# *Chapter 3*

# *OPTIMIZATION OF PHYSICAL PARAMETERS FOR ALCOHOL PRODUCTION BY ALCOHOL & TEMPERATURE RESISTANT* *Saccharomyces cerevisiae* $Y_{B23}$

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## OPTIMIZATION OF PHYSICAL PARAMETERS FOR ALCOHOL PRODUCTION BY ALCOHOL & TEMPERATURE RESISTANT *Saccharomyces cerevisiae* $Y_{B23}$

Biological reactions run most efficiently within optimum ranges of environmental parameters. The growth of organism may be seen as the increase of cell material expressed in terms of mass or cell number & results from a largely complicated & coordinated series of enzyme-catalyzed biological steps. Growth will depend on the availability & entry of necessary nutrients into the cell & subsequent uptake as well as on environmental parameters, such as temperature, pH & aeration all optimum. The main physical parameters which regulate the growth as well as enzyme production & hence alcohol production are i) initial pH of the fermentation medium, ii) temperature of fermentation, iii) volume of fermentation medium, iv) period of fermentation, v) age of inoculums & vi) cell density. In the following chapter, the optimization of these physical factors is discussed in detail.

*Saccharomyces cerevisiae* is capable of very rapid rates of glycolysis & ethanol production under optimum conditions, ensuring higher ethanol yield. It is evident from previous studies by several groups that nutritional requirements & general metabolic activities of organisms should be studied during fermentation at or near the optimum temperature for growth & pH of the fermentation medium, because it is assumed that cell mass & enzyme activity at that particular temperature & pH are most favorable.

Whilst the temperature of the heat treatment will determine its degree of lethality, the conditions during heating will affect the extent & expression of the heat resistance of a yeast strain. Cerney (310) found that, pH of the medium in which an organism is heated, has a profound effect on heat resistance.

The concentration of hydrogen ions in a fermentation broth affects yeast growth, ethanol production rate, byproduct formation & bacterial contamination control. Usually, industrial ethanol fermentation by yeast has an initial pH value of 4-6 depending on the buffering capacity of the medium. In lightly buffered medium, the

initial pH is 5.5-6.0 & in more highly buffered medium, 4.5-4.7 (311). If the pH value is <5 during fermentation, bacterial growth is severly repressed; the pH values for growth of most strains of *Saccharomyces cerevisiae* are 2.4-8.6, with an optimum of 4.5. Yeast sugar fermentation rates are relatively insensitive to pH values in the range (3.5,6.0) (312). Continuous production of ethanol from beet molasses at 30°C & pH 3.0 are optimum for maximum ethanol production (313,314).

The effect of temperature on the specific growth rate is governed by the Arrhenius equation : $k = A.e^{-Ea/RT}$ where A is a constant called the Arrhenius factor, $E_a$ is the energy of activation of the process, R and T are the universal gas constant and absolute temperature respectively. For yeast growth, the activation energy lies between 12.8 to 17.1 $kcal.mol^{-1}$ (55.23 +/- 10.32 KJ/mole or 13.212 +/- 2.46 kcal/mole) whereas, the energy of activation for the death rate of yeast is in the range of 70 to 90 $kcal.mol^{-1}$(315). The significance of the higher value of energy of activation at high temperature is that with increase in temperature the death rate of fungi increases i.e., the growth rate of the same decreases (316). Snedecor and Cooney (317) proved that the rate of carbon – energy substrate conversion to cell mass in case of the growth of yeast and a mixed population of bacteria is also affected by temperature. Temperature also regulates the rate of metabolic processes in the cell. Due to temperature fluctuation, metabolic activities are altered. Alroy and Tannenbaum (318) showed that both growth rate and RNA content of the cell are strong functions of temperature. Most of Brewer's yeasts have a maximum growth temperature around 39-40°C (319). The maximum growth temperature reported for any species of yeast was 49°C for *Kluyveromyces marxianus* (320). Mesophilic strains of *Saccharomyces cerevisiae* have optimum cell yields & growth rates between 28°C & 35°C. The optimum & maximum temperatures for growth of thermophilic yeast are 40-50°C respectively. These strains have a high maintenance requirement & more complicated nutritional requirements. Some yeasts have an optimum fermentation temperature of 40-42°C. They produce upto 12% of ethanol with yield >90% of theoretical (321-324). Because sugar fermentation is exothermic (586 J of heat/gm glucose) (311) using yeasts that ferment at higher temperature substantially reduces cooling costs of fermentors.

