CHAPTER I

Introduction and Review of Literature
Chapter 1-Introduction

The yeast *Saccharomyces cerevisiae* is the backbone of ethanol fermentation industry owing to its capability for rapid and efficient conversion of sugars into ethanol. During fermentation it is subjected to multiple stress conditions that include high initial substrate concentration, accumulation of high concentration of ethanol and toxic byproducts, and decrease in pH of the medium (Pampulha & Loureiro, 1989, Attfield, 1997). It is also subjected to higher temperature during fermentation, particularly in tropical countries. These conditions, especially ethanol and high temperature (Piper, 1995), have synergistic effects on the viability and efficiency of yeast to produce ethanol. We refer to this ability of yeast cells to withstand these conditions as "Fermentation Stress Tolerance" (FST) as it is not always possible to clearly separate the effect of one stress condition from another prevailing during fermentation. FST is poorly understood due to the lack of plate screen that can simulate the conditions encountered by yeast within the liquid fermentation broth. Thus, isolation of mutants impaired in FST and identification of corresponding genes is quite difficult. To circumvent these limitations, earlier we have reported a method that can simultaneously monitor the fitness of individual transposon insertion mutants of yeast present as a mixed population in liquid broth, and used the same for studying the role of several genes critical for FST (Sharma, et al., 2001). A major limitation of such mutant screens is that it is not possible to get loss of function mutants of essential genes; besides among duplicate (redundant) genes, mutation in a copy often does not give a phenotype. Thus we took an alternative approach of screening a genomic overexpression library of yeast, to identify genes that upon overexpression would prolong the survival of yeast cells during fermentation. Yeast *RPII* (Ras cAMP Pathway Inhibitor) gene was identified in such a screen (Puria, 2006).

*RPII* gene was initially identified as putative inhibitor of cAMP pathway, which down regulates the activity of Ras protein activity in *S. cerevisiae*. Over-expression of *RPII* suppresses the glucose induced cAMP signal and its deletion results in heat sensitive phenotype (Kim & Powers, 1991). Large scale screening for yeast genes that are regulated by transcriptional activator Yap1p identified *RPII* as one of the target genes, which was found to be down regulated during the early phase of growth but up-regulated in the stationary phase or under oxidative stress (Dumond, et al., 2000). *RPII* was also identified as a multicopy suppressor of glucose-induced loss of heat resistance.
in hsp104Δ-hxx2Δ-tps1Δ triple mutant of yeast (Versele & Thevelein, 2001). Later, it was also identified as a multicopy suppressor of cell lysis defect associated with the loss of function of the MPK1 of the cell wall integrity pathway (Sobering, et al., 2002). It was shown that RPII is not directly associated with Ras cAMP pathway, but helps the cells in preparation for stationary phase. Besides this, it was also reported as a putative transcription factor, as it resides in the nucleus, possesses a C-terminal transcriptional activation domain, and enhances the mRNA levels of a subset of genes involved in cell-wall metabolism (Sobering, et al., 2002). Previous studies from our lab showed that overexpression of RPII, prolongs the viability of yeast cells and its disruption dramatically decreases cell survival during ethanolic fermentation without any effect on growth or viability of yeast cells during normal growth (Puria, 2006).

Although the above studies convincingly proved that RPII is critical for FST, the actual mechanism involved in stress tolerance was not clear. Thus, we focused our studies on this gene and tried to understand how it mediates its phenotype. So, the broad objective of this study is to understand the molecular mechanism of stress tolerance mediated by RPII under fermentation conditions. We addressed this by two approaches. In one approach, we tried to identify and characterize tolerance conferred by RPII to individual stress conditions encountered by yeast cells during fermentation, such as high concentration of ethanol, high temperature, low pH and high osmolarity. Besides, we also studied the effect of RPII on cell wall integrity (Chapter 3). We hoped that these will clarify the precise protection conferred by RPII and can provide further insight into its mode of action. Since Rpi1p was suggested as a putative transcription factor (Sobering, et al., 2002), we took another approach, of microarray expression profiling, to identify and characterize genes regulated by Rpi1p. The differential expression of many identified genes was further validated by quantitative reverse transcription PCR. Attempts were also made to identify upstream regulators of RPII gene, in order to better understand its transcriptional regulation, and biological process in which it is involved (Chapter 4). Besides the molecular characterization of RPII, we have also characterized several other important FST genes identified through genome scale fitness profiling strategy (Chapter 5). We envisage that characterization of these genes is likely to provide additional insight into stress tolerance during ethanolic fermentation.
Chapter-1 Review of Literature

1.1 Yeast and Ethanol production

Ethanol fermentation is the biological process by which sugars such as glucose, fructose, and sucrose are converted into cellular energy along with the formation of ethanol and carbon dioxide. Ethanol fermentation carried out by *Saccharomyces cerevisiae*, is classified as anaerobic process, since it does not require oxygen (Fig.1.1). It is responsible for the rising of bread dough, production of ethanol in alcoholic beverages, and for industrial production of ethanol for use as a fuel.

![Fermentation Diagram](image)

**Figure 1.1. Fermentation.** During the process of fermentation, glucose is converted into two molecules of pyruvate by glycolysis. The pyruvate is de-carboxylated to ethanol and CO₂. The overall process generates two molecules of ATP and NAD⁺ which is further recycled into the process.

1.1.1 Historical Perspective

Yeast is a simple, unicellular fungus belonging to the phylum Ascomycota of the Kingdom Fungi (Whittaker, 1970). The word "yeast" comes from the Indo-European root yes-, meaning *boil*, *foam*, or *bubble*. It has been used as leavening agent in bread making and in fermentation of alcoholic beverages from prehistoric times. The most common forms of yeast are baker's and brewer's yeast – which are strains of species *Saccharomyces cerevisiae*. The history of fermentation dates back to 4000 years, referred in Egyptian's paintings and drawings related to baking chambers and breweries. However, the organism was first seen microscopically in 1680 by Dutch naturalist and Father of Microbiology, Antoine van Leeuwenhoek. It is Antoine-Laurent de Lavoisier in
1789 (Father of Modern Day Chemistry) for the first time described the process of fermentation, in which he described how sugar is converted into carbonic acid gas and spirit of wine, and coined the term which is related to Arabic word ‘alcohol’ (Lavoisier., 1793). Later French chemist Joseph Gay-Lussac investigated fermentation (1815) his findings are astonishingly close to present-day estimates of conversion of sugar to ethanol and carbon dioxide.

\[
\text{Yeast} \\
\text{Sugar} & \rightarrow 2\text{Ethanol} + 2\text{CO}_2 \\
(100\text{g}) & (51.34\text{g}) & (48.66\text{g})
\]

It was in 1857 French microbiologist Louis Pasteur proved in the paper "Mémoire sur la fermentation alcoolique" that alcoholic fermentation is a biological process and performed by living yeast cells rather than chemical reaction which was earlier believed by his predecessors. Pasteur showed that bubbling oxygen into yeast broth increases cell growth but at the same time inhibits the process of fermentation - this observation was later called as Pasteur effect (Barnett, 2003). The use of pure yeast culture in alcoholic beverage, which revolutionized the fermentation industry, was by Emil Christian Hansen from the Carlsberg Brewery in 1883. Later use of pure yeast culture in wine fermentation was reported by Muller-Thurgau from Germany in 1890 (Dequin, 2001). Since then fermentation industry has flourished and extensive research and technological development has been accomplished in this particular field (Stanbury, et al., 2003)

