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# OPTIMIZATION STUDIES OF YEAST FEED FOR ENHANCED GROWTH FOR ETHANOL PRODUCTION BY SHAKE FLASK METHOD

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**Abstract**

Yeasts are the industrially used microorganisms to provide the best possible combination of characteristics for the biological reactions with the given substrates. Yeast nutrition refers to how cells feed; translocate water and essential in-organic and organic nutrients from the ecosystem. *Saccharomyces cerevisiae* of bakery origin (strain S5) and soil origin wild strain (strain S6) were cultivated on nutrient agar and were activated on Saboraud's dextrose agar and Czepkdox agar, potato dextrose agar and malt extract agar by three subsequent inoculations for the maximum growth. Test strains S5 and S6 were optimized for their growth requirements in malt extract broth as initial medium; pH was maintained at 5.0 and incubated at 37°C for 72 hours, 110 rpm, 1.0 ml inocula size. Growth studies were under taken in three replicates after every 4 hours till 96 hours. Number of nutritional requirements were provided at the optimized concentrations in the form of yeast media 5 and 6 respectively and finally the growth was observed at 600 nm. The test strains grown on growth media (5 and 6) were also subjected for their tolerance to various chemical agents at the respective concentrations (ml v/v). Present study revealed the higher growth rate of *S. cerevisiae* strains S5 on Gm5 as compared to the strain S6 on Gm6 containing vitamins and amino acids as growth promoting factors at 110 rpm, pH 4.0, 0.5 and 1 ml. inocula size after 72 hours incubation by shake flask method which indicates that strain S5 is the best candidate for ethanol production.

**Keywords:** Yeasts industrially, Yeast nutrition, Growth for ethanol production shake flask method

## 1. Introduction

The industrial society developed by the accumulation of the scientific knowledge, the spread of technological innovations, and the exploitation of enormous natural resources (King and Clevedon, 1980). In the current epoch, the utilization of microorganisms in industrial processes or in process in which, their activities may become of industrial or technological significance that relates to the production of desired products including pharmaceuticals, organic acids, enzymes, organic solvents and synthetic fuels and also utilized to prevent their values (Presscott and Dunn, 1959, Zertuche and Zali, 1982). Yeasts are truly fascinating organisms, having 7000 species reported (Boekhout and Kurtzman, 1996; Hawksworth and Mouchacca, 1994). Yeasts, *Saccharomyces cerevisiae* are able to rapidly convert sugars to ethanol having a high fermentation property, high show, high ethanol tolerance and osmotolerance, low pH, and high temperature optima, have high glycolytic, invertase activities and genetic stability (Codon and Bentez, 1985).

## 2. Methodology

*A-Isolation of cultures*

*Saccharomyces cerevisiae* cultures were received from local bakery (Strain-5) and the wild strain was isolated from 20 soil samples. Bakery strain was isolated by mixing bakery dough in the sterilized malt extract (ME) broth and incubated at 37°C for 12 hr. Later a loop full was inoculated on Sabouraud's dextrose agar (SDA) plate containing mycological peptone 10, dextrose 40, yeast extract 3 and agar-agar 20g / liter of distilled water, pH 5.6. Plates were incubated at 37°C for 24 hr to observe the

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Khoc cho nho thuong voi trong long, khoc cho noi sau nhe nhu khong. Bao nhieu yeu thuong nhung ngay qua da tan theo khoi may bay that xa... growth. Colonies were identified and maintained as pure culture in SDA slopes. Soil originated wild strain (S6) was isolated by pour plate method of Khan and Mahmood, (1992). 1ml of each suspension was poured over the surface of SDA agar plates containing 400mg of griseofulvin/liter antifungal agent (Hantscahke, 1968).

**B-Activation of cultures.**

All test strains were activated on Sabourad's dextrose agar (SDA) and Czapekdox agar (CZPA) composed according to Demain and Solomon, (1986) with yeast extract 4, sucrose 15, sodium nitrate 2, ferrous sulfate seven hydrate 0.01, di-potassium hydrogen phosphate 0.5, potassium chloride, 0.5, magnesium sulfate 0.5, agar-agar 15g / liter supplemented with streptomycin 30 mg / liter of distilled water. These plates were incubated at 37°C for 24 hours. The sub culturing of these strains were continued for five days in fresh media plates (Ghumro *et al.*, 2001) and finally the master plates were maintained for further studies.

