Chapter 8

NUTRITIONAL REQUIREMENTS OF Saccharomyces cerevisiae Y_{Bmax} *FOR THE PRODUCTION OF ACETIC ACID IN SHAKE FLASK FERMENTATION*

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Growth (that is, the increase of biomass owing to macromolecular synthesis) constitutes a fundamental process in a living cell. It results from the catabolism of available nutrients, yielding metabolic intermediates and energy for the synthesis of cellular constituents. In order to survive in a variety of different environments, a unicellular microbe must be able to regulate the myriad pathways that lead to growth in response to the external nutrient supply (408-411). For the maintenance and sustenance of life, all living organisms require energy. In terms of energy sources, organisms are of two types – phototrophs and chemotrophs. Both the types have certain basic requirements of raw materials for their nutrition. Among them, carbon occupies a unique position, as compounds having carbon carbon linkage are the characteristic features of the animal world as a whole. Some of the other important substances are nitrogen, hydrogen, oxygen, sulphur, phosphorous, sodium, potassium, calcium, magnesium, iron, manganese, zinc, copper, cobalt and molybdenum. Unlike the autotrophs, the non-chlorophyllous organisms including fungi entirely depend upon the autotrophs for meeting their carbon requirement since the heterotrophs are unable to utilize the inorganic sources of carbon. Chemotrophic organisms are also dependant on chemical energy sources & employ organic compounds as the principal carbon source (412)

Like the rate of chemical reaction, the growth rate of the microorganism depends on the concentration of chemical nutrients. Monod expressed this fact by the relationship: $\mu = \mu_m [S / (K_s + S)]$, where μ and μ_m are the specific growth rate and maximum value (h^{-1}) respectively, S is the substrate concentration and K_s is the substrate concentration at $\mu = 0.5\ \mu_m$ (413). The significance of this expression is that the dependence of growth on chemical concentration can be described by two constants – the saturation constant K_s and the maximum growth rate. This is found to be true for a wide variety of nutrients (414).

Knowledge of the factors concerned in the growth of yeasts is of value for theoretical plant science & biochemistry & provides information for practical application in the field of microbiological assays (415). Growth requirements are met before the cell commits to the process of cell division (416). This general understanding of coordination between growth & division implies that growth control, though is nutrient mediated, is not nutrient specific (417).

A generic fermentative process can be considered to be consisting of two principal steps: first, preparation of the inoculum & second fermentation itself. Some conditions are to be imposed for the control of microorganism activity, so that their multiplication & successive functioning can be favored. The first step is the basic objective of this chapter i.e. the preparation of microorganism in appropriate condition so that the second step can be evolved adequately.

A. SELECTION OF SUITABLE CARBON SOURCE FOR ACETIC ACID PRODUCTION BY AN ETHANOL & TEMPERATURE RESISTANT *Saccharomyces cerevisiae* Y_{Bmax} :

Carbohydrates have the chemical structure of either polyhydroxy aldeydes or polyhydroxy ketones. In general, they can be divided into three broad classes: monosaccharides, disaccharides & polysaccharides. Carbohydrates have a central role in biological energetic in that they produce ATP. The progressive breakdown of polysaccharides & disaccharides to simple sugars is a major source of energy-rich compounds (418).

Carbohydrates are excellent sources of carbon, oxygen, hydrogen & metabolic energy. They are frequently present in the media in concentrations higher than other nutrients & are generally used in the range of 0.2-25.0%. The availability of the carbohydrate to the microorganism normally depend upon the complexity of the molecule. It generally may be ranked as:

Hexose>Disaccharides>Pentoses>Polysaccharide. Amides

Carbon is a component of both structural and functional constituents of cell. It comprises about fifty percent of the total mycelial dry weight in fungi. A multitude of organic constituent of fungal cell, like carbohydrates, proteins, nucleic acids, enzymes

etc.are all made of carbon. All the important components of cell wall like cellulose, chitin and pectic substances contains carbon in varying form and concentration, and thus provides the structural frame – work of the fungal cell.

Monosaccharides usually are easily assimilable form of carbohydrate; among these glucose has been reported to be the most efficient source of carbon and energy for most of the fungi. Hasija and Wolf (419) reported that in *Aspergillus niger*, glucose is present always as a constituent of mycelium irrespective of the carbon source. According to Tandon (420), the comparative study with four hexoses showed that glucose and fructose are good carbon sources for most of the fungal species, galactose is after glucose and fructose but mannose is a poor carbon – source. It has been reported that compounds having more than three carbon atoms are better nutrients for fungi (420). This is due to the fact that after one or two initial reaction, most of those compounds are oxidized through glycolysis and TCA cycle.