1.1.2 Fermentation Technology in India

Production of alcoholic beverages, as mentioned in the literary texts, is more than 3000 year old in India. Sweet substance known as Soma juice prepared by the Vedic Aryans is supposed to be the first product of fermentation in India. The Rig-Veda (1500 BC) indicates that fermentation technology originated with the preparation of Soma juice and later with another drink, known as Sura (wine/beer), prepared by fermentation. These two preparations were used in different formulations of medicine, surgical applications and in many chemical and alchemical operations (Forbes, 1965). In the beginning, fermentation was mainly associated with the preparation of spiritual drinks, but later it was used for other purposes (Mahdihassan, 1981). It is also believed that acetic acid fermentation was known to Indians since the early times. Curd is another very popular fermentation product described in the Rig-Veda and it is still a popular food. The
technology of curdling milk is also found in a number of texts associated with *Yajurveda*.

### 1.1.3 Uses of *Saccharomyces cerevisiae*

**Model Eukaryotic organism:** The alternating haploid/diploid life cycle, growth in defined media, shorter generation time of about 90 min, and amenability to easy genetic manipulation by conventional genetic crosses and recombinant DNA technology make *S. cerevisiae* a popular choice for molecular and cellular biologists. It is also considered to be one of the model eukaryotic organisms. The Nobel Prize for medicine in the year 2001 was given to Leland H. Hartwell, R. Timothy Hunt and Paul M. Nurse for their seminal discoveries regarding the cell cycle and cancer (Hartwell worked on *S. cerevisiae* and Nurse on *S. pombe*). The yeast cells divide mitotically by bud formation and the progression of the cell cycle can be easily monitored by bud size, which was used to isolate large number of cell division cycle (*cdc*) mutants (Nurse, 2002). Since this organism can be grown on completely defined media, many nutritional auxotroph have been isolated (Ausubel, *et al.*, 2002). This has not only permitted the analysis of the complex metabolic pathways but also provided a large number of mutations useful for genetic analysis. Moreover, with the availability of complete genome sequence of this organism in year 1996 (Goffeau, *et al.*, 1996), over 20,000 knockout strains have been created by consortium of yeast labs under the Saccharomyces Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). Four different mutant collections of strains (haploids of both mating types, homozygous diploids for non-essential genes and heterozygous diploids for the essential and non-essential ORFS) were constructed under this project and are available to all researches, which further simplify working with yeast.

**Baking:** The most common yeast used in baking is *S. cerevisiae*. It is used as leavening agent, where it converts the fermentable sugar in the dough into carbon dioxide (CO₂). As CO₂ rises it expands the dough and leaves behind pockets or holes. When the dough "sets" the pockets remain and give the soft and the spongy texture to the baked products.

**Beer:** It is a fermented product derived from fermentation of malt of germinated barley grains, contains 4 to 5% ethanol. There are two types of beer, ale type (top fermenting) uses strains of *S. cerevisiae* and the lager type (bottom fermenting) uses strains of *S. pastorianus*. Another type of beer, also called as Belgian beer, involves species of *Brettanomyces*. 
Wine: It is a distilled beverage, derived from fermented juice of grape, contains 10 to 15% ethanol. The fermentation can be done with endogenous wild yeast, but pure yeast cultures, mostly *S. cerevisiae*, is preferred to get reliable and predictable product.

Fuel Ethanol: It is basically ethyl alcohol, mainly used as fuel or bio-fuel alternative to gasoline. It is used with blended gasoline and widely used in Brazil and USA. Two technologies are used for the mass production of fuel ethanol, one is bio-ethanol, usually derived from fermentation of sugar, and the other is derived from hydration of ethylene (CH$_2$=CH$_2$) or petroleum products. However current interest for the mass production of bio-ethanol has shifted to the use of plant materials which are rich in cellulose and starch.

Besides *S. cerevisiae*, other organisms that can be used for ethanol production include strains of *Kluyveromyces marxianus*, *Zymomonas mobilis* and *Thermoanaerobacter ethanolicus*. These organisms have certain advantages over *S. cerevisiae*, like fermentation at higher temperature and utilization of lingo-cellulosic material (Singh, *et al.*, 1998, Dien, *et al.*, 2003). However *S. cerevisiae* is still a preferred organism, due to its faster growth, tolerance to high concentration of ethanol, low pH and growth under anaerobic conditions.

### 1.2 Fermentation Stress Tolerance

During the process of fermentation yeast cells experience a number of stress conditions such as osmotic stress due to high initial substrate concentration and toxicity due to ethanol produced and decrease in pH of the medium. The exposure of yeast cells to these stress conditions is responsible for decline in cell vitality and viability, which has substantial impact on ethanol productivity.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Ethanol, Temperature, gradual accumulation of metabolities, Toxic acids (acetic acids), sulphites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutritional deficiencies</td>
<td>Nitrogen, Minerals, Vitamins, Ergosterol and unsaturated fatty acids</td>
</tr>
<tr>
<td>Antagonism</td>
<td>Other fungi, lactic acid bacteria and “killer” toxins</td>
</tr>
<tr>
<td>High initial substrate concentration</td>
<td>Molasses, Cellulosic materials</td>
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Fermentation stress increases the lag period, decreases rate of fermentation and limits the life span of re-pitched (recycled) yeast. All these adverse conditions can lead to stuck or suboptimal fermentations (Ivorra, *et al.*, 1999), where growth of yeast cells and ethanol production can come to a virtual standstill. The causes of stuck fermentation are listed...

1.2.1 General Stress Response

To survive different environmental fluctuations, and to maintain the internal steady state homeostasis, cells have developed adaptive stress tolerance mechanisms. These cellular responses lead to change in gene expression and require signal transduction pathways to communicate from the sensors on cell surface or cytoplasm to transcriptional machinery located in the nucleus to elicit stress response (Gasch et al., 2002). The molecular basis of these responses have been extensively studied in *S. cerevisiae* and other related yeast species which reveal that each species responds to environmental changes with a great deal of precision in terms of the genes affected, the magnitude of their expression and the kinetics of the response. Yeast cells respond with a common gene-expression programme, referred to as environmental stress response (ESR) (Ruis & Schuller, 1995). The ESR was found to be transient and involves genes controlled by STRE (Stress Response Elements) and HSE (Heat Shock Elements). The ESR was originally described in *S. cerevisiae* as a set of ~300 genes whose expression is induced and ~600 genes whose expression is repressed in response to diverse types of stress (Causton, et al., 2001, Gasch & Werner-Washburne, 2002). Induced ESR genes were found to be regulated by transcription factors Msn2p/Msn4p. These include genes involved in trehalose bio-synthesis and degradation, oxidative and intracellular redox homeostasis and DNA damage and repair process; other identified genes encode several chaperones and transport proteins. The genes which are mostly repressed are involved in ribosomal RNA transcription and processing, and ribosomal proteins, which probably explains the energy saving and growth arrest adopted by cells.

