Mannan Md. Amin-Ul (2009). ***Studies towards understanding the molecular mechanism of stress tolerance mediated by RPI 1 yeast.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
Supervisor: Dr. K. Ganesan **Acc. No. : Th-210**

SUMMARY OF THE THESIS

Fermentation Stress Tolerance (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 in our lab a method was developed and used in combination with DNA microarrays, to simultaneously monitor the fitness of thousands of yeast mutants present as a mixed population in liquid broth. As an alternative to mutant approach, a second strategy based on gene overexpression was developed, which was also used with DNA microarrays. The overexpression strategy lead to the identification of several genes which provide survival advantage under fermentation conditions (Puria, 2006). The yeast *RPI1* (**R**as cAMP **P**athway **I**nhibitor) gene, identified in such a screen, was found to be critical for fermentation stress tolerance. Introducing an additional copy of *RPI1* with its native promoter helped the cells to retain their viability by over 50-fold better than the wild-type parent strain, after 36 h of fermentation at 38°C. Moreover, disruption of *RPI1* drastically reduced the cell viability during fermentation, further confirming the role of this gene in FST.

Since *RPI1* is important in FST, we focused our studies on this gene and tried to understand how it mediates its phenotype under fermentation conditions. Sobering *et al.* (2002) have suggested that *RPI1* prepares the cells for stationary phase by modulating the expression of cell wall biosynthetic genes. Thus, it is possible that during ethanolic fermentation also *RPI1* modulates cell wall integrity and thereby enhances the survival of yeast cells. To examine this, cells were recovered from fermentation broth and tested for sensitivity to widely used cell wall perturbing agents, calcofluor white and Zymolyase. While *RPI1* overexpression strain showed slight increase in resistance to calcofluor white, the resistance of disruption strain was comparable to that of WT strain. However, the *RPI1* overexpression strain was highly resistant to Zymolyase treatment compared to the WT strain, while the *RPI1* disruption mutant was more sensitive than the WT strain. These results indicate that *RPI1* mediates FST, at least partly, through cell wall modification. Since ethanolic fermentation is a complex process that involves multitude of stress conditions, we have studied phenotypes of *RPI1* over-expression (*RPI1*↑) and disruption (*rpi1*Δ) strains under various stress conditions encountered by yeast cells during fermentation. Results from plate-based assays show that *RPI1* is likely

to be involved in temperature, oxidative stress and low pH tolerance but not in ethanol or osmotic stress tolerance. These phenotypes also could be a reflection of change of cell wall integrity. *RPI1* was not reported in other screens for genes involved in ethanol tolerance (Takahashi, *et al.*, 2001, van Voorst, *et al.*, 2006, Yoshikawa, *et al.*, 2009), indicating that it is not involved in providing ethanol tolerance, but its role becomes evident only under fermentation conditions where cells experience a combination of stress conditions.

Rpi1p is a putative transcription factor (Sobering, *et al.*, 2002). Thus we have performed expression profiling to identify the genes that are differentially expressed in *RPI1*↑ strain compared to WT strain, as well as in *rpi1*Δ strain compared to WT strain. Out of the total 6200 yeast genes, many genes that are known to be involved in important cellular processes are differentially expressed. We have validated microarray data with quantitative real time PCR (RT-qPCR) and found that mRNA expression levels measured by microarray and RT-qPCR are correlated. Several genes involved in cell wall biogenesis (*PIR3*, *GAS1*, *EXG1*, and *YGP1*) are differentially expressed, which is consistent with phenotypes observed with cell-wall perturbing agents. These results provide additional evidence that it is indeed cell wall modification which is responsible for prolonged viability of *RPI1* overexpression strain during fermentation. We also observed that genes involved in osmotic stress response (*RHR2, SIP18* and *HOR2*) and the gene encoding plasma membrane H^+-ATPase (*PMA1*) are up-regulated; indicating that combinatorial expression of these genes perhaps provides survival advantage during fermentation.

The expression profiling also shows that genes involved in mitochondrial genome maintenance (*MRF1, NCA3* and *ICY1*) are induced and genes involved in oxidative phosphorylation (*AI4*, *COB*, *BI2* and *BI3*) are repressed. This is in accordance with several other studies in which integrity of mitochondrial genome was attributed to fermentation stress tolerance (Jimenez & Benitez, 1988, Costa, *et al.*, 1997, Ibeas & Jimenez, 1997). The down-regulation of oxidative phosphorylation genes is consistent with the preference of yeast to fermentation when fermentable sugars are present. The study also led to the identification of several genes of unknown functions that are induced (*ICY1*, *YKL065W-A*, *ECM1*, *YMR118c*), or repressed (*ICY2*, *YPR196w*, *YIL216c*, *YJL213w*) due to overexpression of *RPI1*. Since the molecular function and biological roles of these genes are not known, so it is not clear how they are associated with the phenotypes conferred by *RPI1*. Moreover, the results indicate that glucose

transporters genes (*HXT2*, *HXT5*, *HXT6*, *HXT15* and *HXT17*), genes involved in ion transport (*HOL1*), arginine uptake (*BTN2*), magnesium transporter (*ALR1*) and genes involved in cell cycle (*TOS8*, *SPL2*) are repressed in *RPI1* overexpression strain. It is known that the expression of genes involved in stress response and growth promotion are inversely correlated. Thus, the down-regulation of these genes probably explains the energy saving and growth arrest mechanism adopted by cells during fermentation.