Disaccharides like sucrose, maltose, cellobiose, lactose and melibiose have got greater applicability in fungal nutrition. Plant pathogenic fungi preferentially use sucrose as a carbon source among different disaccharides. Sucrose is metabolized when it breaks down into glucose and fructose. Bilgrami (421) reported that among the above two monosaccharides, the utilization of glucose fraction is comparatively rapid.

Generally the organic acids, like citric acid, succinic acid, fumaric acid, lactic acid, maleic acid which are products of glucose metabolism, are poor carbon sources. This phenomenon is due to the impermeability of the cell due to low pH level caused by organic acids.

All nutrients supplied to the medium have to be utilized for proper growth and activity of the microbe. Thus, keeping in view the need for maximum production of acetic acid, efforts were made to modify the chemical composition of the medium.

Relatively few studies have been made to determine the range of organic compounds which can serve as sources of carbon for yeast growth. The most comprehensive studies among them was that of Wickerham & Burton (422), whose findings concerning the more common compounds may be summarized as follows:

a) Those compounds not utilized by any yeast include cyclohexane, cyclohexanol, maleic acid, kojic acid, ethylene glycol, methanol & acetone.

b) Those used by a few yeasts include malonic, levulinic & ascorbic acids.

c) Many yeasts can utilize fumaric, malic or glutamic acids.

d) Those used by many yeasts include xylose, arabinose, adonitol, dulcitol, mannitol, sorbitol, citric acid, succinic acid, lactic acid, cellibiose, melezitose, salicin & salts of gluconic acid.

e) Those compounds used by the majority of yeast include ethanol, pyruvic acid & glycerol.

Yeasts can also be trained to utilize particular compounds by being repeatedly subcultured in the media containing the compounds in low concentration. Active growth of *Saccharomyces cerevisiae* may occur in media containing acids involved in the Kreb's cycle only after the yeast has been trained for several generations (423); after the yeasts become adapted to such substrates, acetic acid, citric acid, fumaric acid, lactic acid, malic acid, oxaloacetic acid, pyruvic acid & succinic acid can be more or less readily assimilated. Recently certain compounds have been isolated from seawood, found to act as carbon sources for a number of yeasts after a period of training; these compounds include fucocidin, fucose, laminarin & sodium alginate (424).

Eduardo, V.S. has reported the influence of aeration & glucose concentration in the flocculation of *Saccharomyces cerevisiae*. The influence of glucose concentration & aeration rate on the flocculation ability of *Saccharomyces cerevisiae* was studied. A positive effect was achieved with a high glucose concentration (425).

Moulin, G. have demonstrated that, when lactose & glucose were present in the medium with a small amount of alcohol, a synergistis effect on the rate of fermentation appeared. This phenomenon considerably limits the rate of fermentation (426).

Bruno & Vitolo have studied the effct of viscosity of sucrose hydrolysis catalysed by invertase obtained from *Saccharomyces cerevisiae*. It has been observed that the initial reaction rate of sucrose hydrolysis catalysed by invertase (EC 3.2.1.26) slows down when the substrate concentration is higher than 12.0% (w/v) (427).

Xianzhen has improved ethanol production from beet molasses by soy flour supplementation. 11.7% ethanol was obtained by supplementing inoculum medium with soy flour & the fermentation time was reduced by more than 15.0% (428).

M.C. Berottolini et al. isolated new yeast strains from alcoholic fermentation from samples collected from Brazilian alcohol factories at the end of the sugarcane crop season. They were selected by their capacity of fermenting concentrated sugar cane syrup as well as high sucrose concentrations (30.0-33.0%) in synthetic medium with conversion efficiency of 89.0-92.0%. The strains were identified as *Saccharomyces cerevisiae* (429).

Vitolo & Yassuda have studied the effect of sucrose concentration on the invertase activity of intact yeast cells (*Saccharomyces cerevisiae*). They found that soluble & cell bound invertase activity varied by around 10.0% against sucrose concentration ranging from 80.0 gm/L to 200.0 gm/L (430).

Parekh & Cheryan studied the four strains of *Clostridium thermoaceticum* for their ability to produce acetate from glucose & found that, a 35 gm/L glucose medium was fermented completely in 140 hrs, yielding 29 gm/L acetate with conversion efficiency of 83% (167).

