1.1 Weak acid stress in yeast:

The budding yeast *Saccharomyces cerevisiae* is subjected to multiple and changing stress conditions during its growth. These conditions include hyperosmotic shock, nutrient limitation, temperature variation, accumulation of various inhibitors in media, reduction in pH etc. Weak organic acids such as acetic acid, lactic acid, propionic acid, sorbic acid and benzoic acid are inhibitory to growth of *Saccharomyces cerevisiae*. Since various natural habitats of yeast and fungi contain high concentration of weak acids as a result of bacterial fermentation as well as secreted by certain yeasts such as *Brettanomyces* and *Dekkera* (Pretorius, 2000). *S. cerevisiae* has evolved various mechanisms to grow at substantial concentrations of such acids in growth media. Yeast cells are also subjected to acid stress caused by weak acids produced as byproducts of fermentation (Lafonlafourcade et al., 1983) as well as when subjected to fermentation of acid hydrolyzed lignocellulosic materials (Liu, 2006). So studying the process of acid stress as well as mechanism of adaptation to weak acids in yeast, *Saccharomyces cerevisiae*, is of importance. A good understanding of such mechanisms of weak acid tolerance will help to improve our strategies to address the problem of food spoilage as well as efficient utilization of non-fermentable sugars for production of bioethanol.

1.2 Ethanolic fermentation and weak acid stress:

Ethanolic fermentation is accompanied by drastic reduction of pH and weak organic acids like acetic acid, levulinic acid and formic acid are produced as by-products. Weak acids can also be produced in fermentation media by lactic and acetic acid bacteria, which happen to be common contaminants of yeast ethanolic fermentation broths. These byproducts of fermentation, besides ethanol, reduce ethanol yield as well as growth of fermenting yeast (Lafonlafourcade et al., 1984; Viegas et al., 1989). While ethanol produced during fermentation does not accumulate inside the cell (Loueiro and Ferreira, 1983), weak acids such as acetic acid accumulate inside the yeast cells at low pH, which in turn results into inhibition of fermentation. In the presence of ethanol, the inhibitory effect of acetic acid is more drastic and can be observed at slightly higher pH, causing synergistic inhibition of fermentation (Pampulha and Loueirodias, 1989). This synergistic inhibitory effect is thought to be a result of inhibition of glycolytic enzymes, such as enolase (Pampulha and Loueirodias, 1990). Moreover acetic acid, at concentrations occurring in wine fermentation, induces cell death (Pinto et al., 1989).
As interest in alternative energy sources is rising, the concept of biomass conversion to ethanol has become attractive. In recent years, there has been a rapid increase in ethanol production that has been derived from sucrose (in Brazil) and corn starch (in the USA). However, utilization of common crops for ethanol production is hugely debatable as it results into increase in food prices and demand for arable land. Lignocellulosic biomass as substrate for ethanolic fermentation is an attractive option, as it is generated from agricultural or forest wastes thus have least associated cost. However, one major barrier in implementing this process is inhibitory compounds generated during biomass pre-treatment using dilute acid hydrolysis to release monomeric sugars, interfere with microbial growth and subsequent fermentation (Liu, 2006). Acid pretreatment of lignocellulose results in hydrolysis of hemicellulose, followed by enzymatic or chemical hydrolysis of the remaining cellulose (Sun and Cheng, 2002). The release of monomeric sugars during the pretreatment is accompanied by the generation of inhibitors of fermenting yeasts, which strongly affects the fermentation performance (Klinke et al., 2004; Palmqvist and Hahn-Hagerdal, 2000a, b).

The lignocelluloses mainly compose of cellulose, hemicelluloses and lignin, though composition varies from one plant source to another. Pretreatment of lignocellulosic biomass generate a broad range of compounds. D-glucose is mainly obtained from hydrolysis of cellulose. D-galactose, D-mannose, D-rhamnose (hexoses) as well as D-xylose and L-arabinose (pentoses) and uronic acids are released from the hemicellulosic fraction. Hydrolysis of lignin and further degradation of monomeric sugars generate three major categories of compounds that inhibit fermentation. These are furan derivatives, weak acids and phenolics (Almeida et al., 2007). Further details of their type, source and inhibitory effects are as summarized in table 1.1 and figure 1.1 and 1.2.

1.2.1 Furan Derivatives:

The furan compounds 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde are formed by dehydration of hexoses and pentoses, respectively (Ulbricht et al., 1984; Williams and Dunlop, 1948). HMF and furfural decrease the volumetric ethanol yield and productivity, as well as inhibit the growth or give rise to a longer lag phase. These effects depend on the furan concentration and on the yeast strain used, moreover synergistic effects of HMF and furfural have been demonstrated (Taherzadeh et al., 2000). In vitro studies have shown that HMF and furfural directly inhibited alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH), aldehyde dehydrogenase (ALDH) and glycolytic enzymes hexokinase and GPDH. Moreover
<table>
<thead>
<tr>
<th>Inhibitor Class</th>
<th>Inhibitor name</th>
<th>Source</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furan Derivative</td>
<td>5-hydroxymethyl-2-furfural</td>
<td>Dehydration of hexoses</td>
<td>Inhibition of glycolytic enzymes and ADH</td>
</tr>
<tr>
<td></td>
<td>2-furfuraldehyde</td>
<td>Dehydration of pentoses</td>
<td>Reduced NADPH and ATP level, etc.</td>
</tr>
<tr>
<td>Weak Acids</td>
<td>Acetic acid, formic and Levulinic acid</td>
<td>Deacetylation of hemicelluloses and HMF breakdown</td>
<td>Uncoupling, intracellular anion accumulation and ATP depletion</td>
</tr>
<tr>
<td>Phenolics</td>
<td>Acetosyringone, Hydroxybenzoic acid and vanillin</td>
<td>Lignin breakdown and Carbohydrate degradation</td>
<td>Disrupt membrane integrity and Inhibit Electron transport chain</td>
</tr>
</tbody>
</table>

Table 1.1: Type, source and inhibitory effect of inhibitors generated from hydrolysis of lignocellulosic biomass.

Figure 1.1: Average composition of lignocellulosic biomass and main derived hydrolysis products (Almeida et al., 2007).
furan reduces intracellular ATP and NAD(P)H levels by affecting glycolytic and TCA fluxes (Horvath et al., 2003).

1.2.2 Weak acids:

Acetic acid, formic acid and levulinic acid are most common weak acids present in lignocellulosic hydrolyates. Acetic acid is formed by de-acetylation of hemicellulloses, while formic and levulinic acids are products of HMF breakdown. Formic acid can additionally be formed from furfural under acidic conditions at elevated temperatures (Ulbricht et al., 1984; Williams and Dunlop, 1948). Weak acids inhibit yeast fermentation by reducing biomass formation and ethanol yields (Larsson et al., 1999). The inhibitory effect of weak acids has been ascribed to uncoupling and intracellular anion accumulation. According to this theory, at pH lower than pKa value of weak acid, it exists in undissociated form which is permeable to yeast plasma membrane. Once inside the cell these weak acids dissociate into protons and anions, which results into acidification of cytosol and accumulation of anions (Russell, 1992). Moreover ethanolic fermentation using xylose as carbon source is more strongly inhibited by acetic acid, as it enhances xylitol formation (Bellissimi et al., 2009).

