DISCUSSION

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Mother strain of *Saccharomyces cerevisiae* can produce ethanol (1.45%) from glucose by zymase. But the parent strain is not so remarkable for its commercialization. It also cannot tolerate the high temperature in ethanol fermentation as it is an exothermic reaction. Investigation on the development of a high yielding ethanol & temperature resistant strain of *Saccharomyces cerevisiae* resulted in the isolation of resistant *Saccharomyces cerevisiae* Y_{B23} which has better alcohol producing capacity & temperature tolerance than the parent strain.

The resistant *Saccharomyces cerevisiae* Y_{B23} was obtained by treating the parent strain consecutively with 5.0%, 7.5% & 10.0% alcohol. After treatment with alcohol, the isolated *Saccharomyces cerevisiae* Y_{B11} was able to produce 3.4% alcohol from glucose which was 31.0% greater than the parent strain. The resistant strain on further treatment with temperature changed to another resistant *Saccharomyces cerevisiae* Y_{B23} which was able to produce 3.4% alcohol from glucose.

Resistant *Saccharomyces cerevisiae* Y_{B23} was maintained in three different media to obtain the most suitable one which can keep the constancy of alcohol production. It has been observed that the *Saccharomyces cerevisiae* Y_{B23} was quite stable in alcohol producing capacity when maintained in a medium containing glucose: 1.0%, $(NH_4)_2SO_4$: 0.5%. KH_2PO_4: 0.1%, $MgSO_4,7H_2O$: 0.025%; $FeSO_4$, $7H_2O$: 0.002%, Biotin: 0.2µg/ml, Agar: 4.0%, pH : 5.0.

Investigation on the optimization of the physical factors revealed that progressive increase in the days of incubation favoured alcohol production as the activity of zymase enzyme increased gradually. Maximum amount of alcohol was produced at 48hour fermentation. Growth of the organism was dependent on temperature and temperature of 30^0C favoured both growth and alcohol production from sucrose. Higher temperature became inhibitory for zymase synthesis by affecting mRNA coding for the enzyme & it also causes vaporization of alcohol (318). A specific temperature was also required for secretion of extracellular enzyme. Since growth of the microorganism depends on pH of the medium, *Saccharomyces cerevisiae* Y_{B23} being an acidophilic organism, the optimum initial pH was determined to be 4.5. pH lower than this (pH 4.0) was inhibitory due to inactivation of zymase. Optimum

volume of the fermentation medium was determined to be 100.0 ml. The right concentration of nutrients was necessary for effective metabolic activity of the yeast. A specific concentration of substrate was required for maximum amount of product formation. Another important factor in fermentation process was growth of micro organism which depends, along with other factors, on the cell density. Density was adjusted to be 2.05×10^5 cells/ml (v/v) and on increasing this the supplemented nutrients were not efficient for growth and optimum activity of the yeast. When the cell density was below a critical value, optimum activity of the yeast did not occur. Optimum age of the yeast was determined to be 48hours; younger or older cultures were found unsuitable since maturity had not occurred in younger culture while older cultures may have proceeded to the decline or death phase. Thus, the optimum conditions for alcohol production from sucrose by *Saccharomyces cerevisiae* Y_{B23} using a complex medium are as follows :-

i) Initial pH of the medium : 4.5

ii) Volume of the fermentation medium : 100.0 ml

iii) Period of fermentation : 48hours

iv) Cell density : 2.05×10^5cells/ml

v) Temperature of fermentation : 30^0C

vi) Age of the inoculums : 48hours

Acetic acid is a by-product formed during yeast alcoholic fermentation. The acetic acid producing capability was examined using various ethanol & temperature resistant strains of *Saccharomyces cerevisiae* Y_B & we found that *Saccharomyces cerevisiae* Y_{B23} is the desired strain which produces the maximum amount of acetic acid.

As the production of acetic acid is self inhibitory i.e. it can block its own production by feed-back inhibition mechanism, development of acetic acid resistance is highly desirable.The resistant *Saccharomyces cerevisiae* Y_{B23} was treated consecutively with 1.0-3.0% concentration of acetic acid. The isolated strain *Saccharomyces cerevisiae* Y_{B100} produces maximum amount of acetic acid (0.2874gm/100ml). Higher concentration of acetic acid inhibits the growth & simultaneously the acetic acid production as acetic acid uncouples energy generation by dissipating proton motive force across the plasma membrane & inhibiting glycolytic enzymes. Acetic acid

accumulates 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.