The fed batch approach of Parekh & Cheryan to the production of acetate from glucose by an improved strain of *Clostridium thermoaceticum* resulted in better performance than the batch fermentation. At pH 6.6, 46 gm/L acetate was produced in 192 hrs with 93.0% substrate utilization (168).

Mehaia & Cheryan used the sugars in date extracts to produce ethanol & vinegar & in a membrane cycle bioreactor, productivity was 10.8 gm/L/hr at an acetic acid concentration of 45.0 gm/L (227).

Okos, M.R. et al, produced acetic acid from anaerobic fermentation of lactose by the coculture of *Streptococcus lactis* & *Clostridium formoaceticum* at 35°C & pHs between 7.0-7.6. The overall acetic acid yield from lactose was 95.0% at pH 7.6 & 90.0% at pH 7.0 (431).

Efficient production of acetic acid from glucose was investigated in a mixed culture of *Zymomonas mobilis* & *Acetobacter* sp. by Kondo & Kondo. The acetic acid yield

from glucose was 0.64gm/gm (95.5% of the theoretical value) under the optimum conditions (247).

The fermentation kinetics of acetic acid production from fructose as a carbon source by *Clostridium formoaceticum* was studied at pH 7.6 & 37°C by Yu Liang Huang. Acetic acid yield from fructose was 1.0 gm/gm, with a final acetate concentration of 78.0 gm/L. The fermentation product acetic acid was found to be a noncompetitive inhibitor to the cells (249).

Young Soo Kim studied the acetic acid production from xylose by *Clostridium thermoaceticum* which requires adaptation to xylose medium. It preferentially consumes xylose over glucose using a mixture of xylose & glucose as a carbon source. The initial concentration of xylose in the medium affects the final concentration & the yield of acetic acid. Batch fermentation of 20 gm/L of xylose results in a maximum acetate concentration of 15.2 gm/L & a yield of 0.76 gm acid/gm xylose (258).

Palleroni et al. found that a local strain of the typical wine yeast *Saccharomyces cerevisiae* var. *ellipsoideus* can accumulate acetic acid in a liquid medium with ethanol under aerobic conditions. Acetic acid accumulation (6.0 gm/L) was maximum in media whose initial alcohol concentration was around 4.0% (v/v) (208).

A new acetic acid producing strain *Acetobacter* sp. RKY4 was isolated from Korean traditional persimmon vinegar by Kim et al. They cultivated the organism in different medium containing various carbon sources (e.g. galactose, maltose, glucose, sucrose, fructose, lactose, starch & glycerol) of 10.0 gm/L of initial concentration. When 10.0 gm/L of glucose or glycerol was used as a carbon source, *Acetobacter* sp. RKY4 produced 33.6 gm/L & 35.6 gm/L respectively. So they selected glycerol as sole carbon source rather than glucose (262).

The influence of available low-cost carbohydrates as carbon sources on *Brettanomyces bruxellensis* growth and on the production of acetic acid & ethanol was studied by Garcia et al. in order to ascertain the viability of this yeast to eventually become an industrial acetic acid producer. Six different raw materials were included as carbon sources (e.g. glucose, sugarcane molasses, refined cane sugar, pineapple, sugarcane & beet juices). The maximum acetic acid yield (0.24 gm/gm) &

productivity (0.14 gm./L/hr) was attained in tests carried out with sugarcane molasses containing 60.0 gm/L sucrose (432).

Inglis et al. studied the effect of increasing juice soluble solids above 40°Brix on wine yeast's ability to grow and ferment the juice, with particular focus on acetic acid production, titratable acidity (TA) changes and the maximum amount of sugar consumed by the yeast. They found that, increasing icewine juice concentration from 40 to 46°Brix increases the proportion of yeast sugar metabolism towards glycerol and acetic acid production to cope with the increased osmotic stress by decreasing yeast growth, sugar consumption rate, the total amount of sugar consumed and the total amount of ethanol produced. The high proportional contribution of acetic acid to titratable acidity in Riesling Icewine may affect acidity perception (433).

Bartley & Polakis measured the activities of the enzymes of the citric acid cycle, the glyoxylate by-pass and some other enzymes acting on the substrates of these cycles at the pH of the yeast cell during the aerobic growth of yeast on different carbon sources and in different growth media. Sugars induced an anaerobic type of metabolism as measured by ethanol production. Glucose was much more effective than galactose in inducing the anaerobic pathways. The production of ethanol by cells grown on pyruvate was very small. Glucose was also a more effective repressor than galactose of the citric acid-cycle enzymes but both were equally effective in repressing almost completely the enzymes of the glyoxylate by-pass. Disappearance of the sugars from the growth medium resulted in an increase in the activities of the enzymes of the citric acid cycle and in substantial activities of the enzymes of the glyoxylate cycle. By contrast, the activities of purely biosynthetic enzymes (glutamate-oxaloacetate transaminase, $NADP^+$-linked glutamate dehydrogenase) and of pyruvate decarboxylase were decreased (434).