1.2.3 Phenolics:

A wide range of phenolics are generated due to breakdown of lignin and subsequent acid hydrolysis of carbohydrate, depending on biomass composition and its chemical complexity (Perez et al., 2002). Inhibitory mechanism of phenolics involves disruption of membrane integrity, which further leads to loss of membrane barrier, destruction of electrochemical gradient across the membrane and loss of enzyme matrices (Heipieper and Debont, 1994; Heipieper et al., 1994).

1.3 Weak acids as food preservatives:

Yeasts are able to grow in foods with a low pH (5.0 or lower) in the presence of sugars, organic acids and other easily metabolized carbon sources. During their growth, yeasts metabolize some food components and produce metabolic end products. These products change the physical, chemical, and sensible properties of a food which in turn causes food spoilage (Kurtzman, 2006). For centuries variety of substances such as natural herbs, spices and weak acids, have been used as preservatives to protect food from spoiling. Weak organic acids like benzoic, acetic, sorbic and propionic acids are widely used food preservatives in
<table>
<thead>
<tr>
<th>Weak acid</th>
<th>Typical food use</th>
<th>Concentration ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid</td>
<td>Semi preserved fish product</td>
<td>300-1000</td>
</tr>
<tr>
<td></td>
<td>Pickles, mustard</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Fruit juice concentrates</td>
<td>4000</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Pickles, Chutneys, Sauces</td>
<td>A few thousand up to % levels in vinegars</td>
</tr>
<tr>
<td></td>
<td>Salad creams and dressings</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vinegars</td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>Bread</td>
<td>2000-5000</td>
</tr>
<tr>
<td></td>
<td>Flour confectionary</td>
<td>1000-3000</td>
</tr>
<tr>
<td></td>
<td>Jams, tomato puree</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>Non –emulsified sauces</td>
<td>1000</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>Non alcoholic drinks</td>
<td>100-1000</td>
</tr>
<tr>
<td></td>
<td>Alcoholic drinks</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Semi-preserved fish product</td>
<td>500-2000</td>
</tr>
<tr>
<td></td>
<td>Processed fruits and vegetables</td>
<td>500-2000</td>
</tr>
<tr>
<td></td>
<td>Fruits and dairy based desserts</td>
<td>500-1000</td>
</tr>
<tr>
<td></td>
<td>Sugar based confectionary</td>
<td>500-2000</td>
</tr>
<tr>
<td></td>
<td>Bakery products</td>
<td>1000-2000</td>
</tr>
<tr>
<td></td>
<td>Mayonnaise and sauces</td>
<td>1000-2000</td>
</tr>
<tr>
<td></td>
<td>Salads</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>Fat based spreads</td>
<td>100-2000</td>
</tr>
<tr>
<td></td>
<td>Mustard</td>
<td>250-1000</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>Non-alcoholic drinks</td>
<td>100-500</td>
</tr>
<tr>
<td></td>
<td>Alcoholic drinks</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Semi-preserved fish product</td>
<td>100-4000</td>
</tr>
<tr>
<td></td>
<td>Fruit products</td>
<td>500-2000</td>
</tr>
<tr>
<td></td>
<td>Vegetables, pickles, preserves</td>
<td>250-2000</td>
</tr>
<tr>
<td></td>
<td>Sugar and flour based confectionary</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>Mayonnaise and sauces</td>
<td>250-2500</td>
</tr>
<tr>
<td></td>
<td>Salads mustard</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>Fat based spreads</td>
<td>100-1000</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>Fermented meat and dairy products</td>
<td>A few thousand up to % levels</td>
</tr>
<tr>
<td></td>
<td>Carbonated drinks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Salad dressings</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pickled vegetables</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sauces</td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>Non-alcoholic drinks</td>
<td>A few thousand up to % levels</td>
</tr>
<tr>
<td></td>
<td>Jams and jellies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bakery products</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cheese</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Canned vegetables</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sauces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Packet dry soup and cake mixes</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2: Major food grade weak acids and conditions of use (Stratford and Eklund, 2003).
Weak Acids | Structure | log P | pKa | Inhibitory mechanisms
---|---|---|---|---
Formic acid | HCOOH | 3.77  |
Acetic acid | CH₃COOH | -0.24 4.76 | Induce apoptosis, inhibit metabolic pathways, generate oxidative stress and ATP depletion
Propionic acid | CH₃CH₂COOH | 0.32 4.87 | Inhibit metabolic pathways, generate oxidative stress and ATP depletion
Butyric acid | CH₃CH₂CH₂COOH | 0.83 4.87 | Inhibitory action not well studied, lies between propionic and octanoic acid
Octanoic acid | CH₃(CH₂)₆COOH | 3.05 4.88 | Disrupt plasma membrane structure, inhibit oxidative phosphorylation and generate oxidative stress
Sorbic acid | CH₃(CH)₄COOH | 1.63 4.76 | Same as above
Benzoic acid | C₆H₅COOH | 1.71 4.2 | Same as above

Table 1.3: Chemical structure, dissociation constant (pKa), lipophilic tendency (log P) and inhibitory action of some weak acids.

Figure 1.2: Schematic view of known inhibition mechanism of furans, weak acids, and Phenolics in yeast *Saccharomyces cerevisiae* (Almeida et al, 2007).
large scale food and beverage preservation (Stratford and Anslow, 1996). Weak acids are also present naturally in a number of foods, notably fruits and fruit juices, such as citrus food contains citric acid. Use of weak acids as preservatives is controlled by various regulatory agencies worldwide and restricted to certain limits based on legislation, taste and cost (Table-1.2). For instance sorbic acid and benzoic acids are accepted as having GRAS (Generally recognized as safe) status in USA and their use in soft drinks is limited by only taste considerations, whereas in European Union they are regarded as preservatives and permitted at conc. not exceeding 300 and 150 ppm respectively. Similarly, in European Union, acetic acid is equally accepted as acidulant, preservative at higher concentration or as flavor compound whereas formic acid is only accepted as preservative. These acids inhibit microbial growth at different concentration depending on their polarity and carbon chain length. Furthermore, the presence of weak acids can increase the efficacy of physical preservative treatments, such as heat or ultra high pressure. The heat resistance of microbes is greatly reduced in acidic conditions, thus shortening the duration of food processing (Stratford and Eklund, 2003).

1.4 Mechanism of inhibition of yeast growth by weak organic acids:

In general organic acids including acetic acid are weak acids and do not dissociate completely in water, where the strong mineral acids do. Dissociation and re-association of proton and anion in such weak acids depends on the pH of the solution. The pH at which there exists equilibrium of dissociation and re-association between proton and conjugate base of weak acid (HA $\rightleftharpoons$ A$^{-}$ + H$^{+}$) is called pKa value. The organic acids have pKa values below 5, such as formic acid (pK$_{a}$ 3.75 at 20°C), acetic acid (pK$_{a}$ 4.75), sorbic acid (pK$_{a}$ 4.76) and benzoic acid (pK$_{a}$ 4.19) (Table-1.3). The antimicrobial effects of weak organic acids at low pH are result of intracellular acidification and anion accumulation (Russell, 1991; Salmond et al., 1984); though mechanism of action may not be identical for different weak acids (Figure-1.3). The comparative studies of antimicrobial action of acetic acid and sorbic acid have shown that acetic acid mainly acts through acidification of cytosol and anion accumulation, whereas sorbic acid mainly acts by disruption of membrane integrity and oxidative stress caused by associated effects on respiratory chain function (Bracey et al., 1998; Piper, 1999). Such a different mechanism of action defines quite high concentration of acetic acid (80-150mM) is required to completely inhibit the growth of S. cerevisiae at pH 4.5. In contrast, highly lipophilic sorbic acid with almost identical pK$_{a}$ value, 1-3mM concentration was
Figure 1.3: Schematic model for weak acid induced stress responses in yeast *Saccharomyces cerevisiae.*
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enough to cause similar effect. Thus, as the weak acids become more lipophilic, its antimicrobial ability is enhanced due to destructive effects on yeast plasma membrane in comparison to less lipophilic monocarboxylic acids (Holyoak et al., 1999; Piper et al., 1998; Stratford and Anslow, 1998).

