Chapter 5

DEVELOPMENT OF ACETIC ACID RESISTANT STRAINS OF Saccharomyces cerevisiae Y_{B23}

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Acetic acid is a by-product formed during yeast alcoholic fermentation; the production of acetic acid is self-inhibitory i.e. it can block its own production by feed-back inhibition mechanism. Recent evidence has revealed the occurrence of an apoptotic phenotype in *Saccharomyces cerevisiae* that is inducible with oxidative stress. Exposure of *Saccharomyces cerevisiae* to 20-200 mM acetic acid for 200 min at pH 3.0 resulted in cell death (339). Acetic acid uncouples energy generation by dissipating proton motive force across the plasma membrane & inhibiting glycolytic enzymes (340-342). But if the cells are adapted to the acid stress, it can protect the organism from acid induced programme cell death (343).So in our study, we have made our strain *Saccharomyces cerevisiae* Y_{B23} resistant to acetic acid depending on the following view points, so that, it can protect itself from the acid based inhibition.

Pampulha et al found out that, during acetic acid production, lipophilic weak acids, such as, acetic acid, may accumulate inside yeast cells. The undissociated form of acetic acid diffuses into the yeast cells where it dissociates, inducing an acidification of the cytosol. Acetic acid was also found to induce cell death (344).

Narendranath N.V.et al. & Elleonora et al. studied that, specific growth rate of *Saccharomyces cerevisiae* decreased exponentially as the concentration of acetic acid increased in minimal media at 30°C. Moreover, the period of the lag phase of the growth curve also increase (345,346).

Pereira et al. reported that, acetic acid activates a mitochondria-dependent death process in *Saccharomyces cerevisiae* and that the ADP/ATP carrier (AAC) is required for mitochondrial outer membrane permeabilization and cytochrome *c* release. Mitochondrial fragmentation and degradation have also been shown in response to this death stimulus (347-350).

Graves et al. examined the effects of lactic and acetic acids on ethanol production by *Saccharomyces cerevisiae* in corn mash & found that, inhibition by acetic acid increased as the concentration of solids in the mash increased and the pH of the

medium declined. Ethanol production was completely inhibited in all mashes set at pH 4 in the presence of acetic acid at concentrations >=0.8% w/v (351).

D.V. Silva et al. used sugarcane bagasse hydrolysate as a fermentation medium for the conversion of xylose into xylitol by the yeast *Candida guilliermondii* FTI 20037. Acetic acid (2.0 gm/L) was added in the medium at different time intervals to evaluate its effect on the bioconversion process. Addition of acetic acid in the medium after 12 hours of fermentation exhibited strongest inhibition of the yeast metabolism by chemical interference with the phosphate transport through the cell membrane, which requires expenditure of ATP (352).

According to Lawford & Rousseau, acetic acid toxicity is related to the ability of undissociated (protonated) weak acid to traverse the cell membrane & to act as a membrane ionophore, which causes acidification of cytosol (353).

Roe et al. showed that, In the presence of 8 mM acetate the specific growth rate of *E. coli* Frag1 (in MacIlvaine's minimal medium pH 6<0) is reduced by 50%. Hey also found that, Acetate inhibition of growth arises from the depletion of the intracellular methionine pool with the concomitant accumulation of the toxic intermediate homocysteine and this augments the effect of lowering cytoplasmic pH (354).

Limtong et al studied the growth of some xylose fermenting yeasts, *Candida shehatae, Pichia stipitis* CBS5773, fusant F101and fusant F198 & found that the growth was completely inhibited in xylose medium added with 0.5% v/v acetic acid which caused the reduction of pH to 4.1. Only one xylose fermenting strain, *Pachysolen tannophilus* NRRL-Y2460, showed relatively low growth and ethanol fermentation. However, in the medium with 1.0% v/v acetic acid (pH 3.7), all of these strains were completely inhibited. In glucose medium with 0.5% v/v acetic acid, various strains of *Saccharomyces cerevisiae*, M30, Sc90, N1, G/3, G/5, G/2, TJ3 and SH1089, grew with lower specific growth rate and provided lower maximal cell concentration rate than in medium without acetic acid (pH 6.2) (355).

Keeping all these findings in mind, we made our strain *Saccharomyces cerevisiae* Y_{B23} resistant to increasing concentrations of acetic acid (1.0% to 3.0%) so that it can adjust itself with the low pH of the fermentation media & produce higher amount of acetic acid.

MATERIALS & METHODS:

Medium & Cultural conditions: Medium used for the production of acetic acid consisted of sucrose 12.0%, KH_2PO_4 0.1%, $(NH_4)_2SO_4$ 0.5%, $MgSO_4$, $7H_2O$ 0.05%, yeast extract 0.1% & pH was adjusted to 4.5. 5ml of yeast cell suspension containing 2.05×10^5 cells/ml was added to the 100ml of medium in 250ml Erlenmeyer flask & it was incubated at 30°C for 48hours.

Determination of acetic acid: Acetic acid was measured by the same procedure described in Chapter 4.

