress in yeast

The physiologic role of glutathione is multifaceted and has been implicated in a multitude of cellular functions, such as transport of amino acids, synthesis of proteins and nucleic acids, maintenance of enzymes in active forms, regulation of the hexose monophosphate shunt, protection against radiation and endotoxin exposure, and amelioration of septic and cardiogenic shock. Glutathione participates in trans hydrogenation reactions that are involved in the formation and maintenance of the sulphhydryl groups of other molecules (e.g., coenzyme A, various enzymes and other proteins). It provides reducing capacity for several reactions and plays an important role in detoxification of hydrogen peroxide, other peroxides and free radicals; moreover, glutathione also functions in the detoxification of a variety of xenobiotics (Pastore et al., 2003, Zhang et al., 2010).
2.6.1.4.6 Role of glutathione in sulphur and nitrogen metabolism

When cells of *S. cerevisiae* are totally deprived of any external sulfur source, GSH is apparently able to serve as an endogenous sulfur source until it reached a residual concentration of about 10% of its normal value (Elskens *et al.*, 1991). A strong derepression of γ-GT and of the enzymes of the Transsulfuration pathway is observed during sulfur starvation and as a consequence the turnover rate of GSH increased. When *S. cerevisiae* is subjected to nitrogen starvation, more than 90% of the cellular GSH shifted toward the central vacuole of the yeast (Mehdi and Penninckx, 1997). γ-GT induces during nitrogen starvation and translocated from the Golgi toward the vacuolar membrane via a pathway alternative to the prevacuolar compartment possibly similar to the vacuolar delivery route of alkaline phosphatase (Piper *et al.*, 1997).

2.6.1.4.7 Glutathione and yeast dehydration tolerance

A crucial strategy for survival in dried anhydrobiotes is the control of membrane dynamics (Hoekstra *et al.*, 2001). Under dehydration, cell membranes become fluidized and perturbed, as well as being more susceptible to ROS attack (Crowe *et al.*, 1989). ROS often cause an extensive peroxidation and de esterification of membrane lipids at intermediate ranges of water loss (Senaratna *et al.*, 1987). Glutathione induce the reduction in peroxide and removal of many lipophilic xenobiotics from the cytosol (Penninckx, 2002), so the role of GSH is important in protecting cell membranes under anhydrous conditions. Dehydration increases the levels of lipid peroxidation in both control strain and gsh mutants. However, the levels of lipid peroxidation in the glutathione deficient mutants, even in fresh cells, present two- to four-fold higher than in the control strain. Similar to oxidation during dehydration, gsh1 cells shows the highest levels of MDA. It is observed in many studies that, after dehydration, there is a significant enhancement of the intracellular GSSG content in the control strain; confirm the key role of glutathione in maintaining the redox homeostasis of dried cells. Fresh and dried yeast cells shows similar glucose-6-phosphate dehydrogenase (G6PDH) and glutathione reductase (GLR) activities. In seeds and desiccation resistant plants, intracellular low availability of
water reduce the rates of chemical reaction and, therefore of metabolic activity (Grant et al., 1997). Although ROS increases with drying, the enzymatic antioxidant systems can be activated only under conditions of sufficient water and, in the dried state, only molecular antioxidants (e.g., glutathione and sugars) can alleviate oxidative stress. Glutathione also regulate GLR activity in dry conditions (Espindola et al., 2003). The importance of GSH in desiccation tolerance is confirmed by the addition of glutathione monoethyl ester (GME) to glutathione depleted cells, which restored the levels of survival to those of the control strain. The drug GME is a cell-permeable derivative of GSH that undergoes hydrolysis by intra-cellular esterases, thereby, increasing intracellular GSH concentration (Anderson, 1998).