1.4.1 Uncoupling and intracellular anion accumulation:

At low pH weak acids exist predominantly in undissociated form, a form in which they are potent growth inhibitors. The undissociated acid, being uncharged, readily diffuses across the cell membrane only to further dissociate in the higher pH environment of the cytosol. Such dissociation of weak acid generates protons and the acid anion in cytosol, resulting in acidification of cytosol (Krebs et al., 1983). Reduction in S. cerevisiae intracellular pH (pHᵢ) has been demonstrated following the addition of acetate (Ameborg et al., 2000) and benzoate (Krebs et al., 1983), though reduction in pHᵢ is not always a feature of organic acid stress. The S. cerevisiae maintains a relatively constant intracellular pH by pH homeostasis machinery of cell. The decrease in intracellular pH is compensated by the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis. Consequently less ATP is available for cellular metabolism and cells encounter shortage of energy pool for growth and multiplication (Palmqvist et al., 1999; Russell, 1991; Verduyn, 1991). Continuous inflow of undissociated acid into the cell by diffusion across plasma membrane results into gradual acidification of cytosol (Bracey et al., 1998). The acidification of cytosol inhibits the activity of cellular machinery, as optimal pH required for enzymatic activity is disturbed. The presence of acetic acid in yeast cell negatively regulates the activity of glycolytic enzymes. The activity of enolase is most severely affected along with inhibition of hexokinase and phosphofructokinase, while glucose transport is not affected (Pampulha and Loureiro, 1989).

Dissociation of weak acid also releases anions into the cytosol; anions being negatively charged are relatively membrane impermeant and accumulate intracellularly to very high level. This high anion accumulation may generate an abnormally high turgor pressure, resulting in free radical production and severe oxidative stress. The anion accumulation also causes protein aggregation, lipid peroxidation, inhibition of membrane trafficking and perturbation of plasma and vacuolar membrane (Piper et al., 2001; Teixeira et al., 2007). This in turn further affect the yeast cells ability to grow and ferment carbon substrate into ethanol, such inhibition of fermentation is synergistically affected by ethanol. Weak acids have also been shown to inhibit yeast growth by reducing the uptake of aromatic amino acids from the
medium, probably as a consequence of strong inhibition of Tat2p amino acid permease (Bauer et al., 2003). Moreover in presence of weak acids at low pH, acidification of external environment may affect the cell wall structure and alter the conformation of proteins on the outer side of plasma membrane (Stratford and Booth, 2003).

1.4.2 Acetic acid mediates apoptosis in yeast:

The role of acetic acid has been also implicated in yeast apoptosis and chronological life span. Acetic acid induced apoptosis in yeast results in mitochondrial swelling, reduction in cristae number and formation of myelinic bodies. Additionally, mitochondrial ROS accumulation, a transient hyperpolarization followed by depolarization, and a decrease in the activity of COX affecting mitochondrial respiration is seen. Mitochondria become permeabilized, allowing the release of lethal factors like cytochrome c (Ludovico et al., 2002). However, acetic acid induced programmed cell death (AA-PCD) may occur without cytochrome c release, as AA-PCD is significantly observed in yca1Δ and presence of Yca1p is essential for cytochrome c release (Guaragnella et al., 2010). Acetic acid produced by fermentative growth in stationary phase induces oxidative stress, a factor previously implicated in chronological aging of yeast and other organisms (Burtner et al., 2009). The accumulation of acetic acid in stationary phase cultures inhibits growth arrest of cells in G1 and is preferentially toxic to cells that fail to undergo a G1 arrest. In budding yeast and other fungi acetic acid mediated intracellular acidification activates the same highly conserved Ras2 and cAMP-dependent signaling pathways that respond to glucose. Therefore in stationary phase, when glucose is completely consumed, nutrient depleted cells are subjected to acetic acid-induced growth signals that promote entry into S phase. The absence of nutrients or the regulatory mechanisms required for the synthesis of dNTPs and efficient DNA replication result in replication stress and induction of apoptotic manifestation (Burhans and Weinberger, 2009).

1.5 Mechanism of weak acid tolerance in yeast:

Despite all these toxic and inhibitory conditions encountered by yeast cells in presence of acetic acid, they are able to survive and adapt to significant concentration of acetic acid and other weak acids. Adaptation to low pH and high weak acid concentration or inherent acquisition of tolerance to acid would require yeast cells to modify their cellular machinery to minimize the accumulation of acids within the cell. As earlier described, different weak acids
have different inhibitory action depending on lipophilicity. Thus, yeast cells use different resistance mechanism to adapt against stress caused by different weak acids (Figure-1.4).

1.5.1 Preadaptation and weak acid tolerance:

Yeast cells exposed to acetic acid show a decrease in cell viability in a concentration and time dependent manner. On exposure to 80 mM acetic acid in YPD at pH 3.0, cell viability decreases to 60% after 60 minutes, 30% after 90 min and virtually all cells are unviable after 200 min (Giannattasio et al., 2005). But it is well-known that pretreatment with one kind of stressing agent may induce cross-resistance against another type of stress agent in yeast (Evans et al., 1998). Therefore, exponentially growing yeast cells preincubated in YPD medium at pH 3.0 for 30 min remained fully viable after 200 min exposure to 80 mM acetic acid. Resistance to acetic acid observed in such pretreated cells was attributed to high intracellular level of both catalase and super oxide dismutase activities (Giannattasio et al., 2005). Moreover, when exponentially growing yeast cells encounter inhibitory concentrations of weak acid, growth is arrested and cells enter a period of growth latency. During this lag phase cell viability is reduced and growth resumes after extended period of time. However, when these adapted cells are reinoculated into a fresh-growth medium at identical pH and supplemented with the same weak acid concentration, no delay in cell growth is observed (Carmelo et al., 1998; Teixeira and Sa-Correia, 2002). Therefore, yeast cells previously adapted to weak acids or other stress have better tolerance to weak acid stress.