It has also been previously shown that majority of tricarboxylic acid cycle (TCA) genes are subjected to glucose repression (DeRisi, et al., 1997), even at an extracellular glucose concentration as low as 0.1 g/l (Yin, et al., 2003). During the process of fermentation *S. cerevisiae* does not require fully functional mitochondria because sufficient energy is provided by glycolysis and fermentation. It was found that expression of genes encoding mitochondrial ribosomal proteins (MRPs), those encoding cytoplasmic ribosomal proteins (CRPs) and those encoding the rRNA processing proteins are not coordinated, instead MRP expression was correlated with the expression of stress genes induced during the slower respiratory growth in non-fermentable carbon
sources, such as glycerol and ethanol (Ihmels, et al., 2005). However there are reports in which it is highlighted that the relative in vivo respiratory activity of tricarboxylic acid cycle may increase even at high glucose concentration, provided the growth rate or glucose uptake rate are impaired by stress or other environmental parameters (Blank & Sauer, 2004). Similar studies with metabolic flux analysis with hexose sugars showed that there is a strong correlation between growth rate and tricarboxylic acid cycle activity, suggesting that yeast alters its physiology from almost exclusive fermentation to partial respiration even in the presence of high concentration of glucose (Mensonides, et al., 2002).

1.2.2 Stress Signaling in Yeast

1.2.2.1 The cAMP-PKA Pathway

The cAMP protein kinase (PKA) pathway mediates cellular function in response to external glucose. It is the major glucose sensing pathway involved in many cellular processes including nutrient sensing, regulation of yeast cell proliferation, carbon storage and stress response (Thevelein, 1994, Thevelein & de Winde, 1999). The cAMP protein kinase (PKA) pathway is composed of GTP binding protein Ras2p and GTP hydrolyzing Ras1p protein which in turn regulates the activity of adenylate cyclase (Cyr1p). Ras proteins, shuttling between the GDP and GTP bound forms, play diverse physiological roles by mediating signaling for receptor protein kinases and tyrosine kinase-associated receptors. The cAMP generated activates downstream proteins which comprise of a regulatory subunit, encoded by the Bec1p and three catalytic subunits encoded by the Tpk1p, Tpk2p and Tpk3p (Fig. 1.2). The cAMP protein kinase (PKA) regulates cellular responses to the STRE-mediated stress (Belazzi, et al., 1991, Hasan, et al., 2002), the Pos9/ Skn7-mediated oxidative stress (Charizanis, et al., 1999), hyperosmotic stress (Hirata, et al., 1995), and high temperature stress (Norbeck & Blomberg, 2000). The role of the cAMP protein kinase (PKA) pathway as sensor of the nutritional status of the cell has been suggested by the phenotypes shown by mutants in this pathway (Matsumoto, et al., 1985). The activation of cAMP protein kinase (PKA) causes transient changes in several key molecules, including trehalose and glycogen metabolism, glycolysis and gluconeogenesis, which contain components controlled by PKA-mediated phosphorylation. It was found that there is a strong correlation between the activity of PKA and the extent of thermo tolerance; cells with low PKA activity are remarkably thermotolerant whereas cells with high constitutive PKA are thermosensitive under all
conditions (Ruis & Schuller, 1995). The STRE which is controlled by PKA pathway has core consensus sequence element, AGGGG and so far 186 genes have been reported to be regulated by STRE in yeast genome (Ruis & Schuller, 1995). Besides these, in relation to trans-acting factors involved in STRE-mediated gene expression two zinc finger proteins Msn2p and Msn4p have been reported (Smith, et al., 1998, Watanabe, et al., 2007). These two transcription factors switch between nucleus and cytosol and are negatively controlled by PKA in response to stress (Gorner, et al., 1998, Garreau, et al., 2000). Recent findings suggest that mRNA export of Msn2/4p and Hsf1 proteins, on exposure to stress like temperature and ethanol, is controlled by Rsp5p, an essential protein with multi functional E3 ubiquitin ligase complex (Haitani & Takagi, 2008).

Figure 1.2. Protein Kinase A signaling pathway. The PKA mediates cellular function in response to nutrient status. The signaling component involves various transporters and G-protein coupled receptor (involved in glucose sensing) located in the plasma membrane. The effectors branch includes GTP binding and hydrolyzing proteins RAS1/2, which in turn activates adenylate cyclase (Cyr1p). The PKA gene is characterized by regulatory and catalytic subunits of Tpk1p and Bcy1p. The processes which are activated by PKA, shown as ↑ and down-regulated as ↓. Recent evidence indicates that Sch9p and proteins involved in nitrogen catabolism showed cross-talk with PKA pathway. (Adapted from Tamaki, 2007, and Hasan et al., 2002)
1.2.2.2 Tor Pathway

The target of rapamycin (TOR) is phosphatidylinositol related conserved Ser/Thr kinase that controls cell growth by activating an array of anabolic processes including protein synthesis, transcription and ribosome biogenesis, and by inhibiting catabolic processes such as mRNA degradation and autophagy (Fig. 1.3). It was originally identified by mutations in yeast genes TOR1 and TOR2, which confer resistance to the growth-inhibitory properties of the immunophilin-immunosuppressant complex FKBP (FK506 binding protein)-rapamycin (Heitman, et al., 1991). The TOR complex 1 (TORC1) modulates translational initiation, inhibits protein turnover, represses transcription of specific genes and consists of either Tor1p or Tor2p, together with Kog1p, Lst8p and Tco89p (Inoki, et al., 2005).

![Figure 1.3. TOR pathway.](image)

**Figure 1.3. TOR pathway.** Target of rapamycin pathway is involved in diverse growth related process. It activates genes related with growth and cell proliferation, like tRNA synthesis and ribosome’s biogenesis shown as ↑ and down regulates genes involved in stationary phase or autophagy shown as ↓. (Duvel and Broach, 2004)

The TOR complex 2 (TORC2) is involved in regulating actin cytoskeleton, cell polarization during cell cycle, cell wall integrity and contains only Tor2p along with other accessories proteins viz., Avo1p, Sml1p (Reinke, et al., 2004). It is only TOR complex 1 which is sensitive to drug rapamycin while TOR complex 2 is insensitive to the drug because rapamycin-Fp11p does not bind to Tor2p when it is present with TORC2 (Zheng, et al., 1995, Loewith, et al., 2002). The signaling of TOR to the translation machinery is via Tap42p, an essential protein, which physically associates with the protein phosphatase 2A (PP2As) and the SIT4 protein phosphatase catalytic subunits, in a nutrient dependent manner (Hall, 1996, Duvel & Broach, 2004). Tap42p has been demonstrated to be directly phosphorylated by Tor2p and this phosphorylation promotes formation of the Tap42p-PP2A complex (Jiang & Broach, 1999). Tap42p also
nutrient conditions, TOR promotes binding of Sit4p to Tap42p and thereby maintains Sit4p inactive. Upon nutrient limitation or rapamycin treatment, conditions that inactivate TOR, Sit4p dissociates from its inhibitor Tap42p. Liberated and activated Sit4p in turn dephosphorylates and activates targets such as the transcription factor Gln3p, the kinase Npr1p, and scaffold protein Tip41p. Gln3p and Npr1p are involved in scavenging or synthesizing alternative nutrient sources. However, dephosphorylated Tip41p, as part of a feedback loop, binds and inhibits Tap42p, and thereby further amplifies Sit4p activity (Duvel & Broach, 2004, Nakashima, et al., 2008). Thus, TOR negatively regulates Gln3p, Npr1p, and Tip41p by inhibiting Sit4p (Fig. 1.4).