2.6.1.4.8 Glutathione and cryopreservation

It has been proposed that injury caused by freezing and thawing is the result of several factors such as ice nucleation and dehydration (Mazur, 1970). Damage to membrane structures by freezing and thawing has been observed by electron microscopy (Komatsu et al., 1987). Cells can also suffer biochemical damage such as oxidative stress by reactive oxygen species formed during the thawing process (Hermes-Lima and Storey, 1993; Park et al., 1998). However, it has been shown that heat shock treatment makes yeast cells resistant to damage caused by freezing and thawing (Komatsu et al., 1990). This is possibly due to pre-induction of heat shock proteins and oxidative stress scavengers. Heat shock proteins can rescue damaged cells. Glutathione and trehalose are shown to be induced by heat shock treatment and play the role of oxidative stress scavengers during thawing process. (Sugiyama et al., 2000; Benaroudj et al., 2001; Odani et al., 2003). Zhang et al. (2007) evaluated the basis of glutathione thermodynamic characteristics and protection of baker’s yeast during cryopreservation at -30°C. The thermodynamic characteristics and protection of baker’s yeast of glutathione are similar to those of known antifreeze proteins, such as carrot antifreeze protein and holly antifreeze protein. These properties included lowering the freezing point at about 0.20°C non-colligatively, decreasing freezable water content, controlling the movement of free water for its strong hydrophilicity, and improving baker’s yeast survival during the simulated processing of frozen dough.
2.6.1.4.9 Glutathione and heat stress tolerance

Heat causes a lot of catastrophic changes including the excess increase in the oxygen respiration rate of the yeast *S. cerevisiae* (Sugiyama *et al.*, 2000), which resulted in increased intracellular oxidation levels. Therefore, heat-shock stress may be, in some part, equivalent to oxidative stress. The induction of *GSH1* and *GSH2* in *S. cerevisiae* increases the intracellular GSH content, increase after heat stress (Sugiyama *et al.*, 2000). GSH seems important in delaying cell death at lethal temperature. Insufficient ability to scavenge reactive oxygen species in mitochondria may cause accumulation of oxidative damage in the mtDNA that results in the generation of mutants. Piper (1999) reported that a null mutation of *SOD2* caused a higher frequency of mitochondrial mutation. Sugiyama *et al.* (2000) suggested that mitochondrial dysfunction with GSH depletion triggers the catastrophic process. Madeo *et al.* (1999) reported that depletion of GSH induced apoptosis in *S. cerevisiae*. They showed that accumulation of reactive oxygen species caused by depletion of GSH in the cell triggered the apoptotic pathway in this micro-organism and the hypoxia or scavenging of the radicals prevented apoptosis. As heat shock also causes oxidative stress in yeast so glutathione plays an important role as a free radical scavenger to protect cells from the apoptosis.

2.6.1.4.10 Glutathione as an antioxidant in oxidative stress tolerance

Oxidative stress occurs when cells are exposed to high levels of ROS such as peroxides, including H$_2$O$_2$ and alkylhydroperoxides (ROOH), and the superoxide anion (O$_2^-$). Many investigators have shown that GSH plays an important role in the response of yeasts to oxidative stress, in particular to ROOH such as cumene hydroperoxide, t-butyl hydroperoxide or endogenous lipid hydroperoxide (LOOH) generated in biological membranes from unsaturated fatty acids (Grant and Dawes, 1996; Stephen and Jamieson, 1996; Carmel-Harel and Storz, 2000). High levels of GSH in yeast are apparently important for surviving acute peroxide stress but not for growth adaptation under moderate peroxide concentration (Spector *et al.*, 2001). Glutathione peroxidase (GPx: 2GSH + ROOH$\rightarrow$ GSSG + H$_2$O + ROH) is a key enzyme in the defence mechanisms against hydroperoxides. It is found to be induced
by oxidative conditions in *S. cerevisiae* (Galiazzo et al., 1987). GPx is purified from Hansenula mrakii and found to be localized in both the cytoplasmic membrane and the inner membrane of mitochondria where large amounts of ROS are generated (Horiguchi et al., 2001). The Pmp20 antioxidant system relies on GSH as an electron donor and may function within peroxisomes as a GPx. Three GPxs, named Gpx1p, Gpx2p and Gpx3p, were found in *S. cerevisiae* (Inoue et al., 1999). A genetic and phenotypic analysis has shown that GPX1 is induced by glucose starvation. GPX2 is induced by oxidative stress, although apparently not playing a significant role in the response to the stress against \( \text{H}_2\text{O}_2 \) and t-butyl hydroperoxide. The GPX3 gene is found to be expressed constitutively. Apparently Gpx3p is the major GPx that scavenges peroxides in the yeast. Recently it is shown that the GPx genes of *S. cerevisiae*, previously reported to encode classical soluble GPxs, encode partially membrane-associated phospholipid hydroperoxide GPxs (Avery and Avery, 2001). A thioredoxin (Trx)-dependent reduction system (Holmgren, 1985) is also closely involved in the response of *S. cerevisiae* against ROS (Inoue et al., 1999; Carmel-Harel and Storz, 2000). Several studies have clearly implicated the Yap1p and Snk7p transcription factors in regulating the expression of genes induced by oxidative stress (Carmel-Harel and Storz, 2000). These target genes include GSH1 (\( \gamma \)-GCS), GPX2 (GPx2), GLR1 [glutathione reductase (GLR): GS-SG + NADPH + H\(^+\) \(\rightarrow\) 2GSH + NADP\(^+\)], TRX2 (Trx) and TRR1 (thioredoxin reductase (TR)). A Yap1p-dependent induction of GSH synthesis in heat shock response of *S. cerevisiae* is reported. Apparently heat shock stress enhances oxygen respiration, which in turn results in the increase of ROS in the mitochondria, necessitating the operation of a detoxification mechanism (Sugiyama et al., 2000). The adaptive response of *S. pombe* to ROS also involves systems similar to those found in *S. cerevisiae* (Lee et al., 1995), but differences also exist. For example only one gene GPX1 encoding a GPx was found in the fission yeast. As found for *S. cerevisiae* GPxs, the *S. pombe* enzyme does not contain selenocysteine at its active center but rather a cysteine residue (Yamada et al., 1999; Avery and Avery, 2001; Penninckx, 2002).
2.6.1.4.11 Glutathione in xenobiotics and heavy metal stress

