

*Saccharomyces cerevisiae* is an ethanol tolerant microbe that is widely employed in distilleries/breweries for alcohol production. However, the increasing ethanol concentration during fermentation adversely affects its growth and production profiles, especially so at high temperatures usually prevalent in tropical countries like India. This is mainly due to the synergy between heat-induced and ethanol induced damages. Though much research has been done on these stress conditions when studied independently, there is hardly any report which deals with these in totality under the natural fermentation conditions. Lack of extensive studies could be due to the inherent "difficult-to screen" phenotype which can be monitored only in fermentation broths and not through convenient plate screens. Hence, the major goal of this dissertation has been to try to understand the genetic and molecular mechanisms of ET under fermentation conditions. We envisaged that a better understanding of these phenomena would offer a vast potential for rational designing of improved fermenting strains for the distillery and brewing industry. In our pursuit, we could identify few genes, both known and novel, which have a significant bearing on the ET of yeast cells under the natural fermentation environment.

While trying to gain an insight into the complex phenomena of stress tolerance under fermentation conditions our main aim was to construct improved fermentative strains. Kim *et al* (1996), had earlier reported that strains deleted in acid trehalase gene (*ATH1*) with *URA3* as the selection marker showed increased cell densities and stress resistance under fermentation conditions. We created similar *ATH1* mutants in both distillery and standard laboratory strains using *URA3* and *kanMX4* module, respectively. When tested against the appropriately constructed  $Ura^+/Ura^-$  control strains we failed to see any enhanced tolerance in  $\Delta ath1$  strains. However, our results contradicted the findings of Kim *et al* (1996) when we proved by detailed

Acc. No.: TH-91

growth and fermentation studies that the higher cell densities attained in  $\Delta ath1::URA3$  strains as compared to the parent strains (uracil auxotrophs but WT for *ATH1*) were due to the incorporation of *URA3* rather than the disruption of *ATH1*.

Our results pertaining to *ATH1* disruptions highlighted the importance of appropriate prototrophic control strains in yeast disruption studies. We constructed relevant auxotrophic strains (and corresponding prototrophs) for *LEU2*, *LYS2*, *HIS3*, and *TRP1* selection markers besides the *URA3* gene. The role of these auxotrophies was studied under various growth and fermentation conditions. The results pointed out a large growth deficit for these auxotrophs in rich YP-based fermentation media as they showed only 60% – 80% growth as compared to the prototrophic strains. Even in the standard YPD medium (with 2% glucose) commonly used for yeast studies, uracil auxotrophs could attain only 80% growth of the prototrophic controls. These growth differences were further highlighted under conditions like high temperature especially when the auxotrophic strains failed to utilize the extra nutrient supplements added to the medium. These results stress upon the need of constructing appropriate prototrophic controls while studying genes disrupted with the auxotrophic markers. This is vital to the proper interpretation and reproducibility of experimental results.

In our endeavor to isolate mutants having increased thermotolerance under fermentation conditions we generated many promising strains. These mutants showed more than 100-fold better survival than the controls while producing the same amount of alcohol at a fairly good rate. Genetic analysis revealed these mutations to be recessive and non-allelic. As the standard method of complementation could not be used to isolate these mutated genes from the

fermentation broth, we employed Ty-mutagenesis to identify the thermotolerant mutations. However, this approach was not successful because the Ty element did not show the expected co-segregation with the thermotolerant phenotype. This made us to believe that probably point mutations in essential genes were responsible for conferring thermotolerance and hence the absence of any relevant Ty-tagged mutation. Ty-insertion mutagenesis was done in diploids heterozygous for thermotolerance mutation and then selected for thermotolerant diploids after continuous fermentation and selection. Genetic analysis of these by tetrad dissections and further genetic crosses was complicated by many factors like the non-viability of spore clones. The genetic complexity of the distillery strain background posed further problems in the fruitful analysis of these mutants.

The problems faced by us in identifying the genes mutated in thermotolerant fermenting strains highlighted the absence of a suitable screening method for such "difficult-to-screen" mutants which could be analysed only in fermentation broths and not on agar plates. In this context, we optimized different parameters of a novel molecular method "Quantitative Target Display", which was already initiated in our laboratory. We used this method for the simultaneous monitoring of 20 different transposon-disrupted HSP mutants to evaluate their role in fermentation stress. Our results suggested the involvement of *HSP104*, *TPS2*, *UBI4*, *YDJ1*, *HSP26* and *HSP35* genes in conferring tolerance to yeast cells during fermentation at 30°C and/or 38°C. Also, we found that mutants of *SSA2*, *SSA3*, *SSA4* and *SSB2* genes had a higher survival relative to other mutants. This is consistent with the earlier literature where HSP70 is shown to be a negative regulator of HSP expression. These results not only confirm the

established roles of some HSPs in fermentation stress but also implicate the involvement of *HSP26* for which no biological role could be attributed earlier inspite of extensive efforts.

In order to further improve upon this strategy, we standardized the colony-hybridization method. This was used for the simultaneous monitoring of more than 1000 transposon-disrupted yeast mutants in high temperature fermentation. This method helped us in identifying several genes that appeared critical for survival during high temperature fermentation. Three of these, namely, *YER182W*, *YFR017C* and *YCL049C* are novel yeast genes whose functions are yet unknown. Besides, we also identified two known genes, *URA10* and *APAI*, which also seem to play a significant role during fermentation. However, we need to confirm these results by further experimentation.

In yet another investigation using the "Overexpression Strategy" we could identify many genes conferring enhanced tolerance when overexpressed under fermentation conditions. For this purpose, a yeast genomic library under a constitutive ADH promoter was used to transform a standard yeast strain. The transformants were screened for increased survival after enrichment through continuous fermentation both at 30°C and 38°C. This led to the isolation of genes *RP11*, *WSC4*, *PDR13*, *CAP2* and *YIL055C* which seem to play a significant role in stress tolerance during fermentation. Though we need to subclone and confirm these results before reaching any firm conclusion, yet the presence of the *RP11* and *WSC4* genes in several independent clones is sufficient to prove their involvement in fermentation stress tolerance.

Thus, this study has not only identified important genes involved in fermentation stress but also standardized a novel molecular method for studying the role of genes conferring subtle or "difficult-to-screen" phenotypes. In depth studies of the identified genes would help in unraveling the molecular genetic mechanisms of ET and help in the development of improved yeast strains for the fermentation industry.