1.5.2 Plasma and vacuolar membrane ATPases reduce intracellular acidification:

Diffusion of weak acids into the yeast cell causes acidification of cytosol by increased H⁺ influx. To avoid dissipation of plasma membrane potential and maintain the intracellular pH homeostasis to allow physiological activities of cell, yeast cells activate plasma membrane H⁺ ATPase (Pma1p) to efflux protons in ATP dependent manner (Mira et al., 2010c). Plasma membrane ATPases also play important role in activity of H⁺ antiporters efluxing acid anions from cytosol, thus reducing the stress caused by anion accumulation (Tenreiro et al., 2002). H⁺ ATPases present in the vacuolar membrane (V-ATPase) are also been proposed to play similar role. By seizing protons into the vacuolar lumen, V-ATPase activity helps in maintaining pH homeostasis and membrane potential across vacuolar membrane (Carmelo et al., 1997; Marcantoni et al., 2007). For maximal tolerance to weak acids, role of V-ATPases is also implicated in endocytosis, targeting of newly synthesized lysosomal enzymes,
intracellular trafficking, Pma1p sorting to the plasma membrane and compartmentalization of metabolites (Caba et al., 2005; Mira et al., 2009; Mollapour et al., 2004; Teixeira et al., 2005).

1.5.3 Membrane transporters efflux weak acid anion:

Influx of weak acids into the yeast cells also results into anion accumulation. Removal of counter anions is therefore essential and several specific transporters have been implicated in efflux of such anions from yeast cytosol. Pdr12p, a plasma membrane transporter of the ATP binding cassette superfamily is involved in the active efflux of propionate, sorbate, or benzoate anions (Holyoak et al., 1999; Piper et al., 1998). On the other hand, Pdr12p was found to play no detectable role in the active expulsion of counterions of more lipophilic acids such as octanoic acid, decanoic acid, artesunic acid and 2,4-Dichlorophenoxoacetic acid (2, 4-D) (Alenquer et al., 2006; Piper et al., 1998; Teixeira and Sa-Correia, 2002), or of the more hydrophilic acetate anion (Piper et al., 1998). So Pdr12p is an ABC transporter that provides resistance to those carboxylate compounds that can, to a reasonable extent, partition into both lipid and aqueous phases. Pdr12p exports anions by binding to acid anion incorporated within inner leaflet of plasma membrane, and then acts as a flippase to transport them to periplasmic side of membrane. Once on periplasmic side, the anions are readily protonated in acidic environment and released into external media (Mollapour et al., 2008). A close homolog of Pdr12p, Pdr5p is involved in expulsion of anions of highly lipophilic weak acids such as 2, 4-D, artesunic acid and artemisinic acid (Alenquer et al., 2006; Teixeira and Sa-Correia, 2002). A number of genes encoding drug:H⁺ antiporters belonging to the major facilitator superfamily (MFS) have also been found to contribute to yeast tolerance to different weak acids: AQR1 and AZR1 genes are required for maximal tolerance to acetic and propionic acids (Tenreiro et al., 2002; Tenreiro et al., 2000). TPO1 gene was found to be a major determinant of resistance to 2,4-D, artesunic and mycophenolic acids (Alenquer et al., 2006); whereas TPO1 close homologues TPO2 and TPO3, were found to provide protection against acetic, propionic, and benzoic acids (Fernandes et al., 2005). The role of MFS–MDR transporters in reducing the intracellular concentration of different weak acid counterions might also result from the transport of endogenous metabolite(s) that may affect the partition of the weak acid between the exterior and the intracellular environment, through an alteration of intracellular pH and/or plasma membrane potential (Sa-Correia et al., 2009).
1.5.4 Alteration in cell wall and plasma membrane structure:

Cell wall is major barrier that protects cell from rapidly changing external environment. Cell wall avoids the bursting of cells due to outer osmotic changes and maintains an intracellular water activity that is appropriate for biochemical reactions. Cell wall has a dynamic organisation, which changes for different cellular and physiological requirements and on induction of stress. Yeast cell wall is layered structure, with inner layer composed of glucan polymers and chitin and outer layer of highly glycosylated mannoproteins (Levin, 2005). Cell wall assembly is regulated by cell surface sensors coupled with cascades of signalling molecules which activates a set of effector molecules in response to external conditions (Heinisch et al., 1999). On encountering inhibiting concentration of weak acids, yeast cells activate cell wall integrity pathway and so modulate the cell wall architecture to reduce its porosity and entry of undissociated acid into the cell. Exposure to lipophilic weak acids like benzoic acid and 2,4-D results in increased expression of glycosylphosphatidylinositol cell wall protein (GPI-CWP) Spi1p resulting into cell wall remodelling (Abbott et al., 2008; Schuller et al., 2004). The Spi1p mediated cell wall remodelling reduces the porosity of cell wall and reduced plasma membrane damage (Kapteyn et al., 2001; Simoes et al., 2006). At low pH, when glucose repressed S. cerevisiae cells are challenged with high concentration of acetic acid, acid floods into the cells through Fps1p aquaglyceroporin an open glycerol channel of plasma membrane. This transiently activates Hog1p MAP kinase, which in turn phosphorylates the Fps1p at two 12 amino acid regions in the cytosolic surface of membrane that are implicated in turgor mediated channel closure. This phosphorylation is signal for Fps1p to become ubiquitinated and then endocytosed to the vacuole (Mollapour and Piper, 2007). Exposure to lipophilic acid may also result in altered lipid composition; an increase in the ratio of saturated membrane fatty acids over unsaturated one was reported (Viegas et al., 2005).

1.5.5 Weak acids induced metabolic changes:

Low concentration of acetate and propionate can be utilized by yeast via acetyl-CoA and propionyl CoA pathways of central carbon metabolism. Moreover, low concentration of acetic acid is known to enhance the yield of ethanol during fermentation (Pampulha and Loureirodias, 1990). However in the presence of fermentable sugars like glucose, S. cerevisiae cannot use acetate as carbon source and yeast cells as such cannot metabolize sorbate or benzoate (Mollapour et al., 2008). Transcriptomic and proteomic analyses have
Low pH
Cell wall structure remodeling

Increased saturation of fatty acid in Plasma membrane

Neutral pH

Vacuole

Nucleus

Acetate and propionate

RCOO⁻ (C3-C8)
E.g. Sorbate, ADP

Highly lipophilic Acids, e.g. 2,4-D

Pdr12p

Pdr5p

Fps1p

CH₃COOH

H⁺

RCOO⁻ + H⁺

H⁺

CH₃COO⁻ + H⁺

HOG pathway

ER

Vesicle mediated transport

Activation of Vma1p

Acetate and propionate

H⁺

Activates of Pma1p

ATP

ADP

H⁺

RCOO⁻

RCOO⁻

RCOO⁻

RCOO⁻

RCOO⁻

RCOO⁻

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shown that exposure to acetic acid results in activation of the TOR (Target of Rapamycin) pathway, which in turn regulates the yeast response to nutrient starvation. Presence of weak acids results in upregulation of genes encoding proteins involved in amino acid uptake and biosynthesis. Weak acid stressed yeast cells also show increased expression of genes encoding enzymes involved in glycolysis and kreb's cycle (Almeida et al., 2009; Mira et al., 2009; Mira et al., 2010b; Mira et al., 2010c). Such responses are proposed to compensate for increased energy consumption (Holyoak et al., 1996) and inhibition of glycolytic enzymes (Pampulha and Loureirodas, 1990).