GSH plays a key role in cellular defence against reactive electrophiles such as halogenated aromatics. Many xenobiotics can react either spontaneously with the thiol moiety of GSH to form GSH S-conjugates, or via GSH S-transferases (GST: GSH + RX → GS-X + RH) (Vuilleumier, 1997). Ycf1p appears as the major GSX vacuolar transporter in S. cerevisiae, but yeasts may also transport GS-X outside the cells (Zadzinski et al., 1996). A study of the global response of S. cerevisiae to methyl methanesulfonate (MMS) has shown that the transcripts of 325 ORFs induced over four-fold by the reagent (Jelinsky and Samson 1999). One of the strongest inductions observed (28.8-fold) was for GTT2, a gene encoding a putative GST. Moreover CIS2 encoding γ-GT (Mehdi et al., 2001) and PDR10 encoding plasma membrane ABC transporter proteins were induced respectively 7.7- and 8.8-fold in the same conditions. MMS is an alkylating agent that could react with GSH, and it is thus tempting to speculate about the existence in yeast of a prototype of the mammalian and plant mercapturic detoxification pathway (Meister and Anderson, 1983). As(III) detoxification in S. cerevisiae involves two independent transport systems for the removal of arsenite from the cytosol (Ghosh et al., 1999). Acr3p is a plasma membrane protein mediating arsenite extrusion from the cells and Ycf1p catalyzes the ATP-driven uptake of the GSH conjugate As(GS)3 into the central vacuole. On a chemical reactivity basis it is expected that GSH able to form chelation complexes with heavy metals and in this way, involved in detoxification. Studies of the cadmium response in yeasts have shed new light on the mechanisms of intervention of GSH in heavy metal detoxification. The story starts with the NCY S. pombe and Torulopsis glabrata, where it is shown that exposure to Cd2+ salts resulted in the biosynthesis of cadystins [phytochelatins (PhCs)]. PhCs are polymers of general structure (γ-Glu-Cys)n-Gly (Rauser, 1990; 1995). These polypeptides, also found in some plant species, can chelate Cd2+, Cu2+ and Zn2+ ions in the cytosol (Rauser, 1995). The complexes are then conveyed by an ABC transporter into the vacuole where they are deposited after bonding with acid-labile sulfide (Ortiz et al., 1992). Mutants of S. pombe deficient in GSH have lost the ability to neutralize Cd2+ and are also shown to be more sensitive to Cu2+, Zn2+ and Pb2+ ions (Coblenz and Wolf, 1994). The nature
of the phytochelatin synthetase (PCS) in S. pombe has been debated. PCS genes have been identified in Arabidopsis and S. pombe (Ha et al., 1999, Clemens et al., 1999) Disruption of the PCS gene in S. pombe leads to cadmium sensitive mutants lacking PhCs (phytochelatin). Expression of the protein in S. cerevisiae leads to PhC production and the recombinant purified S. pombe protein displayed PCS activity Wolf and collaborators have shown that GSH2 encodes a bifunctional enzyme able to catalyze both the synthesis of GSH by adding glycine to γ-Glu-Cys and the synthesis of PhCs (Al-Lahham et al., 1999). However, the precise enzymatic mechanism of PhC synthesis remains to be determined. S. cerevisiae has evolved a cadmium sequestration mechanism independent of PhCs. In yeast Cd(2+) (GSH)2 complexes are transported into the vacuole by Ycf1p (Li et al. 1997). Challenge of the cells with Cd(2+) resulted in the induction of γ-GCS and eight other enzymes of the sulfur amino acid biosynthetic pathway (Vido et al., 2001). The induction of these nine proteins is dependent on the Yap1p, Met-4, Met-31 and Met-32 transcription factors (Dormer et al. 2000). Trx and TR also seem to play an important role in cadmium tolerance, which indicates that the two cellular thiol redox systems are essential for defense of S. cerevisiae against cadmium, as was similarly observed in the response to oxidative stress.