A synthetic medium has been prepared for acetic acid production by the acid resistant *Saccharomyces cerevisiae* Y_{B100} as complex medium has no definite composition. It has been observed that the *Saccharomyces cerevisiae* Y_{B100} produces less amount of acetic acid in a synthetic medium containing sucrose: 12.0%, $(NH_4)_2SO_4$: 0.5%. KH_2PO_4: 0.1%, $MgSO_4,7H_2O$: 0.05%, pH : 4.5. *Saccharomyces cerevisiae* Y_{B100} was named *Saccharomyces cerevisiae* Y_{Bmax} for convenience.

Investigation on the optimization of the physical factors revealed that progressive increase in the days of incubation favored acetic acid production as the activity of aldehyde dehydrogenase enzyme increased gradually. Maximum amount of acetic acid was produced at 96hours of fermentation. Overoxidation of acetic acid to CO_2 & H_2O occurs during longer incubation. Growth of the organism was dependent on temperature and temperature of 30^0C favored both growth and acetic acid production from sucrose. At high temperature, structural & functional inertness of aldehyde dehydrogenase diminishes the acid content. A specific temperature was also required for secretion of extracellular enzyme. Since growth of the microorganism depends on the pH of the medium, *Saccharomyces cerevisiae* Y_{Bmax} being an acidophilic organism, the optimum initial pH was determined to be 4.5. pH lower than this (pH 4.0) was inhibitory due to inaction of aldehyde dehydrogenase. Optimum volume of the fermentation medium was determined to be 85.0 ml. The right concentration of nutrients was necessary for effective metabolic activity of the yeast. A specific concentration of substrate was required for maximum amount of product formation. Presence of excess nutrients may be toxic for the growth of microorganism & can interfere with, or sometimes inhibit, the function of the enzymes. Another important factor in fermentation process was growth of micro organism which depends, along with other factors, on the cell density. Density was adjusted to be 10.25×10^5 cells/5ml (v/v) and on increasing this the supplemented nutrients were not efficient for growth or optimum activity of the yeast. When the cell density was below a critical value, optimum activity of the yeast did not occur. Optimum age of the yeast was determined to be 48hours and younger or older cultures were found unsuitable because maturity had not occurred in younger culture and older cultures may have

proceeded to the death or decline phase. Thus, the optimum conditions for acetic acid production from sucrose by *Saccharomyces cerevisiae* Y_{Bmax} using a synthetic medium are as follows

i)	Initial pH of the medium	:	4.5
ii)	Volume of the fermentation medium	:	85.0 ml
iii)	Period of fermentation	:	96hours
iv)	Cell density	:	10.25×10^5cells/5ml
v)	Temperature of fermentation	:	30^0C
vi)	Age of the inoculums	:	48hours

Yeast cannot synthesize their own food due to lack of green pigment chlorophyll. For this reason, all yeast require C, O, H, P, N etc. for their survival and growth. A study on the nutritional requirements of *Saccharomyces cerevisiae* Y_{Bmax} revealed that proper concentrations of the appropriate carbon and nitrogen sources supplied were necessary for active proliferation and optimum metabolic activity of the yeast. Glucose at 10.0% concentration was the best utilized carbon source as it is readily assimilable & can be easily oxidized via glycolysis and TCA cycle and also satisfies the condition of biosynthetic precursors necessary for growth of the microorganism. Glucose was utilized more efficiently than at lower concentrations (437). Ammonium sulphate (0.5%) was the most effective nitrogen source. Moreover, utilization of ammonium sulphate by *Saccharomyces cerevisiae* Y_{Bmax} reflects a linear relation with cell growth enzyme synthesis and acetic acid production.

The optimum growth of the yeast *Saccharomyces cerevisiae* Y_{Bmax} and its activity required the presence of various minerals like phosphate, sodium, potassium, magnesium, calcium, zinc, manganese, iron etc., into the synthetic medium. Many of them have appreciable effect on the production of several commercially important primary and secondary metabolites.

Among the phosphate sources tested, both KH_2PO_4 & K_2HPO_4 were found to be the most effective for enzyme activity & acetic acid production at a concentration of 0.125 g per 100 ml of fermentation medium. Higher concentration (> 0.125%) repressed both acetic acid production & enzyme synthesis. Of the macroelements tested magnesium at a concentration of 0.05% was found to be stimulatory to acetic

acid production & enzyme synthesis. Higher concentration than this inhibited the acetic acid production due to lack of ATP synthesis (472). It was also found that Na^+ became inhibitory above 0.1% due to osmotic imbalance (476), whereas, Ca^{2+} ion (added as $CaCl_2$) was stimulatory to acetic acid production up to 0.01%. Na_2HPO_4 at 1.0% concentration & boric acid at a concentration of 0.5mg/L also stimulated growth & acetic acid production.