Rpi1p is a putative transcription factor that possesses a C-terminal transcriptional activation domain between amino acid residues 270-407 (Sobering, *et al.*, 2002). Our expression profiling results also corroborate Rpi1p as transcriptional regulator, since several genes are differentially expressed. To confirm its transcriptional role further, we monitored recruitment of Rpi1p to the promoters of some of the genes, by in-*vivo* chromatin immuno-precipitation experiment. The results suggest that Rpi1p is recruited to the promoters of *PIR1*, *PIR3* and *ICY1* genes. To identify the target sequences bound by *RPI1*, consensus sequence among the promoters of genes was determined by motif finder algorithm MUSA (**M**otif finding using **U**n**S**upervised **A**pproach) which yielded the sequence ATCGGAAA; however, additional experiments need to be done to confirm if this serves as a binding site for Rpi1p.

RPI1 gene shares its 1875 bp upstream region with the divergently transcribed *RHO3* gene (Saccharomyces Genome Database). The regulation of *RPI1* appears to be complex, since at least 34 different transcription factors bind within 1 kb region upstream of *RPI1*, as reported in YEASTRACT (**Y**east **S**earch for **T**ranscriptional **R**egulators **A**nd **C**onsensus **T**racking) database. Since it is not possible to study all these transcription factors, we have shortlisted only those transcription factors which are directly involved in stress or stress regulated processes for further analysis. The results suggest that Xbp1p, Mot3p, Nrg1p, Rox1p and Yap1p are probable repressors, since disruption of their respective genes increases the transcript level of *RPI1*, as determined by quantitative real time PCR. To further check this at the promoter level, we cloned two different regions of *RPI1* promoter (-1 to -600 bp and -1 to -1000 bp) in a *lacZ* reporter vector and measured the β-galactosidase activity. Under fermentation conditions at 38°C, *lacZ* expression levels indicate that Nrg1p and Rox1p act as transcriptional repressors and Tec1p and Mot3p acts as an activator. There is no marked change in the expression due to disruption of genes encoding Xbp1p and Yap1p. Similar experiments were done for each of the transcription factors under normal growth conditions at 30°C as well,

which showed that Rox1p and Sko1p acts as a repressor and Tec1p and Rlm1p acts as an activator.

The other part of this study deals with the characterization of genes identified through genome-scale fitness profiling. A method for genome scale fitness profiling, named quantitative target display (Sharma, *et al.*, 2001),was earlier developed in our lab for the identification of genes involved in fermentation stress tolerance. The method was further, developed in combination with microarrays to study the relative fitness of thousands of transposon insertion mutants during fermentation at 38°C (Puria, 2006) . Since the studies were done with a pool of mutants in broth, the mutants with subtle fitness differences could be identified. While disruption of some genes (*MHP1, SKN7, EGD1, RRD1,* and *ECM33*) reduced the fitness, disruption of several other genes (*AVO2, SHR5, USV1, DLS1,* and *YPK2*) increased the fitness. The rate of fermentation of all these strains was comparable to WT strain, suggesting that the observed phenotype was not due to any difference in the rate of fermentation. Moreover, the growth rates of these strains were also comparable to WT in YPD medium. The genes identified by this approach were not reported in genome wide studies carried out for identification of genes involved in ethanol tolerance (Alexandre, *et al.*, 2001, van Voorst, *et al.*, 2006, Hirasawa, *et al.*, 2007, Marks, *et al.*, 2008). This suggests that genes identified by us are involved in modulating tolerance to some other condition than ethanol stress or a combination of stress conditions prevailing during fermentation. Mutants of some of these genes were sensitive (*SKN7, RRD1*) or resistant (*AVO2, SHR5*) to cell wall perturbing agents indicating that these genes modulate cell wall integrity, thereby affecting FST.

To conclude, the work described here, is mainly molecular and genetic characterization of *RPI1*. We have shown that *RPI1* is critical for cell survival during ethanolic fermentation. *RPI1* prolongs the survival of yeast cells by fortifying their cell wall, by modulating the expression of several genes involved in cell wall integrity. However *RPI1*