2.6.1.5 Other defence system in the yeast

2.6.1.5.1 Effect of metals (Ca, Mg and Zn) on the ethanol tolerance

Metal ions can impact significantly on the progress and efficiency of industrial fermentations. Metal ions govern several important parameters including the rate of sugar conversion to ethanol, the degree of attenuation/ final ethanol yield, the amount of yeast produced, cell viability and stress tolerance, extent of foaming, and yeast flocculation behaviour. Bulk metals, such as magnesium and potassium, are generally required by growing yeast cells in the millimolar (i.e. hundreds of ppm) concentration range, whilst calcium and zinc are regarded as trace metals being required in the micromolar (sub-ppm) range. However, calcium ions are acknowledged to play a key role in the important process of flocculation in brewing fermentations. Calcium antagonises uptake of magnesium and can block essential magnesium-dependent
metabolic processes, so high levels would be detrimental to brewing efficiency. Other metals such as heavy metals, even at trace level concentrations, may be toxic to yeast (Jones and Gadd, 1990; Walker, 1999).

In addition to impacts on important metabolic enzymes, metal ions may also affect the stability and dynamics of cell membranes. When yeasts encounter changes in environment such as nutrient or ion depletion, metabolite accumulation or temperature variation, the plasma membrane must adapt prior to internal structures. Maintenance of membrane fluidity is a crucial factor in preservation of membrane functions. Modulation of membrane fluidity is a primary response to environmental change. Yeasts are able to very effectively accumulate essential minerals and exclude or detoxify non-essential minerals. Magnesium and zinc are actively translocated by yeast from wort into the cell in order to carry out essential physiological roles, and this may be usefully exploited in alcoholic beverage fermentations.

Magnesium is necessary for the activation of several glycolytic enzymes and, in practical terms, this means that if industrial media is magnesium-limited, the conversion of sugar to alcohol may be suppressed leading to slow or incomplete fermentation processes (Birch and Walker, 2000). Magnesium ions also decreases proton and anion permeability of the plasma membrane by interacting with membrane phospholipids, resulting in stabilization of the membrane bilayer (Walker, 1994; Birch and Walker, 2000). Thus it is probably not surprising that magnesium is implicated in the relief of the detrimental effects of ethanol stress in yeast (Walker 1994). Birch and Walker (2000) and Hu et al. (2003), demonstrated that increased the extracellular availability of magnesium ions, increases physiological protection against temperature and ethanol-stress. Hu et al. (2003) showed that the exposure of yeast cells to 20% (v/v) ethanol for 9 hours resulted in the death of all cells, whereas over 50% of the cell population remained viable in the same ethanol concentration but in the presence of Mg$^{2+}$. Calcium requirements for yeast growth are very low (Youatt, 1993; Walker, 1994). The addition of Mg$^{2+}$ salts reverses the ethanol induced leakage and restores metabolic activity. Mg$^{2+}$ ions play an integral role in the production of ethanol by industrial strains of S. cerevisiae, reflected in enzyme activation and membrane stabilization and in known to protect yeast cells from stress caused by ethanol,
temperature and osmotic pressure (Ciesarova et al., 1996). The protective effect of Ca\(^{2+}\) resulting from increased membrane stability was found to prevent the release of cytoplasmic compounds. Calcium ions increase plasma membrane plasma membrane stability either by decreasing the ethanol induced passive protons influx or stabilizing the ATPase activity inhibited by ethanol (Ciesarova et al. 1996).