![Figure 1.4. TOR effectors and signaling pathway](image)

**Figure 1.4. TOR effectors and signaling pathway.** The TOR genes (TOR1/TOR2) act as response regulators to changes in nutritional status of cell. They mediate signaling through TAP42p (Two A Phosphatase Associate protein) which forms complex with protein phosphatase PP2A complex, which in turn activates (↑) or inhibits (↓) translation or transcription. In normal growth conditions, (TOR1/TOR2) repress activity of stress responsive transcription factor Msn2/4p independent of TAP42 pathway. Tor2p is also involved in cell cycle dependent polarization of actin cytoskeleton, which is mediated through PKC1 pathway (for details refer to text). (Schmelzle and Hall, 2000)

In yeast, TOR complex 2 (but not TOR complex 1) additionally controls cell cycle-dependent polarization of the actin cytoskeleton. Genetic and biochemical evidence has shown that TOR complex 2, via the exchange factor Rom2p, activates the Rho1p GTPase (Schmidt, et al., 1998, Schmelzle & Hall, 2000). Rho1p, in turn, signals to the actin cytoskeleton via its direct effector Pkc1p and a PKC1-activated MAP kinase cascade. The finding that yeast TOR controls both translation and organization
of the actin cytoskeleton indicates that TOR integrates temporal and spatial control of cell growth. TOR also negatively regulates stress responsive genes, starvation specific genes, nitrogen pathway associated genes and retrograde target genes by sequestering several responsive transcription factors in the cytoplasm, namely Rtg1p/Rtg3p and Msn2p/Msn4p, but it is independent of Sit4p (Schmidt, et al., 1998, Beck & Hall, 1999, Komeili A, et al., 2000). The sequestration of Msn2p/Msn4p is mediated post transcriptionally possibly by promoting their association with the ubiquitously conserved cytoplasmic 14-3-3 proteins Bmh1p and Bmh2p which are involved in signal transduction (Inoki, et al., 2005). Beside this TOR pathway is also found to be involved in tolerance to salt stress. The transcription of ENA1, a gene encoding a lithium and sodium ion transporter which is essential for salt tolerance, is strongly induced under TOR inactivating conditions. Moreover, the absence of the TOR controlled GATA transcription factors Gln3p and Gat1p results in reduced basal expression and induced expression of Ena1p under salt stress (Crespo, et al., 2001).

1.2.2.3 Correlation between Target of Rapamycin and cAMP Protein Kinase Pathway

The control of cell growth and morphogenesis is a complex process and involves number of coordinated signaling cascades. Several studies have established that there are cross talks among the different components of signaling pathways. However, despite this cross talk, specialization of these pathways can be restored further downstream in the pathway by coordinating inputs from other signaling cascades and targeting different molecular effectors.

TOR regulates number of genes; in nutrient rich condition, it represses the expression of the nitrogen catabolite-repressed (NCR), retrograde response (RTG) and stress-responsive (STRE) genes. Concomitantly, it activates the expression of genes required for ribosome biogenesis, including the ribosomal protein genes. The extraordinary diversity of genes that are affected by TOR signaling implies that, in almost every case, these genes are also regulated by other signal transduction cascades. TOR and PKA are two prominent, evolutionarily conserved signal transduction cascades that couple nitrogen and carbon source availability and regulate diverse cell responses that ultimately drive cell growth and proliferation (Zurita-Martinez & Cardenas, 2005). Remarkably, TOR and the PKA pathways control a number of common functions, including ribosome biogenesis (ribosomal protein gene transcription and RNA
polymerase I and III activity), and responses to stress. In addition, TOR and PKA signaling converge on the Rim15p kinase to control distinct transcriptional and metabolic traits required for proper entry into G0, a key developmental transition in yeast cell cycle (Cutler, et al., 2001, Pedruzzi, et al., 2003). Since these signaling cascades regulate myriad functions, it is not surprising that these pathways converge to regulate the expression of several genes involved in various processes such as glycogen accumulation, cell division, amino acid metabolism and down-regulation of genes involved in glucose transport (Hxt1p), and STRE regulated genes. Recent evidence indicates that TOR controls the sub-cellular localization of both the protein kinase A catalytic subunit Tpk1p and the RAS/cAMP signaling-related kinase Yak1p, which suggest that PKA may act as a novel TOR effectors branch (Schmelzle, et al., 2004).

1.2.2.4 Mitogen Activated Protein Kinases Pathway

The Mitogen Activated Protein Kinases (MAPK) is a series of three kinase proteins which work in cascades. The cascades activate the phosphorylation of MEKK, which in turn phosphorylates MEK and MAPK. The budding yeast S. cerevisiae has at least five signal pathways containing MAPK cascade: 1) the cell-wall integrity pathway, 2) the spore wall assembly pathway, 3) the filamentous/invasive growth pathway, 4) the pheromone response pathway, and 5) the high osmolarity glycerol (HOG) pathway (Posas, et al., 1998, Saito & Tatebayashi, 2004) (Fig.1.5). Transmission of signals via these cascades is usually initiated by activation of a small G protein, followed by sequential stimulation of several sets of cytosolic protein kinases. Since high osmolarity glycerol (HOG) and protein kinase-C (PKC) signaling pathways are associated with fermentation stress, these are described below.

1.2.2.4.1 High Osmolarity Glycerol Pathway

Exposure of S. cerevisiae to increasing extracellular osmolarity activates the stress-activated high osmolarity glycerol (Hog1p) mitogen-activated protein kinase (MAPK), which is essential for cell survival upon osmotic stress. High osmolarity glycerol (Hog1p) pathway plays a critical role upon osmotic stress and induces expression of very large number of genes (Brewster, et al., 1993, Hersen, et al., 2008). The Hog1p is the key element in the high osmolarity (HOG) pathway which is activated upon by phosphorylation of conserved threonine and tyrosine residues, via two independent upstream mechanisms that converge on the Pbs2p MAPKK. Activated Hog1p rapidly, but transiently, accumulates in the nuclear compartment, where it
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Figure 1.5. Signaling cascade in yeast with MAPK Kinase module. The Mitogen Activated Protein Kinase in yeast is involved in five cellular processes, namely cell wall integrity, spore wall assembly, invasive growth, pheromone response and osmotic stress pathway. It is characterized by membrane sensor proteins (like Wsc1p, Ste2p), upstream kinase proteins (like Pkc1p, Ste20p) and three kinases which work in cascades (Bck1p, Ste11p→Mkk1p, Ste7p→Mpk1p, Ste12p). These kinases in turn activate various transcription factors located in the nucleus (Rim1p, Ste12p, Hot1p) and bring about the phenotype like cell wall construction, mating response or osmoregulation (the details related to Pkc1 and Hog1 pathway are described in the text). (Saito and Tatebayashi, 2004)

There are multiple substrates of Hog1p that are known to mediate these effects. Many of these are transcription factors including Msn2p/Msn4p (Schmitt & McEntee,
Sko1p (Pascual-Ahuir, et al., 2001), Hot1p (Reo, et al., 1999), Smp1p (de Nadal, et al., 2003) and Sko1p (Proft, et al., 2005) (Fig. 1.6).