Of all the trace elements tested, iron, manganese and zinc aided growth and enzyme production. Iron is responsible for activation of various enzymes including cytochrome oxidase & catalase & is an integral part of yeast protoplasm (487). Manganese is known to affect cellular concentration as well as the activity of various enzymes. It is essential for various reductase enzymes as a co-factor (493). Zinc is important in the cell cycle (reproduction), and is a cofactor for alcohol dehydrogenase, the enzyme responsible for alcohol production. Other metal ions cannot substitute in place of zinc. Supplementation of zinc into brewer's worts generally has the effect of speeding up fermentation, as well as preventing stuck fermentations (387). When Fe^{2+}, Mn^{2+} and Zn^{2+} were added to the fermentation medium, the stimulatory effect on enzyme activity & acetic acid production became higher than where Fe^{2+}, Mn^{2+} and Zn^{2+} affect individually. 10 μg/ml of each of $MnSO_4,H_2O$, $ZnSO_4,7H_2O$ and $FeSO_4,7H_2O$ gave the maximum acetic acid production (1.0978gm/100ml). Other metal ions have adverse effect probably due to the fact that either they block the active sites of the enzymes to inactivate them and other essential factors by chelation, involved in the acetic acid production process.

The effect of complex nutrients was studied, as they were not only good sources of nitrogen, but also provide amino acid, vitamin, trace elements etc. Results of the investigation on the effect of complex nutrients on the acetic acid production and enzyme synthesis from glucose by *Saccharomyces cerevisiae* Y_{Bmax} revealed that 0.2% soybean meal extract was stimulatory; whereas rice bran extract, paddy soak liquor, malt–extract, beef extract, yeast extract, wheat bran extract etc. were inhibitory to acetic acid production. Defatted soybean meal is a well-known stimulator of acid production as it is generally a rich source of nitrogen, water soluble vitamins, amino acids, minerals and other growth stimulants.

Amino acids are reported as good nutritional sources for yeast. Investigation reveals that L-glutamic acid, L-tryptophan & L-glycine, each having 0.5mg/ml concentration,

have positive effect on acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase. Among them L-glutamic acid & L-tryptophan were highly significant, as tryptophan is necessary for Coenzyme Q biosynthesis & glutamic acid acts as a source of nitrogen. These amino acids were also found to be growth-promoting factors. Among other amino acids L-cystine, L-arginine-HCl, L-leucine & L-methionine were fairly inhibitory to acid production. Besides the above four amino acids others have slightly inhibitory or no significant effect on acetic acid production.

Vitamins are required in minute quantities and are necessary for the growth and metabolism of microorganisms. They act as co-enzymes or constituent part of co-enzymes of various catalytic reactions (558). Results on the effect of several vitamins on acetic acid production and enzyme synthesis showed that biotin (0.2µg/ml), nicotinic acid (1.0µg/ml), Vit B_{12} (1.0µg/ml), Ca-pantothenate (0.5µg/ml) and Riboflavin (1.0µg/ml) have positive effect on acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase. These vitamins were also found to be growth-promoting factors. Among other vitamins pyridoxine-HCl, folic acid, inositol & p-amino benzoic acid were fairly inhibitory to acid production. Besides the above four vitamins others have either slightly inhibitory or no significant effect on acetic acid production.

It is necessary for acetic acid production to maintain proper growth of the microorganism and their enzyme producing capacity. Thus, any agent, which inhibits the cellular growth and catalytic activity of the enzyme, invariably causes a negative effect on production process. The extent of inhibition shown by a particular inhibitor depends on its rate of uptake, which increases with increase in its concentration and time period of incubation. Investigation on the effect of different metabolic inhibitors on acetic acid production & enzyme activities by *Saccharomyces cerevisiae* Y_{Bmax} revealed that 2,4 DNP, Sodium arsenate and Mercuric chloride were absolutely inhibitory to acetic acid production & activities of alcohol dehydrogenase and aldehyde dehydrogenase when added at the start of fermentation, i.e., on the zero day. That was due to the total inhibition of growth of *Saccharomyces cerevisiae* Y_{Bmax} by them. But the inhibition diminished when they were added to the fermentation medium on the 48 and 70hours. Another reason is that 2,4 DNP acts as an uncoupler of oxidative phosphorylation which prevents ATP synthesis in mitochondria by