Zinc is an essential nutrient for all living organisms, which affects both cell growth and metabolism. It has been reported that suitable zinc supplementation in the culture medium is beneficial for ethanol production (Chandrasena et al., 1997; Tosun and Ergun, 2007). Studies also indicate the protective effect of zinc against ethanol toxicity in self-flocculating yeast SPSC01 (Xue et al., 2008), however, until now, no study on the intracellular metabolic changes resulted from zinc addition in ethanol fermentation is reported (Zhao et al., 2009). Zinc is a trace element that is particularly important in fermentation with regard to its role as activator of the terminal alcohologenic Zn-metalloenzyme, ethanol dehydrogenase. Media deficient in zinc may lead slow or incomplete fermentations, and this has long been recognised as an occasional problem in the brewing industry (Zhao et al., 2009).

2.6.1.5.2 Effects of Mannitol the stress tolerance in yeast

Managbanag and Torzilli (2002) reported the accumulation of mannitol after salt stress in fungus *Aureobasidium pullulans* shows the potential role of this compound in the stress tolerance. The role of mannitol in stress response has been investigated by Chatuverdi et al (1997) who created a new mannitol biosynthetic pathway in an osmosensitive, glycerol-defective mutant of *S. cerevisiae* by transforming this yeast with multi-copy plasmids that encoded the mannitol -1-phosphate dehydrogenase gene obtained from *Escherichia coli*. Using this system, the investigators showed that the transformation restored the ability of the mutant strain to grow in the presence of a high NaCl concentration. Therefore, mannitol is capable of substituting for glycerol as the primary intracellular osmolyte in *S. cerevisiae*.

2.6.1.5.3 Effects of amino acids (proline)

L-Proline is an osmoprotectant (Csonka, 1989; Delauney and Verma, 1993) and a sweet amino acid that protect yeast cells from damage by freezing, desiccation,
or oxidative stress (Takagi et al., 2000; Morita et al., 2002; Morita et al., 2003; Terao et al., 2003). L-Proline enhances the stability of proteins and membranes in environments with low water activity or high temperature (Rudolph and Crowe, 1985) and inhibits aggregation during protein refolding (Samuel et al., 2000). Intracellular L-proline can play a crucial role in reducing ethanol stress by preventing protein denaturation and membrane disorder during sake fermentation. Takagi et al. (2005) confirmed that L-proline increases cell viability in the presence of ethanol during static incubations of both laboratory and sake yeast strains. The unusually high L-proline content does not appear to stress the cells such that the levels of trehalose or other amino acids increase. Further research to identify the mechanism(s) by which L-proline interacts with membrane phospholipids and proteins is needed to understand how L-proline increases ethanol tolerance. Thus, L-proline might serve as a protective agent for industrial microorganisms and enzymes (Takagi et al., 2005).

2.6.1.5.4 Effects of inositol

Inositol is a precursor of numerous phospholipids and signalling molecules essential for the cell. Furukawa et al. (2004) observed that yeast cells which lack inositol during ethanol stress exhibited a higher death rate than inositol enriched cells. It is also observed that inositol deficient cells leaked more intracellular components, such as nucleotides, phosphate and potassium, in the presence of ethanol than the inositol enriched cells. Cells which content less inositol exhibit a lower intracellular pH value than inositol enriched cells. Inositol also affects the plasma membrane H⁺-ATPase activity. H⁺-ATPase maintains the permeability barrier of the yeast membrane, ensuring the homeostasis of ions in the cytoplasm of yeast cells. It is assumed that the lowering of H⁺-ATPase activity due to inositol limitation is caused by the change in lipid environment of the enzyme, which is affected by inositol-containing glycerophospholipids such as phosphatidylinositol (PI), because in the PI-saturated mixed micellar assay system, there is not any difference exist in H⁺-ATPase activity between inositol deficient and enriched cells. Guerin et al. (2009) also reported the induction of the apoptosis after the depletion of inositol in yeast cells. So yeast cellular inositol level is one of the important factors which contribute to the high ethanol tolerance by increasing cell viability in the presence of ethanol. The role of
the inositol synthetic gene INO1 of yeast in sake brewing is to increase cell density by synthesizing inositol, a growth-limiting factor and to increase cell viability in the presence of high concentrations of ethanol in the final stage, leading to a desirable fermentation profile.