**Figure 1.6. Osmotic stress response in yeast.** The osmotic shock in yeast is controlled by HOG1 pathway. It comprises of two transmembrane domain effectors sensor proteins; one is controlled by Sln1p and other by Sho1p. Both of these pathways converge at MAPKK Pbs2p which in turn phosphorylates Hog1p transcription factor. The phosphorylated Hog1p in turn controls other effectors genes like Hot1p, Msn2/4p, Hsp26p, Tdh3p, which are involved in processes like stress response, actin reorganization, glycerol production and cell cycle. (Adapted from Hersen et al., 2008).

Msn2p and Msn4p respond to various environmental stresses, whereas Sko1p specifically responds to osmotic stress (Proft, et al., 2005). Hot1p controls the expression of Gpd1p and Gpp1/Gpp2 genes that are important for glycerol production under high osmolarity conditions (Remize, et al., 2003). Over-expression of Smp1p induces Hog1p-mediated expression of osmoreponsive genes such as Stl1p (de Nadal, et al., 2003). Hog1p recruits Rpd3p histone deacetylase to promote the modification of the chromatin.
at the promoters to activate osmo-responsive genes (De Nadal, et al., 2004). Search for suppressors of hog1Δ lead to the identification of mutated TUP1/CYC8 which are components of general co-repressor (Bone & Roth, 2001). It was found that strain deleted in CYC8 is defective for the repression of Sko1p regulated genes but not other genes regulated by other DNA-binding proteins (Kobayashi, et al., 2008). Besides this, Kim & Shah (2007) identified four novel physiological substrates of Hog1p kinase, namely Krs1p, Tdh3p, Hsp26p and Shm2p which plausibly suggest the mechanisms of actin reorganization, cell cycle arrest and regulation of protein synthesis upon osmotic stress.

1.2.2.4.2 Protein Kinase C1 Pathway

The protein kinase C1 (MAPK) cascade is found in budding yeast as part of the cell integrity pathway. This pathway mediates cell cycle-regulated cell wall synthesis and responds to different signals including cell cycle regulation, growth temperature, changes in external osmolarity, and mating pheromone. Signaling proteins in the pathway include the GTP binding protein Rho1p, the protein kinase C homologue Pkc1p, the Bck1p/ Slk1p (MEKK), the redundant pair Mkk1p/Mkk2p (MEKs), the Slt2p/ Mpk1p (MAPK), and the transcription factor Rlm1p and Sbf1p, the latter being composed of the polypeptides Swi4p and Swi6p (Levin, et al., 1990, Kamada, et al., 1996, Levin, 2005) (Fig. 1.5). Membrane proteins that potentially provide input signals to the cell integrity pathway include Wsc1p/Hsc77p, Wsc2p, and Wsc3p (Gray, et al., 1997, Verna, et al., 1997). The PKC1 was found to be most similar to the classical mitogen-activated ERK1-ERK2 MAPK cascade reported in higher eukaryotes (Blumer, et al., 1994).

Studies with pck1Δ has shown that upon addition of glucose into the medium the initiation of fermentation was delayed, as measured on the basis of ethanol produced, and this was found to be due to repressed activity of glucose transporters Hxt1p, Hxt2p and Hxt4p. This effect was not shared by the downstream cascades and thus suggests the bifurcation in the PKC1 mediated pathway (Brandao, et al., 2002). Besides the role in control of carbon metabolism it was found that Slt2p-mediated MAPK pathway plays an important role in the adaptive response to alkaline pH stress condition. The expression analysis at high pH lead to the identification of several cell wall encoding proteins Fks2p, Dfg5p, Skt5p, and Chr1p, which were induced and are Slt2p dependent. Moreover, it was also established that Wsc1p participates as an important pH sensor for...
adaptive stress response (Serrano, et al., 2006). As described above and reported earlier that PKC1 pathway is induced due to temperature shift (Kamada, et al., 1995), recent report suggests that PKC1 response to heat stress is not due to heat \textit{per se} but due to heat stress induced trehalose synthesis (Tps1p) (Mensonides, et al., 2005). Protein ubiquitylation is well known for protein degradation, however recent reports suggest that it is involved in number of other cellular processes like control of gene transcription, DNA repair and replication, intracellular trafficking and virus budding (Haglund & Dikic, 2005). Recently the role of de-ubiquitinating enzyme Ubp3p and its association of PKC1 mediated signaling pathway were elucidated. Disruption of the UBP3 gene lead to an enhanced activation of the cell wall integrity pathway MAPK Slt2p, when cells were challenged with a variety of pathway activating agents such as pheromone and Congo red. The UBP3 mutants accumulate high levels of Pkc1p, suggesting potential regulation of Pkc1p by Ubp3p (Wang, et al., 2008).

The HOG1 and PKC1 pathways are thought to be regulated in opposite directions by changes in external osmolarity. However, it is observed that under certain conditions, cells require both the pathways for cellular adaptation. The initial osmotic shock during the process of fermentation causes cell shrinkage and activates both PKC1 and HOG1 pathways. Bermejo et al., (2008) pointed out that the activation of cell wall integrity response to the cell wall damage caused by Zymolyase (β-1,3 glucanase), requires both the HOG1 and SLT2 pathways. Zymolyase activates both MAPK and Slt2p and its activation is dependent of Sho1p branch of the HOG1 pathway. Moreover, adaptation to Zymolyase requires essential components of the PKC1 pathway, namely the redundant MAPKKs Mkk1p/Mkk2p, the MAPKKK Bck1p, and Pkc1p but it does not require upstream elements, including the sensors and the guanine nucleotide exchange factor of this pathway. In addition, the transcriptional activation of genes involved in adaptation to cell wall stress, like Crh1p, depends on the transcriptional factor Rlm1p regulated by Slt2p, but not on the transcription factors regulated by Hog1p (Bermejo, et al., 2008).

1.3 Ethanol Toxicity in \textit{S. cerevisiae}

Sugars are the major carbon and energy source for yeast in natural habitats and in industrial fermentation, and ethanol is the major product of sugar catabolism in yeasts. During fermentation glucose is converted to pyruvate, which is further decarboxylated to produce ethanol (Fig. 1.7).
Figure 1.7. Glucose metabolism pathways. The glucose is metabolized inside the cell through glycolysis into pyruvate, which either enters tricarboxylic acid cycle, operative in mitochondria, or metabolized to ethanol through fermentation, operative in cytoplasm. The glyceraldehyde phosphate (Gly3-PO4) is metabolized to glycerol by GPP1/2, genes whereas acetaldehyde is reduced to acetate by action of ALD genes. (Adapted from Nelson & Cox, 2004)

Figure 1.8. Possible targets of ethanol in yeast cells. The main deleterious targets of ethanol are cell membranes including nuclear, mitochondrial, endoplasmic reticulum membranes; it also affects membrane transporter proteins, cytosolic proteins, besides affecting membrane fluidity.