Development of acetic acid resistance: Five different concentrations of acetic acid were prepared, such as, 1.0% to 3.0% each of 9ml. Then 1ml of yeast cell suspension was added first to the 1.0% acetic acid. It was then incubated for 24, 48 & 72 hours. After completion of incubation, the whole process was performed in the same procedure as during the development of alcohol & temperature resistance described in Chapter 1.

RESULTS & DISCUSSION:

Development of Acetic acid resistance strains of *Saccharomyces cerevisiae* Y_{B23} :

A group of yeast strains were isolated after resistance to 1.0% to 3.0% acetic acid for varied time of incubation. From Fig. 4a) it was found that, the strain Y_{B39} has produced maximum amount of acetic acid (0.1584 gm/100ml), which is 1.4% higher than the nonresistant variant. This strain was then made resistant to 1.5% acetic acid, and several strains (YB_{43} toYB_{48}) were isolated, which were then tested for acetic acid production. Fig. 4b shows that, the strain Y_{B46} has produced maximum amount of acetic acid (0.2173 gm/100ml), 27% higher than the previous one. This strain was then made resistant to 2.0% acetic acid, and several strains (YB_{49} toYB_{54}) were isolated, which were then tested for acetic acid production. From Fig. 4c, we can see that the strain Y_{B51} has produced maximum amount of acetic acid (0.2481 gm/100ml), 14% higher than the previous one. This strain was then made resistant to 2.5% acetic acid, and several strains (YB_{100}, YB_{90}, YB_{80}, YB_{70}, YB_{60} and YB_{50}) were isolated, which were then tested for acetic acid production. From, Fig. 4d it is clear that, the strain Y_{B100} has produced maximum amount of acetic acid (0.2874 gm/100ml), almost

16% higher than the previous one & almost 84% higher than the nonresistant variant. This strain was then made resistant to 3.0% acetic acid, and several strains (YB_{101} to YB_{106}) were isolated, which were then tested for acetic acid production. Fig. 4e shows that, the strain Y_{B101} has produced maximum amount of acetic acid (0.2661 gm/100ml). But this amount of acetic acid is almost 7.5% less than the acetic acid produced by Y_{B100} (0.2874 gm/100ml), the 2.5% acetic acid resistant strain. So now, we will use Y_{B100} for our further studies. This strain was named Y_{Bmax} for our convenience in those studies.

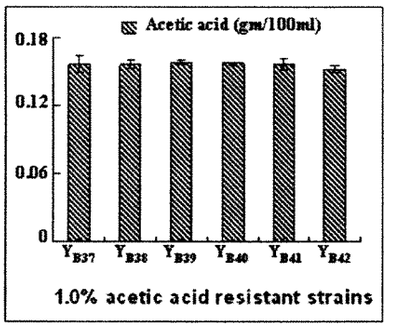

Fig. 4a) Resistance to 1.0% acid

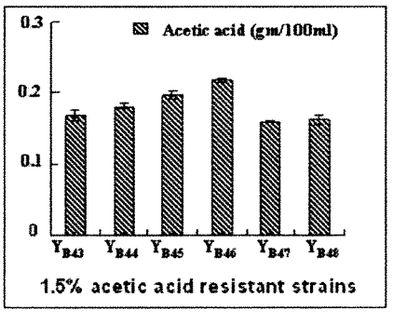

Fig. 4b) Resistance to 1.5% acid

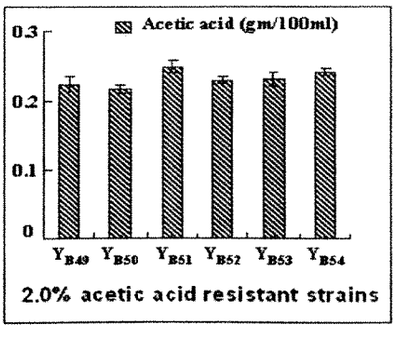

Fig. 4c) Resistance to 2.0% acid

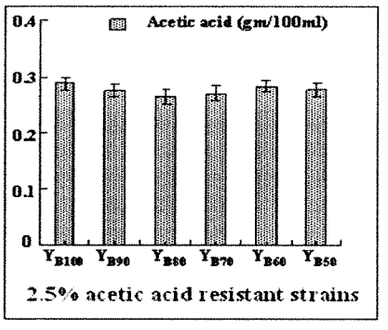

Fig. 4d) Resistance to 2.5% acid

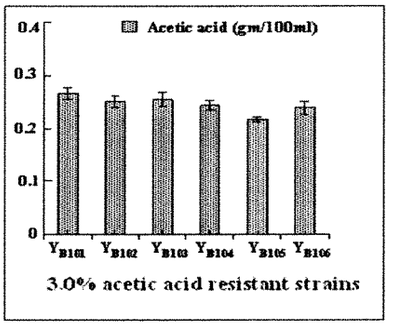

Fig. 4e) Resistance to 3.0% acid

Fig. 4. Development of Acetic acid resistance strains of *Saccharomyces cerevisiae* Y_{B23}

Production values are expressed as mean ± SD.

All values are biologically significant ($p<0.001$).