1.6 Regulation of weak acid tolerance:

The weak acid response in yeast cells result in activation of large number of genes, which make yeast cells resistant to one or few weak acids. Studies show that different weak acids induce different set of genes; however, they may be involved in similar biological processes, such as protein folding, lipid metabolism, cell wall function and multidrug resistance (MDR). In case of MDR protein encoding genes, expression of TPO3, a polyamine transporter is induced by all weak acids studied so far, whereas more lipophilic sorbic acid and others specifically induce expression of PDR5, YOR1, PDR12 and SNQ1, and more hydrophilic acetic and propionic acid activate TPO2 (Abbott et al., 2007; Mira et al., 2010c). Therefore, adaptation to different weak acids requires expression of mostly different set of genes and possibly activated by different regulatory pathways. So far four regulatory pathways, dependent on the transcription factors Haa1p, Rim101p, Msn2p/Msn4p and War1p have been identified in mediating yeast response to weak acid stress (Mira et al., 2010a).

1.6.1 The Msn2p/Msn4p regulon:

Msn2p and Msn4p are two homologous transcription factors involved in yeast response to environmental stress including adaptation to weak acid stress (Gasch et al., 2000). Msn2p/Msn4p complex mediates the induction of genes with STRE elements during acid stress response (MartinezPastor et al., 1996). Most of the genes upregulated by Msn2p and Msn4p in response to weak acids stress encode proteins of the environmental stress response such as molecular chaperones (Hsp26p, Sse2p), enzymes of the carbohydrate metabolism (Hxk1p, Gpd1p), and of the antioxidant defence system (Ctt1p, Gpx1p) (Schuller et al., 2004; Simoes et al., 2006). The induction of Msn2p/Msn4-regulon in response to multiple weak acids provides the cells necessary flexibility to respond to a wide range of challenges,
although the physiological response brought by this activation does not always result in an increased resistance to the weak acid (Mira et al., 2010c).

1.6.2 The Haa1p regulon:

The Haa1p was initially identified as homologous to copper (Cu\(^+\)) requiring transcription factor Ace1p (Keller et al., 2001). Haa1p is involved in yeast adaptation to hydrophilic weak acids such as acetic acid and propionic acid, and its contribution decreases with increase in lipophilicity of weak acid. Haa1p regulates the expression of genes encoding MDR transporters and other plasma membrane and cell wall associated proteins, such as TPO2, TPO3, YGP1, PHM8, and YLR297w. However activation of only Tpo2p, Tpo3p and Ygp1p was found essential for resistance to acetic acid, whereas other Haa1p regulated genes play marginal or no role in adaptation to acetic acid (Fernandes et al., 2005). Based on microarray studies, Haa1p is recently shown to be involved in the expression of SAP30 (Rpd3L histone deacetylase) and HRK1 (a protein kinase involved in phosphorylation of plasma membrane transporters) genes; deletion of these genes severely decreases the ability of yeast cells to adapt to acetic acid (Mira et al., 2010a).

1.6.3 The War1p regulon:

The War1p is a transcriptional regulator involved in expression of ATP binding cassette (ABC) transporter Pdr12p, which effluxes weak acids such as sorbic acid and benzoic acid. The War1p belongs to the family of Zn\(_2\)Cys\(_6\) zinc finger regulators; it recognizes a cis acting weak acid response element (WARE) in the PDR12 promoter (Kren et al., 2003). Deletion of WAR1 leads to hyper sensitivity to moderately lipophilic weak acids such as propionic acid, sorbic acid and benzoic acid, but not to hydrophilic acetic acid (Schuller et al., 2004). War1p activity is controlled by direct binding of weak acid counterions, increasing the affinity of this transcription factor for the promoter region of its target genes. In particular, the ability of sorbate anion to induce a conformational change in War1p structure has been demonstrated to activate PDR12 expression (Gregori et al., 2008).

1.6.4 The Rim101p pathway:

Rim101p transcription factor has been implicated in adaptation of yeast cells to alkaline pH and in cell wall remodelling (Castrejon et al., 2006; Lamb and Mitchell, 2003). Recently it was shown to be required for resistance to weak acid stress at pH 4.0 and its contribution
decreases with weak acid lipophilicity. Rim101p does not protect against low pH as such, when a strong acid is used as the acidulant. Based on the comparison of the transcriptomes of wild-type and rim101Δ cells after weak acid shock, Rim101p was found to induce the expression of known Rim101p regulon. On acid shock Rim101p also induces the expression of new target genes, in particular KNH1, involved in cellwall β-1, 6-glucan synthesis and the uncharacterized gene YIL029c, both of which are required for maximal propionic acid resistance in yeast. Clustering of the genes that provide resistance to propionic acid show the enrichment of genes involved in cell wall function, protein catabolism through the multivesicular body pathway, homeostasis of internal pH and vacuolar function (Mira et al., 2009).

1.6.5 The other weak acid induced regulons:

Besides transcription factors that are solely involved in adaptation to weak acids, there are certain other transcription factors having important role in adaptation to weak acid stress. Pdr1p and Pdr3p transcription factors controlling multidrug resistance in yeast (Gulshan and Moye-Rowley, 2007), are involved in adaptation to lipophilic acids such as 2,4-D and artesunic acid (Ro et al., 2008), by upregulating the expression of Pdr5p and Tpo1p (Alenquer et al., 2006). Based on clustering of genes induced upon weak acid stress with respect to their documented transcriptional regulators using YEASTRACT database (Teixeira et al., 2006), the Yap1p, Sfp1p and Sok2p are also found to be involved in general weak acid transcriptional response. Yap1p is known to play a key role in oxidative stress in yeast; Sfp1p regulates the expression of ribosomal genes in response to nutrient starvation and Sok2p is involved in general stress response in yeast cells. Thus activation of these transcription activators may not be a direct effect of weak acid stress, but a consequence of weak acid induced stress (Mira et al., 2010a).

1.6.6 Genes involved in acetic acid adaptation in yeast:

Genes transcriptionally activated upon acetic acid stress belongs to diverse functional classes like stress response, signal transduction, regulation of carbohydrate metabolism and drug transport, whereas repressed genes belongs to amino acid catabolism and ion transport (Mira et al., 2010b). The *S. cerevisiae*’s transcriptional response to acetic acid stress mainly involves expression of Haa1p regulated genes. Since role of Haa1p in acetic acid tolerance is
discussed in separate section, here other aspects of cellular response to acetic acid stress are discussed.

A genome wide screening of EUROSCARF haploid collection for susceptibility phenotype to acetic acid (70-110 mM acetic acid and pH 4.5 in minimal media) by Mira et al., 2010, has identified 650 determinants of tolerance to acetic acid. Clustering of these acetic acid-resistance genes based on their biological function indicated the enrichment of genes involved in transcription, internal pH homeostasis, carbohydrate metabolism, cell wall assembly, biogenesis of mitochondria, ribosome and vacuole, and in the sensing, signaling and uptake of various nutrients, in particular iron, potassium, glucose and amino acids. Ion and proton transport play important role in maintenance of pH homeostasis in weak acid stress, thus genes of this functional class such as \(\text{PMP}1\), \(\text{VMA}8\), and \(\text{VPH}2\) are critical. Other genes in this class enriched were, those involved in uptake of potassium (\(\text{TRK}1\)), ammonium (\(\text{MEP}3\)), phosphate (\(\text{PHO}88\)) and iron (\(\text{FET}3\) and \(\text{FRE}3\)). Deletion of genes encoding protein involved in glycolysis (\(\text{HXK}2\), \(\text{PFK}1\) etc), Krebs cycle (\(\text{FUM}1\), \(\text{PYC}1\) etc), pentose phosphate pathway (\(\text{ZWF}1\) and \(\text{RPE}1\)), components of respiratory chain (\(\text{ATP}1\), \(\text{ATP}4\) and \(\text{COX}9\) etc) and proteins of mitochondrial ribosomes sensitized yeast cells to acetic acid. Deletion of genes involved in cell wall biosynthesis and remodeling (\(\text{FKS}1\), \(\text{KRE}1\), \(\text{CHS}1\), \(\text{GAS}1\) etc) also makes yeast cell sensitive to acetic acid. A number of genes related to sensing, signaling and uptake of amino acids were also identified as determinants of acetic acid tolerance (\(\text{GTR}1\), \(\text{SLM}1\), \(\text{STP}1\), \(\text{CYS}3\), \(\text{MET}4\) etc.), which is consistent with the observation that acetic acid caused general amino acid limitation. This screen also uncovered 28 transcription factors required for resistance to acetic acid including \(\text{HAA}1\), \(\text{RIM}101\) and \(\text{MSN}2\) whose role in response to acetic acid stress is known (Mira et al., 2010b).