As cells start fermenting there is a gradual build up of ethanol which becomes toxic to the yeast cells, particularly at high temperature. Accumulation of byproducts such as acetaldehyde and organic acids is also toxic to cells. The major effects of these are inhibition of growth, increase in the size, formation of mitochondrial DNA deficient petite mutants, hampered nutrient uptake and reduced plasma membrane ATPase
activity (D'Amore, et al., 1990, Piper, 1995, Kubota, et al., 2004). The main impact of high ethanol concentrations is the disruption of membrane structure, affecting membrane transport systems which lead to increased membrane fluidity, permeability and passive proton influx (Fig. 1.8). Moreover, ethanol is as such permeable to plasma membrane and thus disrupts the osmotic balance of cellular components, and at the same time disrupts the plasma membrane due to its inherent capacity to form hydrogen bonds. There is an associated loss of membrane potential, leakage of electrolytes and amino acids. In addition, ethanol inhibits the activity of key glycolytic enzymes and denatures proteins.

1.3.1 The Yeast Plasma Membrane and Ethanol Toxicity

It has been proposed by several authors that yeast cell membrane is the principal site of action of ethanol. The presence of ethanol around the phospholipid bilayer weakens the water-lattice structure of the membrane and decreases the strength of interactions between fatty acids, thereby disrupting membrane integrity and promoting cell leakage (Salgueiro, et al., 1988, Chi & Arneborg, 1999). Partition of ethanol in the hydrophobic region of the membrane increases its relative polarity and thus increases membrane solubility which in turn perturbs the functions of membrane transporters (Ghareib, et al., 1988), decreases nutrient uptake by the cells (Calahorra, et al., 1987, Brauer, et al., 2008) and increases influx of protons, causing disruption of the proton-motive force (Piper, et al., 1994, Ogawa, et al., 2000) and intracellular acidification (Narendranath, et al., 2001). Ethanol-induced passive proton influx is thought to trigger the activity of the plasma membrane H⁺-ATPase (Pma1p), the enzyme largely responsible for maintenance of the plasma membrane proton gradient (Alexandre, et al., 1994, Aguilera, et al., 2006, Dinh, et al., 2008). Thus it might be expected that the cell would be able to quickly recover its proton gradient following ethanol stress, but it does not happen, perhaps due to Hsp30p synthesis, which is also induced during ethanol stress (Alexandre, et al., 2001), which is an inhibitor of the plasma membrane H⁺-ATPase (Piper, et al., 1994, Piper, et al., 1997)

1.3.2 Ethanol and Cell Wall

The yeast cell wall is a dynamic structure which constantly changes depending on growth conditions and cell cycle. It represents ~30% of the dry weight of the cell and is composed largely of polysaccharides (~85%) and proteins (~15%) (Nguyen, et al., 1998)
Table 2, (Fig. 1.9). It is involved in an array of functions like osmotic homeostasis, physical protection to mechanical damage, cell shape maintenance and morphogenesis and acts as scaffold for extracellular protein (Klis, et al., 2006, Shimma, et al., 2006).

<table>
<thead>
<tr>
<th>Macromolecule</th>
<th>% of wall mass</th>
<th>Degree of polymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannoprotein</td>
<td>30-50</td>
<td>Highly variable</td>
</tr>
<tr>
<td>1,6-β-glucan</td>
<td>5-10</td>
<td>24†</td>
</tr>
<tr>
<td>1,3-β-glucan</td>
<td>30-45</td>
<td>240†</td>
</tr>
<tr>
<td>Chitin</td>
<td>2-6</td>
<td>25†</td>
</tr>
</tbody>
</table>

The macromolecular contents are represented in their order of occurrence from outside to inside of cell wall. †average number of monomers per chain.

Figure 1.9. Yeast cell wall. The yeast cell wall is largely composed of polysaccharides and proteins. The glucose, N-acetylglucosamine (GlcNAc) and mannose residues represent 80 to 90%, 1 to 2% and 10 to 20% of total polysaccharide respectively. Glucose are linked to other glucose residues through β-1,3 and β-1,6 linkages and to GlcNAc via β-1,4 bonds; Mannoproteins are linked through glycosylphosphotidylinositol anchors. The vegetative cell wall has layered by inner layer of glucans and chitins, and outer layers of mannoproteins. (Adapted from Klis et al., 2006)

The major pathway controlling cell wall is PKC1/SLT1 (as described in section 1.2.2.4.2) and Rlm1p as the downstream transcription factor. Besides these, additional signaling pathways may also regulate cell wall assembly; expression profile of deleted mutants of FKS1, GAS1, KRE6 and MNN9 (Terashima, et al., 2000, Lagorce, et al., 2003) reveal up-regulation of clusters of 79 genes as cell wall compensatory. These genes were also found to be up-regulated under stress conditions such as in presence of caspofungin, calcofluor white, congo red and Zymolyase (Boorsma, et al., 2004), all of which affect the cell wall. Ethanol seems to affect cell wall integrity, since ethanol tolerant strains were found to be resistant, and ethanol sensitive strains were sensitive to cell wall perturbing agents Zymolyase and calcofluor white. Studies with ethanol tolerant
mutant of sake yeast suggest that higher content of glycosylphosphotidylinositol (GPI)-anchored cell wall proteins (Spilp, Sedlp) renders cells, resistant to K1 killer toxin and Zymolyase (Ogawa, et al., 2000).

1.3.3 Genes Involved in Ethanol Stress

Several studies have tried to identify and characterize genes involved in ethanol tolerance by mutant screens. Augilera & Benitez (1986) described 21 monogenic ethanol sensitive mutants belong to 20 complementation groups. Studies with transposon insertion mutants in *S. cerevisiae*, under ethanol stress conditions, identified five genes *BEM2, PAT1, ROM2, VPS34* and *ADA2*, which are involved in cell wall integrity (Takahashi, et al., 2001). There are reports in which accumulation of trehalose (Ogawa, et al., 2000), increase in ergosterol content of membrane (Swan & Watson, 1998), increase in monounsaturated fatty acid and ATPase were correlated with ethanol sensitivity (Swan & Watson, 1997, Aguilera, et al., 2006). There are also reports in which disruption of *URA7* and *GAL6* genes was reported to increase the ethanol tolerance and fermentation rate of *S. cerevisiae* (Yazawa, et al., 2007). Besides these, several genome wide studies have identified genes involved in ethanol stress. Alexandre, et al., (2001) have studied the transcriptional response after short term ethanol stress and pointed out that there is a tight regulation of the energy pool at the level of the glycolysis, trehalose and glycerol metabolism. Comparison of ethanol tolerance of parent strain of sake yeast with ethanol tolerant mutants by expression profiling, showed that ethanol tolerant mutant exhibits multiple stress tolerance due to elevated expression of stress responsive genes and accumulation of glycerol and trehalose (Ogawa, et al., 2000). Transcriptome analysis of wine fermentation under enological condition pointed out that general stress response was established only in later stages of growth. TOR nitrogen-sensing pathway plays a key role in modifying transcription patterns by changing the expression of genes involved in nitrogen metabolism, carbohydrate storage, and with down-regulation of the ribosomal protein genes. Another interesting finding was the expression of genes from sub-telomeric regions which include many genes involved in fermentation (*SUC, MAL, MEL*), (Rossignol, et al., 2003).