Laopaiboon et al. investigated the ethanol production from sweet sorghum juice as a carbon source by *Saccharomyces cerevisiae* NP01 under very high gravity (VHG) fermentation and various carbon adjuncts. When sucrose was used as an adjunct, the sweet sorghum juice gave the maximum ethanol production efficiency with concentration, productivity and yield of 120.68 ± 0.54 g/L, 2.01 ± 0.01 g/L/hr and 0.51 ± 0.00 g /g respectively. When sugarcane molasses was used as an adjunct, the juice under the same conditions gave the maximum ethanol concentration,

productivity and yield having the values of 109.34 ± 0.78 g/L, 1.52 ± 0.01 g/L/hr and 0.45 ± 0.01 g/g, respectively (435).

Fasidi & Jonathan carried out experiments to show the effect of simple organic, inorganic and complex compounds on growth of *Psathyerella atroumbonata* (Pegler), a Nigerian edible Mushroom. A number of carbon compounds were utilised and glucose stimulated the best growth followed in order by mannose, cellulose and mannitol. Sorbose and myo-inositol enhanced the least growth (436). The preference of glucose over other carbon compounds may be due to the ease with which this sugar was metabolised to produce cellular energy (437).

Rosma & Cheong used Pineapple waste medium to cultivate yeast, *Candida utilis*. It served as the sole carbon and energy source for the yeast growth (438).

Costa et al. proved that, the constituents of a medium must satisfy the basic requirements for cell biomass and metabolite production, by providing an adequate supply of energy for biosynthesis and cell maintenance. The carbon substrate has a dual role in biosynthesis and energy generation, with carbohydrates being the usual carbon source for microbial fermentation processes (439,440).

Shafaghat et al. investigated the effect of various carbon sources such as- glucose, fructose & sucrose on growth kinetics & ethanol productivity of *Saccharomyces cerevisiae* strain PTCC 24860. The obtained maximum specific growth rates for various substrates of glucose, fructose & sucrose were 11.39, 39.19, 97.82 gm/L respectively. Fermentation with media containing fructose, a maximum ethanol concentration of 14.45 gm/L was obtained. The maximum ethanol productivity using fructose was 1.22 & 1.38-times higher than glucose & sucrose respectively (441).

Li et al. revealed that the yeast can convert glucose into glycerol and ethanol (442).

MATERIALS & METHODS:

Medium & Cultural conditions: For the Selection of a suitable carbon source, the 85ml of medium used contained KH_2PO_4-0.1%, $(NH_4)_2SO_4$-0.5% 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 sterile distilled water. The cell density was adjusted to 2.05×10^5 cells/ml. 5ml of cell

suspension was used for the inoculation of the fermentation medium. Fermentation was carried out at 30°C at B.O.D. incubator for 96 hrs using 48 hrs old culture of *Saccharomyces cerevisiae* $Y_{Bmax.}$

Carbon Sources: A series of carbon sources were used in the medium to observe their effect on cell growth, acetic acid production capability & activities of alcohol dehydrogenase & aldehyde dehydrogenase of *Saccharomyces cerevisiae* $Y_{Bmax.}$ Vide Fig.6a). The sugars used in this study are glucose, galactose, sucrose, maltose, lactose, arabinose, xylose, starch, Na-acetate & glycerol. The concentration of each carbon source was maintained at 12% (w/v) in the fermentation medium. Each carbon source was sterilized separately and added to the above mentioned basal medium aseptically.

RESULTS & DISCUSSION:

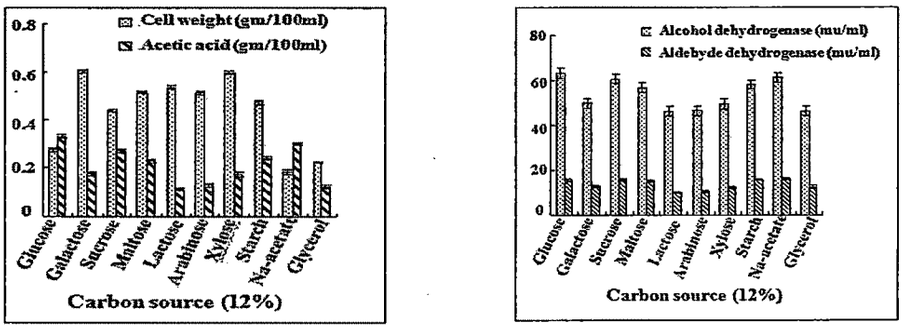

Fig. 6a) Effect of different carbon sources on Acetic acid production and activities of Alcohol dehydrogenase and Aldehyde dehydrogenase :

Production values are expressed as mean ± SD.