2.6.2 Enzymatic defences

Reactive oxygen species are commonly present in aerobic organisms; they generally removed by enzymatic as well as non-enzymatic defence systems. In enzymatic defence system superoxide dismutase catalyzes disproportions of \( \text{O}_2^- \) to \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \), and \( \text{H}_2\text{O}_2 \) thus formed is decomposed to \( \text{H}_2\text{O} \) and \( \text{O}_2 \) by catalase. \( \text{H}_2\text{O}_2 \) as well as (lipid hydroperoxide) \( \text{LOOH} \) are reduced to \( \text{H}_2\text{O} \) and corresponding alcohol by glutathione peroxidase (GPx) (Inoue et al., 1999; Day, 2009).

2.6.2.1 Superoxide dismutase and stress tolerance

In eukaryotic cells, manganese superoxide dismutase is found exclusively in the mitochondrial matrix where it catalytically removes superoxide anion radicals. In addition, eukaryotic cells contain a Cu/Zn superoxide dismutase in the cytosol and the mitochondrial inter membrane space. The two types of superoxide dismutase have different metals at the active site, and different molecular weights, subunit number, and amino acid sequences but catalyze the same reaction (Autor, 1982). Nedeva et al. (2004) reported the presence of SOD in many species of yeasts.

It is suggested by various studies that although the both of SOD (Cu/Zn SOD and MnSOD) shows induction upon the ethanol stress still it is shown by many researchers that MnSOD is more responsible for ethanol stress tolerance due to its location in the mitochondria (Costa et al., 1993). When exponential-phase cells of *S. cerevisiae* growing on glucose are exposed to a sublethal thermal or ethanol stress, antioxidant defences, such as MnSOD and catalase T, are induced and ethanol tolerance increases (Watson and Cavicchioli, 1983; Wieser et al., 1991; Costa et al., 1993; Schuller et al., 1994). The yeast cells can also become more tolerant to ethanol and other stress agents when yeast cells shift from fermentative to respiratory growth (Piper, 1995). Indeed, when fermentation comes to an end, a number of genes down
regulated by glucose are activated, including genes encoding antioxidant defences (Wemer-Washburne et al., 1993; Moradas-Ferreira et al., 1996). Costa et al. (1997) also showed the positive correlation between the activity of CuZnSOD and MnSOD and ethanol tolerance during different phases of growth. The primary target of ethanol induced oxidative stress is the mitochondria so the role of MnSOD is very important in the ethanol tolerance in the yeast. Under ethanol stress conditions, MnSOD prevents the lipids, proteins and nucleic acids from oxidative damage. The H$_2$O$_2$ produced by dismutation of .O2$^-$ catalysed by MnSOD can be decomposed by catalase T (Costa et al., 1997).

2.6.2.2 Catalase and stress tolerance

Catalase is an essential enzyme in the decomposition of intracellular hydrogen peroxide (H$_2$O$_2$). In S. cerevisiae cells, two different types of catalase have been found (Traczyk et al., 1985, Skoneczny and Rytka 2000), which are designated as catalase type A and catalase type T. The two enzymes are encoded by different genes (CTA1 and CTT1 respectively), possessing independent control and being localized in different compartments: catalase T is a cytoplasm enzyme whereas catalase A is localized in peroxisomes (Lapinskas et al., 1993). The molecular weights of the biologically active, homotetrameric enzymes are 170-190 kD and 225-250 kD for catalase A and T respectively (Klei et al., 1990, Petrova et al., 2002). Under conditions of oxidative metabolism in the yeast, S. cerevisiae, catalases protects proteins from oxidation by ROS (Izawa et al., 1996). In particular, it protects G6PDH, which probably is inactivated by hydrogen peroxide or by-products of its metabolism. The role of antioxidant enzymes is in the protection of proteins against oxidation depends very much on selected conditions. Lushchak and Gospodaryov (2005) reported the role of catalases in protection of certain cellular proteins against damage by ROS undergrowth in non-fermentable substrates like ethanol (Izawa et al., 1996).

2.6.2.3 Glutathione peroxidise and stress tolerance

Glutathione peroxidise is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from
oxidative damage. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Galiazzo et al., 1987). Casalone et al. (1988) reported the presence of glutathione peroxidase in many yeast species.

GPx plays a crucial role in the defense yeast against oxidative stress (Tran et al., 1993; Fu et al., 2007). GPx catalyzes the reduction of hydroperoxides using glutathione (GSH) as an electron donor. GPxs have been known to be located primarily in the cytosol and in the inner and outer membrane of mitochondria (Inoue et al., 1995). Purified enzymes reported so far have been mainly obtained from cytosolic fraction (Mannervik, 1985). Ursini et al. (1985) reported a membrane-associated phospholipid hydroperoxide glutathione peroxidase (PHGPx). Glutathione peroxidases (Gpx) are a part of the glutathione system, which is known to be one of the main thiol antioxidant systems in the cell in addition to the thioredoxin system. It has been suggested that the thioredoxin and glutathione systems are maintained independently (Trotter and Grant, 2003), though compensation between these two systems has been observed (Inoue et al., 1999; Missall et al., 2005).