Llewellyn Bowman & Edwin Geiger (316) & R. Sathees Kumar (272) have worked on the optimization of fermentation conditions for alcohol production. The

quantitative effects of carbohydrate levels, degree of initial saccharification, glucoamylase dosage, temperature of fermentation & fermentation time were investigated using a Box-Wilson central composite design protocol. With *Saccharomyces cerevisiae* it was found that the use of a partially saccharified starch substrate markedly increased yields & attainable alcohol level.

R.K. Sadhukhan & D.K. Sengupta (325) have optimized different external factors in ethanol fermentation of sugar cane molasses in batch process. Waste sugar cane molasses (total fermentable sugar 48.6%) was used as substrate & fermentation were carried out in 2000ml Erlenmeyer flasks at various concentrations of molasses (326,327). Preliminary studies confirmed that an optimum temperature 40°C & pH 5.5 & reutilization of distillery effluents in the fermentation of ethanol from cane sugar molasses.

Cecilia Laluce et al. (300) have reported growth & fermentation characteristics of new selected strains of *Saccharomyces* at high temperature & high cell densities. Maintenance of high cell viability was the main characteristic of their new strains of thermotolerant *Saccharomyces*. Total sugar conversion to ethanol was observed for sugar cane fermentation at 38-40°C in less than 10 hour without continuous aeration of the culture. They found that, invertase activity of the cells & optimum temperature for growth, as well as velocity of ethanol formation, were dependant on medium composition & the type of the strain used.

M.A. Agab & M. Collins (328) have reported that the highest survival levels in buffer occurred at pH 6.0 or occasionally at pH 5.4 & the lowest at pH 7.0.

Some mesophilic yeasts & a thermotolerant strain of *Saccharomyces cerevisiae* were found by Midori Yamamura & Yoichi Nagami (329) to grow at 40°C in complex media containing 1.0% yeast extract (6.0%) permitted *Saccharomyces cerevisiae* to grow at 40°C even with a smaller imoculum size ($10^5$ cells/ml).

P.J. Anderson et al (297) have observed that a number of yeast strains, isolated from cane mills & identified as strains of *Kluyveromyces marxianus* var. *marxianus*, were examined for their ability to ferment glucose & cane syrup to ethanol at high temperature. Several strains were capable of rapid fermentation at temperature up to

47°C. at 43°C, >6.0% (wt/vol.) ethanol was produced after 12 to 14 hours of fermentation, concurrent with retention of high cell viability (>80.0%).

The present work was undertaken to study in detail the various factors affecting the production of ethanol by an ethanol & temperature resistant strain *Saccharomyces cerevisiae* $Y_{B23}$, & the factors are: i) initial pH of the medium, ii) period of incubation, iii) volume of the medium, iv) temperature of incubation, v) age of inoculums, vi) volume of inoculums.

## MATERIALS & METHODS:

The optimum conditions for the ethanol production by *Saccharomyces cerevisiae* $Y_{B23}$ were found out by changing one variable at a time within reasonable limits, all the other factors remaining constant. In practice, the factors were varied in order of the medium-i) initial pH of the medium, ii) volume of the medium, iii) period of incubation, iv) cell density, v) temperature of incubation, vi) age of inoculums.

