Yashpal (2011). Studies on gene involoved in acetic acid tolerance of yeast saccharomyces cerevisiae. Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
Supervisor: Dr. K.Ganesan **Acc. No. : Th-264**

SUMMARY OF THE THESIS

Acetic acid is generated as a byproduct of ethanolic fermentation by yeast. Acid hydrolysis of lignocellulosic substrates to be used for ethanolic fermentation also generates acetic acid. Acetic acid inhibits growth and fermentating efficiency of yeast strains and this inhibition is synergistic with ethanol. Acetic acid is known to effect yeast viability through apoptosis and chronological aging of yeast population. Thus, identifying and characterizing genes involved in acetic acid tolerance is essential for a better understanding of the mechanisms of adaptation of yeast to acetic acid stress. The genes identified might be useful for improving yeast strains for higher acetic acid tolerance and ethanol production, particularly from biomass hydrolyasates.

Screening for genes conferring resistance to acetic acid in yeast *S. cerevisiae*

We screened yeast transformants of multicopy yeast genomic library to identify genes that upon overexpression increase the tolerance to acetic acid at pH 3.0 or 4.5. Plasmids from resistant yeast clones were isolated and retransformed in yeast to confirm the acetic acid tolerance, and further sequenced to identify the effector genes. Our screening yielded *HAA1* gene in multiple acetic acid resistant clones, which is already known to be involved in acetic acid adaptation in yeast, thereby validating our approach. The other genes identified provided only marginal improvement in acetic acid tolerance of yeast.

From one such clone we identified *YLR297w*, an uncharacterized gene in yeast, which upon overexpression provided acetic acid tolerance only in late growth phase (24 hours growth in synthetic dextrose media). Moreover, even constitutive overexpression of *YLR297w* under strong GPD promoter did not increase the acetic acid resistance of exponentially growing yeast cells, but only that of cells in late growth phase. *YLR297w* overexpression (unlike *HAA1*) also resulted in resistance to lipophilic acids like octanoic acid and benzoic acid, but only in late growth phase. It is likely that the growth phase specific effect of *YLR297w* depends on the expression of some other protein(s) in late growth phase or stationary phase. Deletion of *YLR297w* rendered the yeast cells only marginally sensitive to acetic acid and other weak acids tested. Thus, *YLR297w* does not appear to be critical for adaptation of yeast cells to weak acids and its function is either redundant or can be bypassed.

We found that *YLR297w* mediated acetic acid tolerance in late growth phase requires acidification of pregrowth media as in SD medium. Pregrowth in YPD medium, media with reduced glucose or buffered to near neutral pH, acidification is not observed, and under these

conditions *YLR297w* did not increase acetic acid tolerance. Burtner et al., 2009, have shown that acidification of pregrowth media in stationary phase is associated with release of organic acids in growth media, and release of acetic acid among them causes chronological aging. Thus we checked the role of acetic acid in pregrowth media in activation of *YLR297w* function. When exponentially grown yeast strains overexpressing *YLR297w* were subjected to acetic acid containing low pH media resembling spent media in late growth phase, *YLR297w* mediated acetic acid resistance was increased. Thus Ylr297wp may increase survival of yeast cells by modulating acetic acid induced apoptosis and chronological aging.

Insights into molecular mechanism of Haa1p mediated acetic acid resistance

Among the multiple clones with *HAA1* gene conferring acetic acid resistance upon overexpression, few had *HAA1* truncated at 3' termini, thus encoding Haa1p as short as 450 aa out of 695 aa sequence of Haa1p These truncated clones were complementing *HAA1* deletion for acetic acid sensitivity and producing shorter transcripts thus further confirming their size. Based on this observation we wished to identify the functional domains of Haa1p important in regulation of acetic acid tolerance. Various deletions of Haa1p missing either C-terminal or N-terminal region were made and expressed under TEF promoter in *haa1Δ* strain. The C-terminal deletions encoding initial 400 or 500 amino acid residues (H400 and H500 respectively) were found to fully complement the *HAA1* deletion; moreover these provided better acetic acid resistance than full-length protein. Like full-length Haa1p, C-terminal deletions can also activate expression of tested Haa1p target genes, namely *TPO2, TPO3* and *YGP1*. N-terminal deletions lacking initial 50 or 100 amino acids (N50 and N100 respectively) which constitute the DNA binding region were partially complementing the *HAA1* deletion and could support expression of *TPO3* and *YGP1* but not *TPO2*. Thus, presence of N-terminal DBD is essential for *TPO2* expression, whereas possibly it is not essential for *YGP1* and *TPO3* expression. Only simultaneous deletion at N-terminus (first 50 or 100 aa) and C-terminus beyond 400 amino acid rendered the protein non functional in complementing acetic acid sensitivity of *haa1Δ* strain, and also in induction of Haa1p target genes. Here it was also seen that expression of *TPO2* is induced upon acetic acid shock, whereas *YGP1* and *TPO3* expression was comparable in with or without acetic acid shocked cells.