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

Fig. 6a) indicates that among all the carbon sources used, glucose is the most suitable carbon source for maximum growth of *Saccharomyces cerevisiae* Y_{Bmax} & consequently helps in maximum acetic acid production & enzyme activities, because-glucose is easily assimilable & also acts as an important source of energy. The effect of other sugars are in the following decreasing order:

Na-acetate>sucrose>starch>maltose>galactose>xylose>arabinose>glycerol>lactose.

B. DETERMINATION OF OPTIMUM CONCENTRATION OF GLUCOSE FOR ACETIC ACID PRODUCTION BY AN ETHANOL & TEMPERATURE RESISTANT *Saccharomyces cerevisiae* Y_{Bmax} :

Since, glucose is the best carbon source for the production of acetic acid by *Saccharomyces cerevisiae* Y_{Bmax}, different concentrations of glucose were tested to determine its optimum concentration to facilitate the acetic acid production & enzyme activities. If the concentration of carbon source is more than the optimum value, then, it will cause a decrease in growth rate. This phenomenon is known as "substrate inhibition effect". This is because of high osmotic pressure of the solution which causes partial dehydration of the cell. But it has also been observed that bacteria are more sensitive to osmotic pressure effect than are the molds and yeasts. Again, if the concentration of carbon source is less than the optimum level, then the unavailability of the essential nutrient will decrease the energy production by the organism, as well as the growth, enzyme activities & finally the acetic acid production. The results are depicted in Fig. 6b).

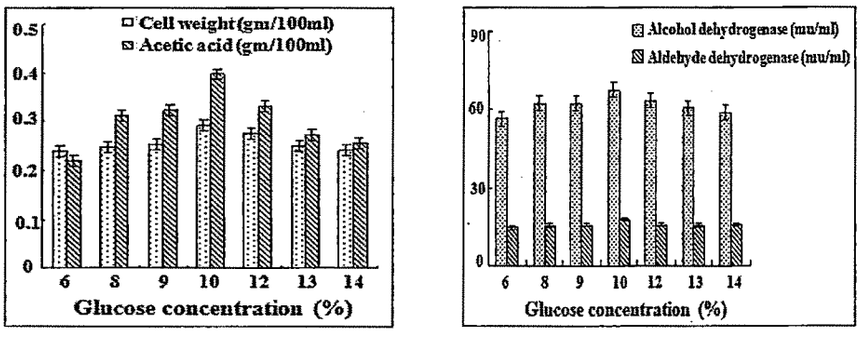

Fig. 6b) Determination of optimum glucose concentration on Acetic acid production and activities of Alcohol dehydrogenase and Aldehyde dehydrogenase :

Production values are expressed as mean ± SD.

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

From fig. 6b), it is found that, glucose at 10% concentration shows maximum production of acetic acid by *Saccharomyces cerevisiae* Y_{Bmax}. Use of other concentrations decreases the acid production.

C. SELECTION OF SUITABLE NITROGEN SOURCE FOR ACETIC ACID PRODUCTION BY AN ETHANOL & TEMPERATURE RESISTANT *Saccharomyces cerevisiae* Y_{Bmax} :

Next to carbon source, nitrogen source is the most important substance of the growth medium. A few organisms utilize nitrogen source as energy source too. Nitrogen is also utilized both for functional as well as structural purposes by fungi. Different forms of nitrogen have profound effects on the metabolism of fungi. Generally, the nitrogen content of fungi is about 14% of its dry weight. An extracellular supply of nitrogeneous material is essential for the continued production of new protoplasm & yeasts generally derive this element from such relatively simple substances as ammonium salts, nitrates, amino acids & amides, although there is evidence that di or even higher peptides may also be assimilated.

Unlike carbon sources, the nitrogen sources are good for both growth and reproduction. Nitrogen is assimilated in the cell as glutamate and glutamine.These two compounds are responsible for the synthesis of many nitrogen containing molecules like asparagine, histidine, tryptophan and purine nucleotides. Some research scholars demonstrated that nitrogen and carbon contents of the medium cause pronounced qualitative and quantitative variations in the amino acid contents of the mycelium (443,444).

