Molecular genetics of stress tolerance during ethanolic fermentation is poorly understood, mainly due to lack of genetic screens for isolating mutants or for cloning genes by complementation. Fermentation stress tolerance (FST) is a quantitative phenotype that cannot easily be monitored by conventional plate screens, since the conditions experienced by yeast colonies on the surface of solid media are not exactly the same as those experienced by cells within fermentation broth. Therefore, the broad objectives undertaken in the present study were the development of genome wide genetic screens to assess the fitness of strains under competitive growth conditions in liquid culture and to further elucidate the function of genes thus identified. We envisaged that identification of such genes would help in gaining insights into FST mechanism, and the genes identified could be used for engineering strains for better performance during fermentation.

To get an insight into fermentation stress, a screening approach named "Quantitative target display" was earlier developed in our lab, and validated with 20 known mutants impaired in various heat shock protein genes. In the present study it was developed further at genome-wide scale, by using this method in combination with DNA microarray to simultaneously assess the fitness of thousands of mutants. This resulted in the identification of many genes that are important for survival of yeast during fermentation. The role of some of these genes (ECM33, MHP1, EGD1, SKN7, RRD1 and PHO23) in fitness was confirmed by creating independent transposon insertion mutants for these genes. The identification of ECM33, MHP1 and SKN7 revealed the active participation of cell wall biogenesis in maintaining the integrity of cell wall under stress conditions. Moreover, SKN7 is also known to be associated with HOG1 and PKC1 pathway. Identification of RRD1 and PHO23 indicated the contribution of TOR pathway (Target Of Rapamycin) and phosphate metabolism in fermentation stress tolerance. This study also resulted in the identification of genes that upon disruption improve the viability of strains, but these are yet to be fully characterized.

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Simultaneously, a complementary approach based on the screening of genomic expression library of yeast was initiated in the lab to get more leads into fermentative stress tolerance. A genomic expression library of yeast under the control of constitutive ADH1 promoter was transformed into yeast. The transformants were screened for increased survival during fermentation at 30°C or 38°C, which led to the identification

of four genes (RPI1, WSC4, SRA1 and YIL055C). In the present study the role of these genes in FST was confirmed by generating independent overexpression and disruption strains. Though only a few genes could be identified, they had a significant role in enhancing the viability of strains under fermentation conditions. Further, it was observed that these genes conferred cross protection at temperatures other than that of selection, though they were initially isolated either at 30°C (WSC4, SRA1 and YIL055C) or 38°C (RPI1) only. This indicated that genes conferring maximum tolerance got enriched at the expense of those conferring only moderate tolerance, and many genes are likely to be missed. Thus, to identify genes contributing even moderately to fitness, the overexpression strategy was further modified and used in combination with microarrays. The fitness contribution of thousands of genes was simultaneously monitored after one or two rounds of fermentation. Abundance of transformants in unselected population was compared to their abundance in selected population. This resulted in the identification of several genes that upon overexpression enhanced the viability of strains in mixed pool. Some of these genes (WSC2, ECM33, ECM39, PBS2, PLP1, YPL206A, ADE16, MKT1 and SOL1) were further confirmed for their role in FST by creating independent overexpression and disruption strains. However, certain genes (ECM39, ADE16, MKT1 and SOL1) failed to show the expected phenotype. Thus, to seek an explanation for their initial identification in our screen, various possibilities were examined such as effect of spontaneous genomic DNA mutations, contribution of partial ORFs, or the effect of mixed culture on fitness of strains. A thorough examination of these possibilities revealed that these genes have their maximum role under competitive mixed fermentation conditions.

An interesting observation from our studies is the role of mixed culture. We observed that genes with moderate contribution under individual fermentation condition had significant role in mixed culture. Moreover, the marginal difference made in fitness upon the disruption of functionally redundant genes (WSC4, ADE16 and SOL1) became more pronounced in mixed culture. Since competitive mixed culture conditions are more like what yeast normally encounters in the natural environment, the genes identified in this study would be important for yeast under natural conditions, particularly in evolutionary time scale. This also indicates that the genome-scale approaches developed here are quite sensitive to identify even genes that make only moderate fitness contributions.

Another interesting aspect of our whole genome analysis was the implication of different signal transduction pathways in conferring fermentation stress tolerance. Many key determinants of these pathways were found to contribute significantly to FST, such as WSC2, WSC4 (signaling molecules of PKC1 pathway), SRA1 (Ras cAMP pathway), RRD1 (TOR pathway), PHO23, ADE16 (Nutrient starvation) and PBS2, SSK2 (HOG1 pathway) (Figure). This suggests that these pathways cross talk with each other to generate an efficient fermentation stress response.

Among the genes identified in this study, RPI1 showed the maximum effect on fermentation stress tolerance. Thus this gene was further studied to elucidate its mechanism of action. Initially dosage suppressor analysis was done in combination with DNA microarray to identify the suppressors of RPI1 deletion. This resulted in the identification of many genes, but none could completely suppress RPI1 deletion phenotype. This indicated that the network of RPI1 regulated genes is large and complex. Several genes associated with cell wall organization and biogenesis (ECM8, ECM33, WSC4, NRK1, SBE22, HSP150, WSC2, ECM39, WSC3, WSC1, KRE6 and RHO1) were observed, pointing towards the exacerbated effect of RPI1 deletion on cell wall organization. Further, to identify genes whose expression is modulated by differential expression of RPI1, expression profiling was also carried out under fermentation conditions at 38°C. This resulted in the identification of several genes that showed differential expression upon addition or deletion of RPI1.

To conclude, this study has not only resulted in the development of high throughput approaches for assessing the function of genes, but has also helped in the identification of several genes that can possibly be used for improving the stress tolerance of strains for industrial application. Further in depth characterization of these genes will lead to a better understanding of molecular genetics of fermentation stress tolerance.