It is reported that the expression of GPX gene under several environmental stress conditions increases to control the oxidative damage. Disruption of the GPX gene enhanced susceptibility to peroxides; thus the Gpx have a major role in scavenging peroxides generated during normal metabolism in S. cerevisiae. Expression of the GPX gene is also reported to induce by oxidative stress in the Yap1p-dependent manner. Presence of STRE (stress response element) on the GPX gene also suggests its role in the stress tolerance (Marchler et al., 1993; Schuller et al., 1994, Ruis and Schuller, 1995; Inoue et al., 1999).

2.6.3 Lipid and yeast stress tolerance

Yeast is a typical eukaryotic organism with respect to the membranous organelles it contains, as well as the lipids that comprise its membranes. Like other eukaryotes, it synthesizes and incorporates sterol into its membranes. In addition, its membranes contain a typically eukaryotic mixture of phospholipids, including
sphingolipids, cardiolipin, phosphatidylerine, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol. The pathways for the synthesis of yeast membrane lipids are increasingly well characterized. In addition, a number of mutations affecting these pathways have been characterized, and reliable methodologies for isolation of some sub cellular membrane fractions are also available. As a result, *S. cerevisiae* is an attractive organism in which to conduct studies on the roles of specific lipids in membrane biogenesis and membrane-mediated processes. A number of independent observations identify the plasma membrane as being critically involved in the transduction of stress into a biological signal (Panaretou and Piper, 1990; Coote *et al.*, 1994; Curran and Khalawan, 1994; Carratu *et al.*, 1996). Lipids play a major role in determining the sensitivity of the heat shock response to stress (Chatterjee *et al.*, 2000). Lipid composition plays a similar role in STRE-mediated activation by both heat and salt stress. The studies on lipids suggest the important role of lipids in the stress related signalling or tolerance.

A decrease in total lipid content of the yeast cells in the presence of ethanol has been reported by many studies (Gupta *et al.*, 1994; Serrano *et al.*, 2001; Malhotra and Singh, 2006). The decrease in total lipid content of ethanol-treated cells may be owing to enhanced lipid peroxidation or alcohol-induced changes in membranes activating the enzymes involved in the hydrolysis of lipids. The reduction in lipid content along with dehydration caused by alcohol alters the specific interactions between lipid and membrane proteins essential for the maintenance of membrane integrity (Hernandez and Cooke, 1997).

2.6.3.1 Phospholipids, Glycolipids and sterols and stress tolerance in yeast

Glycerophospholipids, commonly known as simply phospholipids (PL), are built on a glycerol molecule, which becomes chiral when derived to glycerol-3-phosphate. The backbone of membrane PLs is the L isomer, called sn-glycerol-3-phosphate. With fattyacyl chains in ester linkage on carbons 1 and 2 it becomes phosphatidic acid (PA). Esterification of PA with another alcohol creates the following PLs: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylerine (PS), phosphatidylglycerol (PG) and phosphatidylinositol (PI). In
addition, PG can link through its glycerol headgroup to PA to form diphosphatidylglycerol (CL for its composition name, cardiolipin) (Van der Rest et al., 1995). Altered phospholipid and sterol levels were observed in the various forms of Candida species of yeast (Goyal and Khuller, 1994). Besides affecting phospholipid content, the ethanol also modulates phospholipid composition of yeast (D'Amore and Stewart, 1987). Comparatively, a decrease in phosphatidylethanolamine (PE) and increase in phosphatidylcholine (PC) were also reported after ethanol stress on yeast (unpublished results of our lab). Mizoguchi and Hara (1997) also reported similar decrease in the content of PE of S. cerevisiae and S. pombe cells when treated with ethanol. However, a reverse trend is observed in PC contents; a minor increase in phosphatidylinositol (PI) content and minor decrease in phosphatidylserine (PS) content reported in the yeast after ethanol stress. The change in the phospholipids and glycolipid affects the membrane fluidity and also affects the ethanol stress tolerance capacity of yeast.