A proteomic approach to study role of acetic acid in apoptosis of yeast by Almeida et al, 2009 has revealed number of proteins whose expression is altered in the presence of acetic acid. Upon acetic acid induced apoptosis 53 spots were affected in 2D electrophoresis, with 41 spots showing decrease and 12 spots with increased intensity and subsequent MS analyses of these spots correspond to 28 proteins. These results indicate involvement of several cellular processes in acetic acid induced apoptotic condition. Upon acetic acid treatment chaperones of Hsp70 family (Ssa1p and Ssa2p etc.) decreased in intensity whereas intensity of Hsp90 family chaperone (Hsc82p) increased. Three proteins involved in nucleotide biosynthesis were affected, Ade6p involved in purine nucleotide biosynthesis increased in spot intensity
whereas intensity of spots corresponding to Rnr2p and Rnr4p proteins that supply DNA precursor for replication and repair decreased. These results indicate that upon acetic acid induced apoptosis, nucleotide biosynthesis is downregulated. Among proteins involved in carbohydrate metabolism, Pfk2p, Fba1p and Pdc3p decreased in spot intensity, further validating the inhibitory effect of acetic acid on carbohydrate metabolism. Acetic acid stress also results in amino acid starvation in yeast, as proteins involved in amino acid biosynthesis (Leu1p, Lvl3p and Thr4p), lysyl-tRNA synthetase Ksr1p and ubiquitin activating enzyme Uba1p showed decreased expression upon acetic acid treatment. However, intensity for a fragment of beta subunit of phenyl-alanyl-tRNA synthetase (Frs1p) increased. These results show that acetic acid treatment results in amino acid starvation reduced processivity of tRNAs, fragmentation of proteins and decreased degradation of amino acid permeases. The transcriptional repressor Wtm1p involved in regulation of meiosis and silencing showed increased expression; whereas expression of proteins involved in translation initiation (Tif1p/Tif2p) and elongation (Eft1/2p, Tef1/2p and Yef1p) is decreased (Almeida et al., 2009).

These studies indicate that acetic acid affects diverse cellular processes in yeast. On the one hand acetic acid inhibits many life governing processes, such as carbohydrate metabolism, amino acid biosynthesis and transport and nucleotide biosynthesis. On the other hand it activates many life saving processes, such as cell wall remodeling, ion transport and pH homeostasis, inhibition of growth and increased recycling of cellular resources.

1.6.7 Genes involved in adaptation to other weak acids in yeast:

Schuller et al., (2004) have used a two pronged approach to identify the genes involved in resistance to sorbic acid, by simultaneously screening for sensitivity of yeast deletion mutants to weak acids and by global transcriptome analysis upon exposure to sorbic acid in yeast. Global transcriptome analysis showed differential expression of 100 genes upon induction with sorbic acid, which were further classified based on transcription factors involved in their regulation. War1p regulates expression of at least four genes including PDR12 which is most important determinant of resistance to sorbic acid. Msn2/4p regulates expression of 35 genes (SPII, HSP26, CTTI, HXT7, PDR15 etc.) and the expression of 13 genes is coregulated by War1p and Msn2/4p (GPD1, GLK1, TPS1, LSM4 etc.), 21 other genes are regulated by neither War1p nor Msn2/4p (HSP30, YGP1, TPO2 and TPO3 etc.) (Schuller et al., 2004).
Expression of *YGP1, TPO2* and *TPO3*, which were initially categorized as Warlp and Msn2/4p independent were further shown to be regulated by Haalp transcriptional activator (Fernandes et al., 2005). Moreover mutants identified for sorbic acid sensitivity belong to diverse cellular functions, including energy metabolism (*PFK1, ADH1* etc), mitochondrial function, ergosterol biosynthesis (*ERG2, 3, 6 and 28*), aromatic amino acid biosynthesis (*TRP1, 2, 5 and *ARO2*) and transcription and translation (*SWI3, SSN6, RPB9* etc) (Schuller et al., 2004).

In a study by Mira et al (2009), the deletion of 254 genes made yeast cells sensitive to propionic acid. These genes were further clustered into following functional classes: intracellular trafficking (*VPS16, PEP5* etc), protein catabolism through the multivesicular body system (*VPS28, STP22, SNF8* etc), generation of ATP and energy, ergosterol biosynthesis, posttranslational modification and Rim101 proteolytic processing (*RIM8, RIM13* etc) and pH homeostasis (*VMA1, TFT2, VPH2* etc). Deletion of genes encoding Hog1 kinase (HOG signaling pathway) and Slt2 kinase (Cell wall integrity pathway) also leads to propionic acid sensitivity. Furthermore, gene expression analysis has shown that Rim101p plays essential role in resistance to propionic acid. Upon activation with propionic acid Rim101p regulates expression of 22 new genes which were previously not reported to be Rim101p dependent. The Rim101p dependent genes encode proteins involved in cell wall metabolism, iron homeostasis, transcription factors and few proteins of unknown function (Mira et al., 2009). Immediate adaptation to propionic acid also requires expression of Haalp regulated genes (Fernandes et al., 2005).

**1.7 Haalp, a principle regulator of yeast adaptation to acetic acid:**

**1.7.1 Haalp is yeast transcription factor:**

Haalp was discovered as transcription factor homologous to gene encoding the copper activated transcription factor Ace1p (Keller et al., 2001). It was further implicated in rapid adaptation of yeast cells to hydrophilic weak acids, such as acetic acid and propionic acid (Fernandes et al., 2005). Ace1p is a transcription factor with an N-terminal DNA biding domain (DBD) and C-terminal transactivation domain. The DNA binding domain of Ace1p consists of two sub domains, a domain with 40 residues stabilized by one Zn (II) ion and a 70 residues copper regulatory domain (CuRD) that binds four Cu (I) ions through eight cysteiny1 thiolates forming a polycopper cluster (Graden et al., 1996). DNA binding by Ace1p requires
both the Zn and copper regulatory domains for major groove and minor groove DNA interactions (Dobi et al., 1995). The Haalp transcription factor has a region homologous to both the N-terminal Zn module and copper regulatory domain of Ace1p, but it lacks any similarity to Ace1p beyond DBD (Figure-1.5 (a)). Haalp-GFP fusion is shown to localize to nucleus (Figure-1.5 (b)), when expressed from under GAL1 promoter upon galactose induction. Haalp target genes were shown to code for plasma membrane proteins, MDR proteins and cell wall associated proteins, mostly uncharacterized till then. Expression of Haalp target genes was found to be independent of the copper status of cells and absence of copper metalloregulation is not associated with transactivation domain of Haalp. Nevertheless striking similarity between the CuRD of Ace1p and Haalp suggests that, possibly polycopper thiolate cluster may stabilize the DNA binding conformer of Haalp as it do for Ace1p DBD (Keller et al., 2001).