Screening a collection of yeast deletion mutants (4,847 non-essential gene deletions) by their phenotypes yielded 256 genes that are important for cell growth in the presence of ethanol (Kubota, et al., 2004). Ethanol stress has been associated with increase in cell size and it was due to enhanced expression of Swel (negative regulator...
of Cdc28-Clb kinase). The addition of ethanol caused a delay in the cell cycle and this was associated with transient dispersion of F-actin, resulting in increase in cell size. This suggests that perturbation of actin localization by ethanol might be a primary signal for induction of the cell cycle delay (Kubota, et al., 2004). A screen with collection of deletion pool of yeast mutants, for sensitivity to 6% ethanol lead to the identification of 46 mutants. These mutants were found to be genes involved in vacuolar function, cell wall integrity pathway, and mitochondrial function, subunits of the co-chaperone complex (GimC) and components of the histone acetyl transferase (SAGA) complex. Among the mutants, several were also found to be sensitive to calcofluor white (14 mutants), sorbic acid (9), high temperature (5) and NaCl (3) (van Voorst, et al., 2006). Alper, et al., (2006) described that engineering of global transcriptional machinery Spt15p lead to improve ethanol tolerance and glucose utilization. Comparison of expression profiles of laboratory strains and those used for sake fermentation in presence of 5% (v/v) ethanol showed that genes involved in tryptophan biosynthesis are overexpressed, and addition of tryptophan to the culture medium or over-expression of tryptophan permease gene conferred ethanol stress tolerance to yeast cells (Hirasawa, et al., 2007). A recent study, based on growth of single deletion strains in 8% ethanol, identified 446 genes important in ethanol tolerance; these are involved in several biological processes including tryptophan, tyrosine and phenylalanine synthesis (TRP1, TRP2, TRP3, TRP4, TRP5, ARO1, ARO2 and ARO7) (Yoshikawa, et al., 2009). Genome wide transcriptome studies of strains better suited to wine fermentation showed increased expression of genes encoding Hsp26p, cytosolic Ald6p, and sulfur metabolizing enzymes (Cys4p, Hom6p, and Met22p) (Zuzuarregui, et al., 2006). Microarray analysis of yeast from 15 day wine fermentation identified 223 genes that were differentially expressed and were named as fermentation stress response (FSR) genes. Surprisingly the genes involved in respiration and gluconeogenesis were expressed despite the presence of glucose; these findings were contrary to the glucose repression. Moreover, the authors have suggested that it was ethanol rather than the nutrient depletion which seems to be responsible for the entry of cells to stationary phase under fermentation conditions (Marks, et al., 2008). An ethanol sensing protein Asr1p, which localizes exclusively to nucleus upon alcohol stress, was reported (Betz, et al., 2004). However in other study Izawa, et al., (2006) stated that Asr1p is not indispensable for alcoholic fermentation in Japanese sake brewing yeast. The discrepancy between the two reports might be due to differences in the experimental conditions; in first case the study was done with external
added ethanol in YPD plates while in the second case it was performed under fermentation conditions.

1.3.4 Fermentation and pH Stress

Under normal growth, yeast cells usually maintain their intracellular pH within a narrow range, despite the changes in extracellular pH. But during the process of fermentation the intracellular pH of *S. cerevisiae* showed a dynamic range and adjusted according to changes in external pH of the medium. It is usually maintained between 5.5 to 5.7 when the extracellular pH is 3.0 or 5.9 to 6.7, when the external pH is between 6.0 to 10 (Imai & Ohno, 1995). The main cause of decrease in the pH of the medium is weak acids, like acetic acid and butyric acid that are generated as byproducts of normal fermentation (Keating, *et al.*, 2006). These are also made by acid hydrolyzing bacteria, if present as contaminants in the starting substrates used for fermentation like lignocelluloses materials. These compounds are not metabolized by glucose-repressed yeast cells and enter the cell in the un-dissociated form by simple diffusion. Inside the cell, the acid dissociates and if the extracellular pH is lower than the intracellular pH, this will lead to intracellular acidification, anion accumulation and inhibition of the metabolism including fermentation (Pampulha & Loureiro, 1989, Casal, *et al.*, 1996, Narendranath, *et al.*, 2001). Organic acid stress is toxic, not simply owing to high hydrogen ion concentration, but is also dependent on the chemical nature of the organic acid to which the organism is exposed. Moreover, these acids exert stronger inhibitory effects at low pH, where they are substantially un-dissociated (Bauer, *et al.*, 2003). To maintain the intracellular pH within a physiological optimum range for metabolism, the cells pump out protons at the expense of metabolic energy (ATP), thereby decreasing the growth yield (Meyrial, *et al.*, 1997). It has also been reported that the grams of biomass produced per mole of ATP generated decreased from 14 to 4 when the concentration of acetic acid was increased from 0 to 170 mM at pH 4.5. As the gap between the extracellular pH and the intracellular pH widens, greater stress is placed on the cells and more energy is expended to maintain the intracellular pH within the range that permits growth and survival of yeast cells (Verduyn, 1991, Pampulha & Loureiro-Dias, 2000).

Genome scale expression analysis and functional screening both suggested that the acidic condition affects cell-wall architecture (Mira, *et al.*, 2009). In fact, low pH has been found to induce alterations in yeast cell-wall architecture (Kapteyn, *et al.*, 2001). Cell wall composition analyses revealed that at pH 3.5, yeast cells exhibit high levels of
incorporation of cell wall mannoprotein Cwp1p, O-mannosylated heat shock protein Pir2p, which are dependent on Hog1p kinase pathway. The expression analysis indicated that most of the genes involved in acid adaptation are also involved in metal metabolism and are regulated by Aft1p. Functional screening studies indicate that loss of the V-ATPase and Hog1p proteins caused acid sensitivity (Kawahata, et al., 2006). Moreover, the adaptation of yeast cells to weak acids are regulated by two transcription factors, Haalp and Warlp. Haalp is required for yeast resistance to acetic, propionic and butyric acids (Fernandes, et al., 2005) and Warlp is required for Pdr12p (ABC transporter) induction in response to weak organic acid stress, and its absence causes hypersensitivity to a range of acids that include sorbate, benzoate and propionate (Kren, et al., 2003, Gregori, et al., 2008).