**Medium & Cultural Conditions:** The medium used for the production of ethanol contained- Sucrose-12.0%, $KH_2PO_4$-0.1%, $(NH_4)_2SO_4$-0.5%, yeast extract-0.1% and $MgSO_4$, $7H_2O$- 0.05% & pH was adjusted to 4.5. This solution was sterilized at 121°C & 15lb/inch$^2$ pressure for 15 minutes. The yeast cells were harvested by washing the slants with sterilised distilled water. The cell density was adjusted to $2.05\times10^5$ cells/ml. 5ml of cell suspension was used for the inoculation of the fermentation medium. Fermentation was carried out using 250ml Erlenmeyer conical flasks, each containing 100ml of the medium & incubating them at 30°C for 48hours.

**Determination of ethanol:** Determination of % of ethanol was the same as discussed in Chapter1.

**Determination of pH:** pH was adjusted by pH meter.

**Measurement of Dry Cell weight:** After complete production of alcohol, microorganisms were separated from the production medium by filtering through Whatmann 1 filter paper. Then it was dried in hot air oven for 6 hrs at 115°C to remove the total water content of the cell.

**Statistical Analysis:** Statistical analysis of data were performed according to Chapter 1.

## RESULTS & DISCUSSION:

**Effect of initial pH on ethanol production:** The effect of an increase in pH from 4.0 to 6.0 on ethanol fermentation from sucrose shows that ethanol production becomes maximum (4.1%) at pH 4.5 & progressively decreases with increasing pH values. It was found from fig.3a) that ethanol production increases >20% than the previous one, because pH 4.5 is suitable for both growth & zymase activity of *Saccharomyces cerevisiae* $Y_{B23}$.

**Optimization of volume of medium:** *Saccharomyces cerevisiae* $Y_{B23}$ was inoculated at different volumes of production media ranging from 80ml to 120ml. From fig. 3b) it is evident that, the 250ml Erlenmeyer flask containing 100ml of fermentation medium gives the maximum yield of ethanol (4.1%).

At lower media volume, nutrients were not sufficient for optimum growth or multiplication of the organism. So % of alcohol decreases. At volume >100ml, excess nutrients may be toxic & interfering with the growth of microorganism & so production decreases.

**Optimization of incubation time period:** Alcohol production using *Saccharomyces cerevisiae* $Y_{B23}$ was carried out at different incubation periods ranging from 24hours to 72hours. From fig. 3c) it was clear that, % of alcohol production increased gradually & attained maximum (4.1%) at 48hours, after which it declined.

At lower time period, activity of zymase enzyme was not on full swing, so production of alcohol was lower. But at higher time of incubation, feedback inhibition of alcohol decreases its own production. Vaporization of alcohol is also responsible for its lower yield.

**Optimization of cell density:** From fig. 3d) it is found that, optimum cell density for alcohol production by *Saccharomyces cerevisiae* $Y_{B23}$ is $2.05\times10^5$ cells/ml (v/v).

At lower cell density, sufficient microorganism was not available for alcohol production. When cell density was very high, accumulation of toxic metabolic byproducts also increase, which inhibit the growth of microorganism & decrease the yield of ethanol. At $10.25\times10^5$ cells/5ml cell density, alcohol production was maximum (4.7%) & >14% higher than the previous production.

**Optimization of incubation temperature:** Alcohol production by *Saccharomyces cerevisiae* $Y_{B23}$ was employed at different temperatures ranging from 25-35°C. Fig. 3e) shows maximum alcohol production (4.7%) at 30°C. Temperature lower than 30°C may not be sufficient for activating the alcohol producing zymase enzyme. So

yield of alcohol was lower. At high temperature, vaporization of alcohol & inactivation of zymase decreases the alcohol content.