We also tried to purify the Haa1p and its deletions from yeast as well as *E. coli* hosts. Protein purification from yeast was not fruitful whereas protein purified from *E coli* host was

metal dependent and specific DNA binding ability with *TPO2* and *YGP1* upstream regions, since EDTA inactivated protein was unable to show any DNA binding. Moreover, DNA binding of Haa1p was seen with multiple and overlapping probes from *YGP1* and *TPO2* upstream regions. Thus possibility of existence of multiple Haa1p binding sites in upstream region of target genes is likely. Promoter regions of *YGP1* and *TPO2* were mapped by progressive deletions in 1000 bp upstream region of respective genes to identify essential regulatory elements. We identified that *YGP1* has two upstream regulatory elements, one responding to acetic acid shock and another in untreated cells only, and these were named as Acetic Acid Response Element (AARE) and Non Acetic acid Response Element (NARE), respectively. In *TPO2,* single regulatory element mainly responding to acetic acid shock was identified, thus named as AARE. Thus differential regulation of *YGP1* and *TPO2* by Haa1p can be explained by regulatory elements present upstream of these genes. Moreover, presence of dual DNA binding domain in Haa1p cannot be ruled out as N50 and N100 deletions of Haa1p lacking N-terminal DBD is functional in activating *YGP1* and furthermore two different DBD recognizing different *YGP1* promoter elements cannot be ruled out.

Regulation of Haa1 mediated acetic acid tolerance

We have also studied the regulation of Haa1p activity in adaptation to acetic acid stress. Possible Haa1p interacting proteins were shortlisted from interaction datasets, and acetic acid sensitivity of strains deleted for genes encoding these proteins, were checked. Expression of full-length *HAA1* under constitutive TEF promoter in *ngg1Δ, msn5Δ, fus1Δ* and *ste20Δ* strains resulted in very long lag phase of growth in minimal media, whereas expression of H400 deletion did not cause any growth defect. Growth defect was also seen when full-length *HAA1* was expressed under strong and constitutive GPD promoter, perhaps resulting from excessive Haa1p activity in the absence of regulatory proteins. Based on the preliminary results and known functions of individual Haa1p interacting proteins, we tested their role in regulation of Haa1p function. Since Msn5p is known to function as nucleopherin in yeast, we checked the localization of Haa1p and H400 deletion protein in *msn5Δ.* strain. HA tagged Haa1p and H400 expressed under TEF promoter localize to nucleus only in *msn5Δ* whereas in *haa1Δ* it partially localize to nucleus as significant cytosolic signal is seen. Thus role of Msn5p can be implicated in restricting Haa1p out of nucleus when Haa1p

function is not required. Haa1p is also shown to interact with Pho80p cyclin, which is known to be involved in phosphorylation of Pho4p transcription factor in association with Pho85p CDK. Phosphorylation of Pho4p leads to its Msn5p dependent exit from nucleus. Similarly, Pho85p-Pho80p complex might be involved in Msn5p mediated export of Haa1p from nucleus.

Expression of *HAA1* and its target genes *TPO2* and *YGP1* was checked in strains deleted in genes for Haa1p interacting proteins. In *ngg1Δ* strain expression of *YGP1* is reduced; upon acetic acid shock expression of *TPO2* is substantially increased, but no expression of *YGP1* is seen. Ngg1p is a component of HAT complexes which is known to regulate the activity of many transcription factors in yeast. When *NGG1* or its functional deletions which complement the slow growth defect of *ngg1Δ* strain are expressed in *ngg1Δ* strain, the wildtype like expression of *YGP1* and *TPO2* is seen. Deletion of *HAA1* in *ngg1Δ* strain results in loss of expression of *TPO2* and *YGP1*, indicating that the Ngg1p regulation of *TPO2* and *YGP1* expression is by modulating the activity of Haa1p. Haa1p and its deletions expressed under its own promoter in *ngg1Δhaa1Δ* strain provide better acetic acid tolerance than its expression in *haa1Δ* strain, indicating that Ngg1p may function as repressor of Haa1p activity in yeast growing under normal conditions, but upon acetic acid shock Ngg1p repression is released and Haa1p functions to regulate the adaptation of yeast to acetic acid stress. Like in *ngg1Δ* strain *YGP1* expression is not seen upon acetic acid shock in *pho85Δ* and *pho80Δ* strains, whereas *TPO2* expression is seen. Thus, it is likely that Pho85p-Pho80p complex function upstream of Ngg1p in regulating activity of Haa1p.

Thus, a working hypothesis for Haa1p regulation, which needs to be experimentally proved, is as follows. Pho85p-Pho80p complex may phosphorylate Haa1p in untreated cells leading to its export from nucleus in Msn5p dependent manner, and repression of acetic acid induced transcriptional response Haa1p via Ngg1p. Upon acetic acid shock, increased metabolic cost and ATP consumption may lead to cellular conditions similar to phosphate starvation. Thus, Pho85p-Pho80p complex cannot phosphorylate Haa1p, and unphosphorylated Haa1p is released from Msn5p and Ngg1p mediated repression, leading to acetic acid induced transcriptional response by Haa1p.