Many algae and fungi use ammonium nitrate and sodium nitrate as nitrogen sources; however, yeasts and bacteria have problems utilizing nitrogen in this form. Most of the species comprising the genera *Saccharomyces, Pichia, Hanseniaspora & Debaromyces* fail to assimilate nitrates. In natural media the most common sources of nitrogen easily assimilated by yeasts are the amino acids, purines, pyrimidines, and urea. Depending upon the buffer capacity of the system urea will raise the pH value of the medium. Organic urea is also formed from urea cycle reaction, starting with ammonia (445).

$$NH_3 + ATP + CO_2 \rightarrow H_2NCOOP + ADP$$

Assimilable nitrogen is essential for yeast metabolism and growth. Nitrogen availability is directly related to biomass production during the yeast exponential growth phase at early stages of alcoholic fermentation (446,447). Again, if a culture

medium is starved of carbon source in presence of amino acids, then, the carbon skeleton of amino acids is consumed and ammonia accumulates affecting the cell toxically. Instead of ammonium compounds or amino acids, if nitrate salt is used as a source of nitrogen, nitrite is formed by the partial reduction of nitrate which is toxic to the cell. Therefore, the effect of nitrogen source is to be derived from its metabolic products as well as from its original form.

The accumulated knowledge of the various sources of carbon & nitrogen which can support yeast growth might suggest that synthetic media can be prepared which will stimulate growth to the same extent as the complex undefined media. Since the composition of the production medium largely influences the growth of *Saccharomyces cerevsiae* Y_{Bmax} and the production of acetic acid, it is necessary to search for a suitable nitrogen source for the same. Earlier in this Chapter, we mentioned that 10.0% glucose is the most suitable carbon source for the production of acetic acid. Here, we studied the effect of several nitrogen sources for the same.

Albers et al. cultivated an industrial strain of *Saccharomyces cerevisiae* in batch cultures with different nitrogen sources, e.g. ammonium salt, glutamic acid & a mixture of amino acids, with 20 gm/L of glucose as carbon & energy source & measured the effect of nitrogen source on metabolite formation, cell growth & cell composition. They found that, ethanol yield increased on both glutamic acid & the mixture of amino acids. Glutamic acid has a large influence on the formation of products, e.g. α keto glutaric acid, succinic acid & acetic acid. Cultures grown on amino acids have a higher specific growth rate than cultures of both ammonium grown & glutamic acid grown cells (448).

Johnston et al. found that, during nitrogen starvation, cells of the yeast *Saccharomyces cerevisiae* increased threefold in number, little RNA & protein were accumulated. Both RNA & protein were extensively degraded during starvation (449).

Godard et al. compared the transcriptomes of *Saccharomyces cerevisiae* cells growing under steady-state conditions on 21 unique sources of nitrogen. They found 506 genes differentially regulated by nitrogen and estimated the activation degrees of all identified nitrogen-responding transcriptional controls according to the nitrogen source. One main group of nitrogenous compounds supports fast growth and a highly active nitrogen catabolite repression (NCR) control. Catabolism of these compounds

typically yields carbon derivatives directly assimilable by a cell's metabolism. Another group of nitrogen compounds supports slower growth associated with excretion by cells of nonmetabolizable carbon compounds such as fusel oils, and characterized by activation of the general control of amino acid biosynthesis (450).

Beltran et al. found that, nitrogen limitation is one of the most common causes for stuck or sluggish fermentation (451).

Guillamon et al. showed that, nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentation & the most common method for dealing with nitrogen-deficient fermentation is adding supplementary nitrogen (usually ammonium phosphate). However, it is important to know the specific nitrogen requirement of each strain, to avoid excessive addition that can lead to microbial instability and ethyl carbamate accumulation (452).

Kinetics of alcoholic fermentation by *Saccharomyces cerevisiae* wine strains in a synthetic medium with high sugar content were established by Strehaiano et al. for different nitrogen initial content and are presented for four strains. The composition of the medium was close to grape must except that the nitrogen source consisted mainly in ammonium and was varied from 120 to 290 mgN/L assimilable nitrogen. They found that, the effect of assimilable nitrogen was in general greater on sugar consumption rates than on growth; three kinds of effect on sugar consumption rates were observed: (i) existence of an optimal initial nitrogen level for a maximal sugar consumption rate (inhibition if excess), (ii) no effect of nitrogen beyond the intermediary level (saturation), (iii) sugar consumption rate proportional to the initial nitrogen level (activation). In all cases, the amount of consumed nitrogen increased with its initial concentration and so did the fructophilic capacity of the strains. The optimal requirement varied from 0.62 to 0.91 mgN/g of sugars according to different strains (453).