dissipating the energized membrane state while substrate oxidation and oxygen consumption proceed normally (673). Thus it is expected that the active transport process which requires energy would be inhibited where primary source of ATP generation is oxidative phosphorylation. Inhibition of acetic acid production due to addition of 2,4-DNP indicates that ATP generated by oxidative phosphorylation is required for the growth & acetic acid production by *Sacharomyces cerevisiae* Y_{Bmax}. Sodium arsenate inhibits the mitochondrial activity in yeast cell completely causing the inhibition of cell growth. Mercury has been extensively characterized as an inhibitor of enzymes due to its binding to a number of functional groups. The extracellular β-glucosidase and β-fructosidase of yeast have been shown to be extremely sensitive to mercury. Other inhibitors, such as- 6 – marcaptoethanol, sodium fluoride, malonic acid, sodium arsenite and 2 – thiouracil cause a moderate inhibition of acid production.

The studies on the effect of antibiotics on cell growth, acetic acid production & enzyme activities demonstrate that Chlotrimazole, Terbinafine, Amphotericin B, Streptomycin, Erythromycin and Chloramphenicol, all the six antibiotics had a negative effect. In the cases of all the antibiotics, the extent of inhibition of cellular growth, acetic acid production and enzyme activities were the function of both concentrations of antibiotics as well as the day of its addition to the fermentation medium.

Among the antifungal antibiotics tested chlotrimazole showed a 42.97% inhibition to acid production when added on 50 µg/ml concentration at 0 hour of fermentation. Whereas terbinafine & amphotericin B caused about 37.78% & 19.52% inhibition respectively. Streptomycin inhibited the protein synthesis and chloramphenicol blocked the synthesis of respiratory system of the cell causing the cell death.

The study of the effect of surface active agents on the activities of alcohol dehydrogenase, aldehyde dehydrogenase and acetic acid production from glucose by resistant *Sacharomyces cerevisiae* Y_{Bmax} showed that, all the four surface active agents tested impart a negative effect on acetic acid production. Among them sodium lauryl sulfate was the most inhibitory and Tween-20 was less inhibitory on zero day. Triton X – 100 and Tween – 80 gave lesser negative effect on acetic acid production and enzyme activities. In almost all cases growth of resistant *Sacharomyces cerevisiae* Y_{Bmax} was greatly hampered.

In the present study, the most suitable carbon and nitrogen sources were found to be glucose and ammonium sulfate respectively. It was found from the graph that the rate of glucose uptake increases from 36hours and reaches maximum at 96hours of fermentation. From 36hours to 84hours, the rate of glucose uptake was the highest. After 84hours of fermentation, the rate of utilization of glucose by *Sacharomyces cerevisiae* Y_{Bmax} gradually decreases and after 96hours, the organism shows almost no uptake of glucose. It also appeared from the Table that during the maximum glucose utilization period, the pH of the fermentation medium remained acidic which was due to the liberation of acetic acid & several other organic acids. After that period, pH of the fermentation medium gradually increased which was associated with the breakdown of acetic acid into CO_2 & H_2O due to its overoxidation. It was also observed that maximum utilization of ammonium sulfate occurred from 48 to 96hours of fermentation. However, cell nitrogen increased rapidly upto 96hours, after which the rate decreases. A positive correlation was observed between ammonium sulfate depletion, acetic acid production and ammonia & amino nitrogen in broth.

Immobilization of the resistant strain was done in Ca-alginate & Agar agar for better yield of acetic acid, reusability of the organism & its protection from environmental stress (755). Immobilzation in Ca-alginate increased the acetic acid production almost 28.98%. But in case of Agar agar, the acetic acid production decreases than the free cells. So, it is clear that for sufficient amount of acetic acid production immobilization in Ca-alginate is most preferable.

So, it may be concluded from the study that to obtain acetic acid by fermentation, an acetic acid resistant strain was isolated for better acetic acid production & subsequently the different physical & chemical parameters were optimized for maximum production. Signiicant variations & contradictory findings have been obtained by various scientists throughout the world. This may be attributed to the variations in strains used. Nevertheless, *Sacharomyces cerevisiae* Y_{Bmax} used in our study was found to show increased production from 0.2874 gm/100ml to 1.395 gm/100ml.