The primary sterol of yeast is ergosterol which shares many structural similarities with cholesterol, an important component of animal plasma membrane and which to a lesser extent is also found in their organellar membranes (Park and Casey, 1995; Zinser and Daum 1995). One of the main functions of sterol is to maintain membrane fluidity in the face of environmental changes such as culture medium, temperature, etc. Since sterols are metabolically expensive cellular components, they may have other critical roles in the cell functioning (Bottema et al., 1985; Parks and Casey, 1995). Walker-Caprioglio et al. (1990) reported the role of sterol especially ergosterol in the ethanol stress tolerance. It is also proved that sterol helps yeast cells in maintaining viability during ethanol stress (Walker-Caprioglio et al., 1990; Chi and Arneborg, 1999). It is also reported in laboratory previously that the changes in the phospholipid and glycolipids after ethanol stress in yeast. According to these studies, it seems that glycolipids resist high transition temperatures against environmental stress (Curatolo, 1987, Sorger and Daum, 2003). A relative higher content of glycolipid with ethanol may develop an adaptive response of yeast to alcohol stress (Malhotra and Singh, 2006).
2.6.3.2 Fatty acid and stress tolerance in yeast

Several studies have correlated the role of fatty acid in ethanol stress tolerance in yeast (Alexandre et al., 1994; Ding et al., 2009). Fatty acid composition can greatly influence the physical state of phospholipids and thereby affect membrane fluidity and permeability. Oleic acid (18:1) and palmitoleic acid (16:1), together with trace amounts of palmitic acid (16:0) and stearic acid (18:0), are the principal fatty acyl chains in yeast (Cottrell et al., 1986). The fatty acyl packing of these chains determines membrane fluidity to a large extent. Packing increases as the length of the acyl chains increases and as fluidity decreases. Perturbations of the bilayer that decrease the area of a lipid molecule, such as increased hydrostatic pressure, lowering of the temperature, or addition of sterols to phospholipids, also resulted in a decrease in fluidity (Shinitzky, 1984; Bottema et al., 1985). Yeast grown in the presence of ethanol appears to increase the amount of unsaturated fatty acyl residues in the membrane lipid, which increases the membrane fluidity (Mishra and Prasad, 1989; Mishra and Kaur, 1991). This is a supposed adaption to ethanol stress (Kajiwara et al., 1996). Increased ethanol tolerance with the unsaturated fatty acid oleic also supports the positive correlation of ethanol tolerance with the increased unsaturated fatty acid (You et al., 2003). In the ethanol-tolerant microorganism Zymomonas mobilis, Δ⁹Z-C₁₈:₁ is the most abundant UFA, and there are only minor amounts of Δ⁹Z-C₁₆:₁ and C₁₆:₀ (double bond between carbon atoms 9 and 10 of saturated 18- and 16-carbon fatty acids) (Carey and Ingram, 1983; Moreau et al., 1997; Rupčić and Jurešić 2010). Chi and Arneborg (1999) found the increased unsaturated fatty acid in the more ethanol tolerance yeast species proves the most important role of fatty acids in lipids in the ethanol tolerance mechanism.

2.6.4 Tocopherol and yeast stress tolerance

Tocopherols are a class of lipophilic potential active antioxidant of which many have a vitamin E activity. It is a series of organic compounds consisting of various methylated phenols. Vitamin E activity was first identified in 1936 from a dietary fertility factor in rats. Tocopherols are the primary fat-soluble antioxidants that
help minimize the effects of free-radicals. Of the four compounds - designated as alpha, beta, delta and gamma tocopherols - alpha tocopherol has the greatest nutritional and biological value. Vitamin E protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. It also (alpha tocopherol) prevent polyunsaturated fatty acids from breaking down and combining with substances that may harm the cell. Naturally tocopherol is not present in the yeast (Skinner and Sturm, 1968) although Diplock et al. (1961) and Gupta et al. (1994) reported minor amount of tocopherol in it.

Therefore, yeast can be used to see the protective role of tocopherol in various stresses. Raspor et al. (2005) first time used the chemical Trolox (model compound for α-tocopherol) against the reactive oxygen species in the yeast and found very positive results. Dilsiz et al. (1997) observed the effects of tocopherol on the fatty acids of yeast they found that tocopherol decreases the saturated and unsaturated fatty acids content of the yeast. It is also reported that alpha-Tocopherol abrogated the edelfosine-induced generation of intracellular ROS and apoptosis (Zhang et al., 2007).