1.4 Microarray Gene expression profiling

Microarray analysis has emerged in the last few years as a flexible method for analyzing large numbers of nucleic acid fragments in parallel. It is similar to foundation technologies like recombinant DNA and the polymerase chain reaction (PCR) and is having broad applications in areas like genetic screening, proteomics, and diagnostics. The analysis is based on standard molecular biology, with a principal advantage being higher throughput and greater precision than traditional filter and blotting techniques. Microarray allows the analysis of thousands of genes in multiple samples with relative ease as compared to the traditional techniques like Northern and RT-PCR which are limited to a few samples at a time. The first commercial use of microarray in small glass slide (miniaturization) was done in the lab of Patric O Brown for the yeast genome analysis (Schena, et al., 1995). Microarrays can be seen as a continued development of molecular biology hybridization methods, as an extension of the use of fluorescence microscopy in cell biology, as well as a diagnostic assay using capture to solid surface as a way to reduce the amount of analytes needed. The convergence of ideas and principles utilized in these fields, together with technological advancements in preparing miniaturized collections of nucleic acids on solid supports, all have contributed to the emergence of microarray and microchip technologies (Shalon, et al., 1996, Stears, et al., 2003).
1.4.1 Gene Expression Analysis

A typical microarray gene expression analysis experiment compares the relative expression levels of specific transcripts in two samples. One of these samples is a control and the other is derived from cells whose response or status is being investigated i.e., test. The quantitative gene expression information can be performed in one and two color fluorescent schemes. In one color analysis, expression profiles for each sample are generated on different chips using a fluorescent label (e.g. phycoerythin) and then the different images from two chips are compared. In two color formats, each of the samples is labeled with a different fluorescent dye (Cy3 and Cy5), and equal amounts of the labeled samples are combined and hybridized to the microarray (Fig. 1.10). The fluorescent signals corresponding to the two dyes are measured independently from each spot after hybridization. After normalization, the intensity of the two hybridization signals can be compared. The intensity of fluorescent signal is not only proportional to the number of hybridized fragments but also on the length and number of fluorescent labels in each fragment, which is called as labeling density. If two samples have been labeled under similar conditions, the length and labeling density of specific transcripts will be similar in the two samples, making it possible to compare the relative abundance of the transcripts in the two samples. However microarray analysis does not give information about absolute gene expression levels in the samples.

1.4.1.1 Array Platforms

Broadly there are two types of array platforms on the basis of material spotted on microarray slides, namely complementary DNA (cDNA) and oligonucleotide microarrays. Probes for cDNA arrays are usually products of the polymerase chain reaction (100–3000 bp) generated from cDNA libraries or clone collections, using either vector-specific or gene-specific primers, and are printed onto glass slides or nylon membranes as spots at defined locations. Spots are typically 100–300 μm in size and are spaced about the same distance apart. Using this technique, arrays consisting of more than 30,000 cDNAs can be fitted onto the surface of a conventional microscope slide. For oligonucleotide arrays, short 20–25mers oligonucleotides are synthesized in situ, either by photolithography onto silicon wafers (high-density-oligonucleotide arrays from Affymetrix) (Wodicka, et al., 1997) or by ink-jet technology (licensed to Agilent Technologies). The oligonucleotide targets have several benefits over cDNA targets; it enables robust design of microarray experiment as simultaneously large number of genes
can be analyzed, it can be used to distinguish between alternative splicing variants and offer a precise control over the genetic composition of the arrays. With the judicious choice of oligos it is possible to discriminate between related gene sequence and study different members of gene families simultaneously. Moreover, oligonucleotide targets are readily available from commercial manufactures (Affymetrix, Agilent, Ocimum, Applied Biosystems), (Meltzer, 2001, van Berkum & Holstege, 2001). Alternative to these are spotted microarrays with the pre-synthesized long (50-120 mers) oligonucleotides spotted on the glass slides (Kane, et al., 2000).

Figure 1.10. Schematic representation of microarray. The experimental setup of microarray is broadly classified in three steps; 1) Target & probe preparation, 2) Hybridization, 3) Scanning and Data analysis. Total RNA is isolated from cells or mRNA purified from total RNA are labeled with fluorescent dyes during cDNA synthesis. The labeled cDNA are mixed in equi-molar ratio and hybridized to microarray. The dual colored slides are scanned in array scanner, and with software the fluorescent signal is converted into ratio intensities (the details about isolation of mRNA, labeling, scanning and data analysis is described in Chapter 4).
1.4.1.2 High Throughput Phenotype Screening

Several high throughput screening strategies were developed for studying microbial growth under competitive growth conditions. These usually involve a large pool of transformants overexpressing a library of genes, or a large pool of mutants. In *S. cerevisiae*, most of the studies have mainly been done with mutant pools, either generated by insertional mutagenesis or systematic deletion approach. A functional analysis method that couples insertion mutagenesis termed 'Genetic Footprinting' (Smith, *et al.*, 1995). In this method after *in-vivo* Ty1 transposon mutagenesis on a large population of yeast mutants in a pool were tested for their fitness under various physiological conditions. Strains containing insertions at different places within a gene of interest are scored by PCR, using gene specific primer and a common primer, which targeted a region within the Ty1 transposon. Results of each PCR reaction from the parent population and selected population were compared and quantified. Depletion of the PCR product from the later population ('genetic footprint') shows that the gene is critical for viability of cells under that particular condition (Smith, *et al.*, 1995). The advantage of this method is that the population of genes can be analyzed under competitive growth conditions. However, since only one gene can be detected at a time, large scale analysis is resourceful and labor intensive.

Another insertional mutagenesis method called as ‘Signature Tagged Mutagenesis’, was initially devised to identify virulence genes in human pathogenic bacteria (Hensel, *et al.*, 1995). In this method the transposon insertion mutants in a mixed pool are individually monitored by means of unique sequence tags present in each transposon insertion. Although the method was reasonably successful, it was limited by the size of the pool, and the need for prior introduction of unique sequence tags in mutants. An alternative method to genetic footprinting, referred as ‘Quantitative Target display’, was earlier developed in our lab for simultaneously monitoring the fitness of individual transposon insertion mutants of yeast present as a mixed population in liquid broth (Sharma, *et al.*, 2001). The method does not require sequence tags or gene specific primers, instead the mutants could be monitored in mixed pool by means of the DNA regions flanking the transposon insertion, which could be selectively and quantitatively amplified by ligation mediated PCR. Using this method, *UBI4, YDJ1* and *HSP26* were shown as essential for stress tolerance of yeast during ethanol production. The strategy seems to be less expensive, less labor intensive and can well be used for functional analysis of genes of any microbe amenable to insertion mutagenesis. However, like other
mutagenesis approaches, the screening strategy is not comprehensive and cannot be exploited to identify redundant or essential genes. A similar strategy employed hybridization of PCR amplified DNA flanking transposon insertions to DNA microarrays have been reported (Badarinarayana, et al., 2001). This method was used to assess the fitness contributions of *Escherichia coli* mutants in complex and minimal medium.

An alternative to insertion mutagenesis for functional analysis of genes is use of deletion library approach. The deletion libraries were constructed with the use of deletion cassette containing a kanamycin resistance gene (KanMX4) flanked by gene specific ‘barcode’ sequence, which are further flanked by common priming sites for PCR. Upon transformation the deletion cassette replaces the coding sequence by homologous recombination (Winzeler, et al., 1999). A large pool of yeast mutants can be analyzed in a single experiment by amplifying the bar code and probing over DNA microarray containing sequences complementary to gene specific barcodes. The quantity of each mutant in the culture is determined by the intensity of the spot on the microarray corresponding to the gene deletion. This approach was initially used for analysis of 558 yeast deletion pool mutants for growth, in rich and minimal medium (Winzeler, et al., 1999). Later the entire pool of non-essential gene deletion strains was used for identification of genes which are sensitive to UV radiation (Birrell, et al., 2001) and growth in media containing various fermentable and non-fermentable carbon sources (Steinmetz, et al., 2002). This approach provides a comprehensive collection of null mutants that can be screened for a specific phenotype. However, these mutants do not represent essential genes and are also limited due to deletion of only the annotated ORFs. Nevertheless, they are very powerful since all the mutants can be rapidly screened under a variety of conditions.