1.7.2 Role in adaptation to weak acids:

Fernandes et al. (2005) have shown that Haalp is involved in yeast response to weak acid preservatives, especially in adaptation to hydrophilic weak acids. Haalp mediated resistance to weak acids decreases steeply with the increase of the liposolubility of the weak acids, being maximal for acetic acid and negligible for octanoic acid. Exposure to weak acids is known to result in decreased viability and extended lag phase of yeast cells. Deletion of HAA1 further decreases the viability and increases lag phase duration. Furthermore it was shown that yeast cells upon exposure to propionic acid or octanoic acid upregulate 9 out of 10 Haalp regulated genes described by Keller et al (2001). Among these genes, the rapid activation of TPO2, YGP1, YIR035c, YLR297w, YPR157w, YER130c and YRO2 is essentially dependent on Haalp, whereas expression of PHM8 and TPO3 is partially dependent on the Haalp activation and GRE1 is not expressed in response weak acids. Among Haalp regulated genes YGP1, TPO2 and TPO3 and less significantly YLR297w, YER130c and YRO2 are involved in yeast adaptation to acetic acid and propionic acid (Fernandes et al., 2005).

TPO2 and TPO3 encode highly homologous putative multidrug transporter belonging to major facilitator superfamily (MFS). These putative drug:H\(^+\) antiporters exhibit 12 predicted membrane spanning segments and are required for polyamine transport to alleviate polyamine toxicity (Tomitori et al., 2001; Tomitori et al., 1999). Deletion of TPO2 and TPO3 results in increased growth latency of yeast cells exposed to weak acids, though changes are less drastic as compared to HAA1 deletion. Moreover, the tpo3\(\Delta\) and haa1\(\Delta\) strains show
Figure 1.5: (a) Sequence comparison of the N-terminal segment of Haa1p and the DNA binding domains of Ace1p and (b) Nuclear localization of Haa1p. A GFP tagged Haa1p expressed from GAL11 promoter on galactose induction localizes to nucleus (Keller et al., 2001).
increased accumulation of radiolabelled (1-14C) acetic acid in cytosol compared to wildtype. These results indicate that Tpo2p and Tpo3p are possibly involved in active export of the counter-ion of weak acids towards which they provide protection (Figure-1.7). YGP1 encodes highly glycosylated cell wall protein, which is induced in response to environmental stresses, like nutrient starvation, low external pH and hyperosmomic stress. In Fernandes et al (2005), studies the protective effect of Ygp1p was more evident for the lipophilic octanoic acid and benzoic acid, while negligible for acetic acid and propionic acid. As Ygp1p is involved in reducing cell wall permeability, thereby more protective towards lipophilic acids causing plasma membrane damage (Fernandes et al., 2005). Recent transcriptomic analysis of Haa1p regulated genes upon acetic acid treatment has shown that expression of SAP30 and HRK1 are essential for protection to acetic acid. SAP30 encodes a subunit of a histone deacetylase complex and HRK1 encode a protein kinase belonging to a family of protein kinases dedicated to the regulation of activity of plasma membrane transporters. Deletion of HRK1 leads to increased accumulation of acetic acid into acetic acid stressed cells, so Hrk1p is proposed to be involved in activation of plasma membrane transporter by phosphorylation, to efflux acetate ions to reduce intracellular concentration. Sap30p is proposed to modulate the yeast transcriptional response to acetic acid by affecting the activity of transcription factor involved in this response (Figure-1.6) (Mira et al., 2010a).

Haa1p is also implicated in yeast transcriptional response to acetaldehyde stress. Transcriptional profiling of yeast cells after acetaldehyde shock has shown induction of YRO2, TPO2, TPO3 and TPO4. The expression YRO2, TPO2 and TPO3 requires presence of functional Haa1p, since in haalΔ cells expression of these genes is repressed (Aranda and del Olmo, 2004). The role of Haa1p in acetaldehyde tolerance in yeast should be indirect, as yeast cells have ability to metabolize acetaldehyde into acetic acid and activation of Haa1p may result from subsequent accumulation of acetic acid inside the yeast cells. A recent report suggests that Haa1p acts downstream of Yak1p to regulate acetic acid resistance and FLO11 mediated adhesion in yeast (Figure 1.7). The Yak1p is a member of the family of dual-specificity tyrosine kinases and appears to be part of nutrient responsive signaling pathway that acts in parallel to cAMP-PKA pathway, in antagonistic manner (Garrett and Broach, 1989). Glucose starvation and rapamycin induced inhibition of TOR pathway results in nuclear localization of Yak1p (Moriya et al., 2001). Yak1p effects the expression of few known Haa1p regulated genes; these are GRE1, PHM8, TPO2, YGP1 and YROI and deletion of YAK1 results into acetic acid sensitivity in yeast which can be suppressed by
Figure 1.6: Haa1p mediated transcriptional response to acetic acid in yeast (Mira et al., 2010). (a) Clustering based on biological function, of genes activated in response to acetic acid stress in a Haa1p dependent manner and (b) schematic diagram showing the crosstalk between the regulatory systems controlled by the weak acid responsive transcription factors Haa1p, Rim101p, and Msn2p/Msn4p in yeast response to acetic acid.
Figure 1.7: A schematic model for molecular mechanism Haap mediated acetic acid tolerance in yeast S. cerevisiae.
overexpression of *HAA1*. Moreover upon acetic acid stress, Haa1p activates expression of *FLO11* and possibly Flo11p mediated adhesion in Yak1p dependent manner (Malcher et al., 2011).

1.7.3 Haa1p mediated transcriptional response:

The transcriptional analysis of yeast cells upon acetic acid treatment to wildtype strain *BY4741* and *haa1Δ* strain by Mira et al (2010), have identified several novel target genes regulated by Haa1p that were not identified in earlier studies by Keller et al (2001) and Fernandes et al (2005). Genes transcriptionally activated by acetic acid treatment belong to diverse functional classes, including stress response, signal transduction, regulation of carbohydrate metabolism and drug transport, whereas functional classes of genes downregulated include amino acid catabolism and ion transport. Activation of Haa1p is required for at least 80% (85 out of 112) of genes upregulated upon acetic acid shock. All genes previously reported to be regulated by Haa1p, like *TPO2, TPO3, YGP1* and *PHM8* etc. have reduced expression in acetic acid challenged *haa1Δ* cells, whereas 73 genes were identified as novel to Haa1p mediated acetic acid response in yeast. These novel set of Haa1p target genes regulated in response to acetic acid belongs to following functional classes: environmental stress response (*HOR2, SSE2* etc), carbohydrate metabolism (*NRG1, HKR1, TOS3, PCL10* etc), nucleic acid processing (*SYC1, SAP30* etc), lipid metabolism (*INMI, YPC1, SUR2* etc), cell wall and plasma membrane (*SPI1, PDR12, AQR1* etc), transcription factors (*MSN4, STP3/4, FKH2* etc), amino acid metabolism and at least 40% with unknown function (figure 1.6 (a)). Moreover YEASTRACT database analysis to classify acetic acid induced genes based on documented weak acid induced regulons, has shown that Haa1p specifically regulates almost 45% (51 out of 112 genes) of the acetic acid induced genes, although considerable overlap exists with Msn2/4p (23 genes), Rim101p (3 genes), War1p (1 gene) regulon/s and 6 genes appeared to be controlled by Haa1p, Msn2/4p and Rim101p regulons (Figure 1.6 (b)) (Mira et al., 2010a).