Ethanol production from concentrated sweet sorghum juice was carried out by Tan et al. under very high gravity (VHG) fermentation conditions using *Saccharomyces cerevisiae* yeast strain at 30°C. Different nitrogen sources $CO(NH_2)_2$ or $(NH_4)_2SO_4$, with the concentration of 0.8 g nitrogen/L, were added to promote the ethanol yields and to prevent the byproduct formation (no additional phosphorus). With an initial sugar concentration of 300 g/L, the maximum ethanol concentration and cell number

of the fermentation medium with $CO(NH_2)_2$ as nitrogen source (135 g/L and 5.5×10^8 m/L, respectively) were higher compared to those of the control one (120 g/L and 4.7×10^8 m/L, respectively). However, no positive effects of $(NH_4)_2SO_4$ were observed compared with the control. The yield of main byproducts (glycerol and acetic acid) obtained was 0.027 g/g of consumed sugar when $CO(NH_2)_2$ was used as a nitrogen source, which was clearly lower than those for ammonium-grown culture (0.035 g /g consumed sugar) and the control (0.037 g/g consumed sugar). The lower values of glycerol and acetic acid yields indicate that $CO(NH_2)_2$ succeeds in the efficient balancing of the redox potential and reduces the formation of byproducts during VHG fermentation than when, for example, ammonium salt is the only available nitrogen source (454).

A Shiraz must with low yeast assimilable nitrogen (YAN) was supplemented with two increasing concentrations of diammonium phosphate (DAP) and fermented with one *Saccharomyces cerevisiae* and one *Saccharomyces bayanus* strain by Ugliano et al. with maceration on grape skins (455).

According to Azemi et al. & Loo et al. nitrogen supplementation is essential for the growth of *Saccharomyces cerevisiae* & *Candida utilis* pineapple waste extracted medium, because- it had very little nitrogen content (0.003%-0.015%). So supplementary nitrogen source is needed to increase the nitrogen content in the waste and thus to enhance the yeast growth (456,457).

Berry et al. grown *Saccharomyces cerevisiae* in continuous culture using a control medium and media which contained low levels of ammonium and phosphate. They investigate the effects of medium composition and growth rate on the levels of intermediates of the glycolytic pathways, the tricarboxylic acid cycle and the glyoxylate cycle. The energy charge varied only between 0.7 and 0.9 over the range of dilution rates studied; however, the level of ATP decreased by 50% at higher aerobic growth rates. Intermediates of the Embden-Meyerhof-Parnas pathway were higher at the low aerobic growth rates and decreased as the dilution rate was increased. However, higher levels of these intermediates were also observed at even higher dilution rates at which ethanol formation and fermentative metabolism occurred. Significant differences in levels of intermediates were observed between control experiments and fermentations using the low nitrogen and phosphate media.

The greatest differences were observed in the levels of glucose 6-phosphate, 6-phosphogluconate, pyruvate, citrate and glyoxylate. Twenty-one different steady states were investigated and each was found to have a unique composition (458).

Ernandes et al. showed that, biomass and ethanol production by I ndustrial *Saccharomyces cerevisiae* strains were strongly affected by the structural complexity of the nitrogen source during fermentation in media containing galactose, and supplemented with a nitrogen source varying from a single ammonium salt (ammonium sulphate) to free amino acids (casamino acids) and peptides (peptone) (459).

MATERIALS & METHODS:

Medium & Cultural conditions: For the Selection of a suitable nitrogen source, the 85ml of the medium used contained: Glucose 10%, KH_2PO_4 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 sterile distilled water. The cell density was adjusted to 2.05×10^5 cells/ml. 5ml of cell suspension was used for the inoculation of the fermentation medium. Fermentation was carried out at 30°C at B.O.D. incubator for 96 hrs using a 48 hrs old culture of *Saccharomyces cerevisiae* $Y_{Bmax.}$

Nitrogen Sources: A series of nitrogen sources were used in the medium to observe their effects on the cell growth , acetic acid production capability & activities of alcohol dehydrogenase & aldehyde dehydrogenase of *Saccharomyces cerevisiae* $Y_{Bmax.}$ and the results are shown in Fig.6c). The nitrogen sources used in this study are- $(NH_4)_2SO_4$, urea, $NaNo_3$, NH_4Cl, $(NH_4)H_2PO_4$, NH_4No_3, tri-ammonium citrate, ammonium carbonate & ammonium oxalate. In each of the nitrogen source the N-level was maintained at 0.1059% in the fermentation medium. Each nitrogen source was sterilized separately and added to the above mentioned basal medium aseptically.