**Optimization of inoculums age:** Alcohol production was found to be maximum by 48hour culture of *Saccharomyces cerevisiae* $Y_{B23}$ . From fig. 3f) we can see that, it produces 5.7% alcohol, >21.0% higher than previous one. Before 48hours, cells may be in the lag phase, zymase enzyme was not fully active for alcohol production. Again the cell of death phase (after 48hours) exhibit lower yield of alcohol due to inactivation of zymase & other cellular metabolic activities.

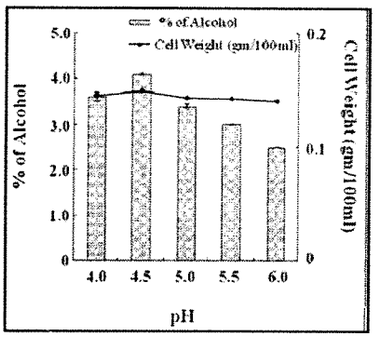


**Fig. 3a) Effect of pH on Alcohol production**

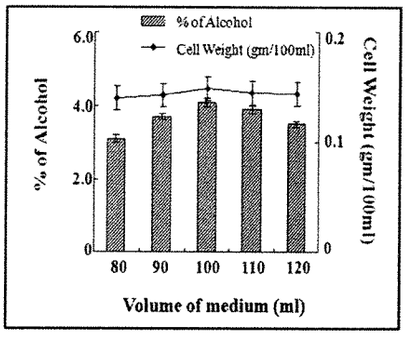


**Fig. 3b) Effect of medium volume on Alcohol production**

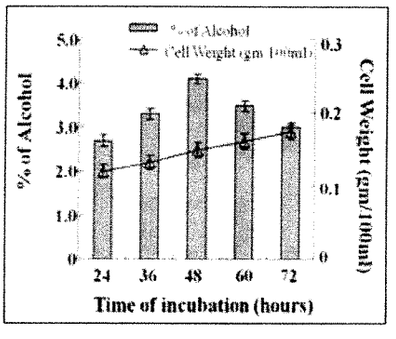


**Fig. 3c) Effect of incubation time on Alcohol production**

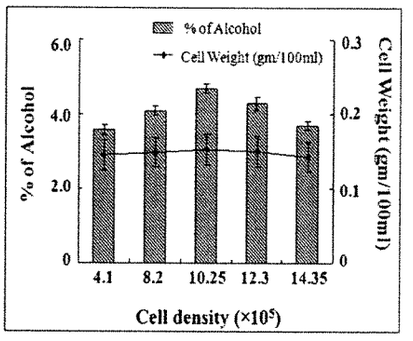


**Fig. 3d) Effect of cell density on Alcohol production**

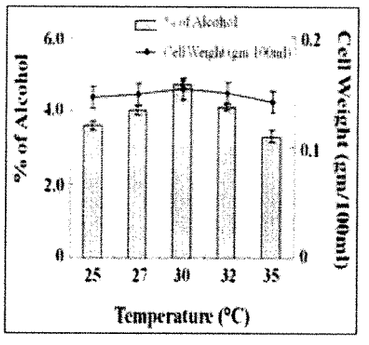


**Fig. 3e) Effect of temperature on Alcohol production**

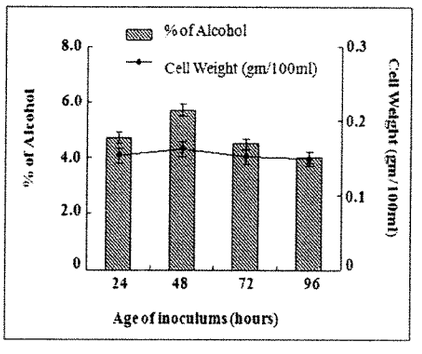


**Fig. 3f) Effect of inoculums age on Alcohol production**

**Fig. 3. Determination of Optimum Cultural Conditions for Alcohol Production by Ethanol & Temperature resistant Strain *Saccharomyces cerevisiae* $Y_{B23}$**

Production values are expressed as mean ± SD.

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