1.8 Stationary phase mediated acetic acid resistance in yeast:

1.8.1 Introduction:

Eukaryotic cell proliferation is controlled by specific growth factors and availability of essential nutrients. If either of these signals is absent, cells may enter a specialized non dividing resting state, known as stationary phase or G0. The yeast *S. cerevisiae* utilizes
fermentable sugars such as glucose as preferable carbon source. When yeast cells are grown in liquid media containing glucose, they metabolize glucose by glycolysis and release ethanol in media. When glucose becomes limited, the yeast cells enter a diauxic shift characterized by decrease in growth rate and metabolic shift from glycolysis to aerobic utilization of ethanol. Once ethanol is depleted from media and no other carbon source is available, yeast cells enter quiescent or stationary phase G0 (Figure-1.8). Similar manifestation of growth arrest is also seen when yeast cells are starved for nitrogen or phosphate (Zhang et al., 2009). Yeasts cells in diauxic shift or stationary phase are stressed by lack of nutrients and by accumulation of toxic metabolites from oxidative metabolism. Such cells are differentiated in such a way that cell viability is maintained under in nutrient limiting conditions. These cellular responses to stress are very much similar to cells undergoing stress response, such as induction of heat shock proteins and accumulation of trehalose (Gray et al., 2004).

1.8.2 Cellular manifestation:

The yeast cells in stationary phase are undividing, unbudded and contain unreplicated nuclear DNA. In such cells rate of transcription is reduced by 3-5 fold, whereas translation decreases to 0.3% of proliferating cells (Galdieri et al., 2010). Stationary phase cells exhibit thick and more rigid cell wall structure and increased resistance to cell wall perturbing agents (de Nobel et al., 2000). On approaching stationary phase, yeast cells accumulate storage sugars like glycogen, trehalose, triacylglycerol and polyphosphates. Accumulation of glycogen begins before depletion of glucose and peaks at diauxic shift; it is further utilized as storage polysachharide, whereas accumulated trehalose protects cells against stress. Entry into stationary phase also results in condensed chromosome structure, more abundant mitochondria and induction of autophagy (Gray et al., 2004). Stationary phase cells are also more resistant to heat shock, osmotic shock and toxic drugs and are able to induce transcriptional response to different stresses as exponentially growing cells (Galdieri et al., 2010). Most importantly stationary phase cells have ability to respond to nutrient availability and reenter the mitotic cycle of cell division (Martinez et al., 2004).

1.8.3 Regulation of entry into stationary phase:

Entry into stationary phase is highly regulated process and involves protein kinase A (PKA), TOR, Snf1p and Rim15p signaling pathways that signal availability of nutrients (Figure-1.9). PKA pathway plays inhibitory role in transition from exponential growth to diauxic shift and stationary phase. Cells with increased PKA activity fail to enter stationary phase and die early.
Figure 1.8: Growth phase exhibited by *S. cerevisiae* on glucose based media (Herman et al., 2002).

Figure 1.9: Overview of signaling pathways that control transcription during transition from exponential growth to diauxic shift and stationary phase in yeast (Galdieri et al., 2010).
on nutrient limitation, whereas cells with decreased PKA activity shows stationary phase like features even when glucose is abundant (Zaman et al., 2008). In yeast, PKA signaling pathway is activated by Ras proteins (Ras1/2p) and it further inhibits Msn2p transcription factor and Rim15p protein kinase (Gorner et al., 2002; Reinders et al., 1998). TOR (target of rapamycin) pathway also inhibits transition into stationary phase. In yeast, Tor1p or Tor2p and three other proteins comprise TOR1C complex which responds to growth conditions and availability of nutrients. Inhibition of TOR1C by rapamycin or nitrogen starvation results in decreased protein synthesis, induction of autophagy and entry into G0 state (Shamji et al., 2000). Inhibition of TOR1C results in activation of genes responsive to limitation of nitrogen and carbon by regulation of Gln3p and Gat1p transcription factors. On the other hand inhibition of TOR1C induces Sch9p (homolog of mammalian PKB/Akt kinase) dephosphorylation and inactivation, which in turn triggers nuclear localization of Rim15p that regulates entry into stationary phase (Swinnen et al., 2006). Snf1p and Rim15p dependent signaling pathways positively regulate entry into stationary phase. The Snf1p complex has Snf4p as regulatory subunit and Snf1p as catalytic subunit, in presence of glucose Snf4p binds Snf1p and inactivates its function. In absence of glucose Snf1p is released from Snf4p and results into activation of transcription via Mig1p and Adr1p. Mig1p is involved in transcriptional repression in presence of glucose and Snf1p mediated phosphorylation of Mig1p releases this repression. Adr1p is transcription factor involved in activation of genes involved in catabolism of nonfermentable carbon sources. Rim15p also positively regulates the entry into stationary phase and functional Rim15p is required for smooth transition from exponential to stationary phase of growth. Rim15p activates transcription factors Msn2p, Msn4p and Gis1p, which in turn regulates the expression of genes required for entry and survival into stationary phase (Galdieri et al., 2010).

1.8.4 Acetic acid tolerance:

Yeast cells on entering the stationary phase show resistance to many stress conditions, such as oxidative stress and heat shock. Recently Burtner et al (2009) have shown acetic acid acts as a mediator of cell death during stationary phase of growth. When yeast cells growing in minimal media (SC media) enter stationary phase, pH of media decreases to acidic range. Reduction of pH is further shown to result from release of acetic acid, malic acid, citric acid, pyruvic acid and oxalic acid in media from yeast cells. Among these acids accumulation of acetic acid at low pH causes reduced cell viability and chronological life span (CLS) of yeast.
cells in stationary phase. When yeast cells are grown in media with dietary restriction (reduced glucose content, from 2% to 0.5% or 0.05%) reduction in pH by accumulation of acetic acid is not observed, thus yeast cells in stationary phase have increased viability and CLS. This increase in viability and CLS is also observed when yeast cells are grown in media with 2% glucose with some osmotic stabilizer, such as sorbitol or NaCl, though acidification of media occurs. These results indicate that viability of yeast cells in stationary phase can be increased by either reducing the accumulation of acetic acid or providing yeast cells resistance to oxidative stress caused by acetic acid. Furthermore, deletion of $SCH9$ and $RAS2$, known to be involved in progression of stationary phase, results in increased resistance to acetic acid. Resistance of $sch9\Delta$ to acetic acid depends on presence of Rim15p and Gis1p, which are known to be involved in yeast cell’s entry and survival in stationary phase (Burtner et al., 2009). These results indicate that resistance to acetic acid in yeast cells is fundamental to survival in stationary phase. Therefore when yeast cells enter the stationary phase it activates several stress responsive mechanisms that result into resistance to acetic acid.