RESULTS & DISCUSSION:

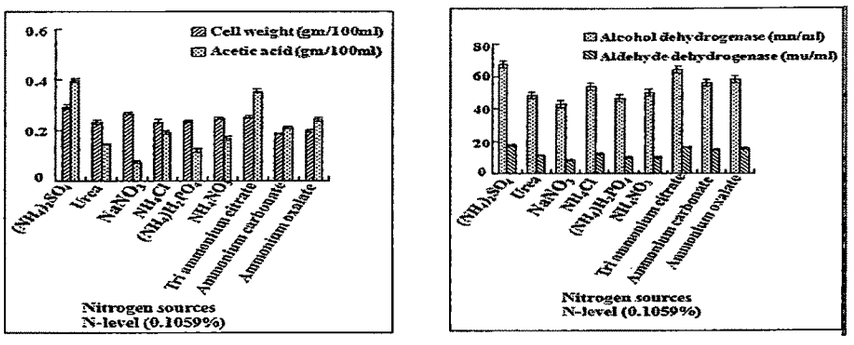

Fig. 6c) Effect of different nitrogen sources on Acetic acid production and activities of Alcohol dehydrogenase and Aldehyde dehydrogenase :

Production values are expressed as mean ± SD.

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

Fig. 6c) indicates that among all the nitrogen sources used, $(NH_4)_2SO_4$ is the most suitable nitrogen source for maximum growth of *Saccharomyces cerevisiae* Y_{Bmax} & consequently helps maximum acetic acid production & enzyme activities.

D. DETERMINATION OF OPTIMUM CONCENTRATION OF $(NH_4)_2SO_4$ FOR ACETIC ACID PRODUCTION BY AN ETHANOL & TEMPERATURE RESISTANT *Saccharomyces cerevisiae* Y_{Bmax} :

The optimum concentration of nitrogenous substance is important for optimum growth and reproduction of the cell. Besides, accumulation of toxic metabolites and the pH changes in the medium due to its nitrogen content are much sharper than those due to carbon sources and this may also be a reason for limiting good sporulation to a narrower range of nitrogen concentration. As $(NH_4)_2SO_4$ is found to be the most suitable nitrogen source for the growth of *Saccharomyces cerevisiae* Y_{Bmax}, it is important to optimize the suitable concentration of $(NH_4)_2SO_4$ to achieve maximum growth of the biomass and consequently the maximum production of acetic acid. Different concentrations of $(NH_4)_2SO_4$ were used for the acetic acid production to determine the optimum concentration for maximum cell growth and production of acetic acid. The result are shown in Fig. 6d).

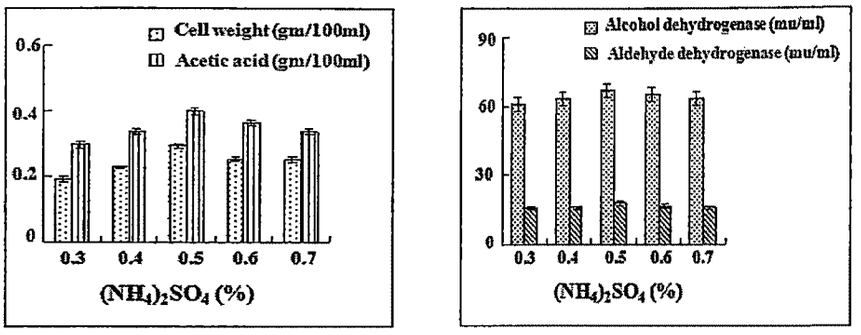

Fig. 6d) Determination of optimum $(NH_4)_2SO_4$ concentration on Acetic acid production and activities of Alcohol dehydrogenase and Aldehyde dehydrogenase :

Production values are expressed as mean ± SD.

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

It was observed from the Fig. 6d) that maximum cell growth and acetic acid production is achieved at 0.5% $(NH_4)_2SO_4$. With the higher or lower concentration of $(NH_4)_2SO_4$, the rate of cell growth and acid production significantly declines.

Thus from the present study, it can be concluded that:

Most suitable carbon source: Glucose.

Optimum concentration of glucose: 10%.

Most suitable nitrogen source: $(NH_4)_2SO_4$.

Optimum concentration of $(NH_4)_2SO_4$: 0.5%.