**C-Growth studies.**

Growth studies were under taken in three replicates after every 4 hr till 96 hr at room temperature, 37°C in static incubator and orbital shaker at 120 rpm and were examined at OD 600 (Choudhary *et al.*, 1986). Further optimization of various sizes of inocula, pH, temperatures and agitation speeds in ME broth.

**D-Preparation of growth media (Gm) for the growth of test strains.**

After optimization studies of various nutritional factors, synthetic growth media (5 and 6) for the growth of test strains were prepared according to the optimized concentrations.

**E-Growth studies of test strains on growth media.**

Fresh cultures of test strains into 300 ml conical flask containing 100 ml of growth media, flasks were incubated at 30°C for 72 hr in orbital shaker at 110 rpm. Absorbance of all test strains was determined and compared after every 24 hr incubation with the absorbance of test strains grown in the malt extract broth and later total viable count (Sherply, 1960; Noor and Hameed, 1996; Ghumro, 1996; Birch *et. al.*, 2003) was obtained.

**F-Growth studies of test strains under stressed environments**

i - *Effect of ultraviolet (UV) radiations*

Fresh inocula of test strains (S5 and S6) were separately inoculated in four sets of conical flasks containing 100 ml (v/v) of growth media and incubated at 30°C for different time periods at 260 nm UV lamp. The irradiated cultures were sub cultured on fresh growth media and kept at 110 rpm, 120 rpm in orbital shaking incubator at 30°C for 72 hrs at OD 600.

ii- *Effect of moist heat at various temperatures*

Fresh inocula of test strains were separately inoculated in seven sets of conical flasks containing 100 ml (v/v) of growth media and kept in water bath at various temperatures and various intervals. Heat treated cultures were sub cultured in fresh growth media (5 and 6) and kept at 110 rpm in orbital shaker incubator at 30°C and 36°C for 72 hr respectively and finally the absorbance was checked at OD 600.

iii - *Effect of mutagenic chemicals*

Fresh inocula of test strains (S5 and S6) were separately inoculated into seven sets of conical flasks containing 100 ml of growth media and mercuric chloride, ferric chloride, phenol at the concentration (v/v). All culture flasks were incubated for 72 hrs in shaking incubator at 30°C, 110 rpm, OD 600.

## 3. Results

This research work has been performed at the Enzyme and Fermentation Biotechnology Laboratory, Institute of Biotechnology and Genetic Engineering, University of Sindh Jamshoro, Pakistan. Accordingly the test strains S5 and S6 grown well during 72 hr incubation at 37°C in orbital shaker incubator (at 120 rpm) as compared to the incubation at room temperature and in the static incubator (**Fig. 1**). Test strains S5 and S6 showed the maximum growth at 0.5 and 1.0 ml inoculum size, pH 4.0, temperature 30°C and 110 rpm agitation speed respectively (**Table-1**). During optimization studies for the yeast growth it was revealed that the mineral requirement of yeast growth ranges from 0.2 to 0.6% (w/v) in ME broth (**Fig. 2**).

The optimized concentration of minerals supports and the growth of test strains increases

at 30°C, pH 4.0, 0.5-1.0 ml inoculum size after incubation at 110rpm (Fig. 3-4). *Saccharomyces cerevisiae* strains S5 and S6 were tested for their tolerance to various stress conditions such as UV radiations, moist heat (in water bath) in various intervals and were also tested at various concentrations of mercuric chloride, ferric chloride and phenol, ethanol and methanol.. This study has revealed that both S5 and S6 strains survived for 15 minutes with little decrease in growth and thereafter a drastic decrease in growth occurred. Chemical stress revealed the tolerance at 0.2 ml v/v concentrations of mercuric chloride, ferric chloride and phenol. It was also observed that these test strains have a tolerance in ethanol at 11 and 06 ml and methanol at 6 and 04 ml v/v of the broth medium (Table- 2-3, Fig. 5).

**Table -1. Growth of test strains (*S5 and S6*) of *S. cerevisiae* at *1.0 ml inoculum size* with various sizes of inocula (ml v/v), *pH values*, temperatures °C and agitation speeds (rpm) in shaking incubator at 37°C after 72 hr incubation**

| Sizes of inoculum (ml v/v) | | | pH values | | | Temperatures °C | | | Agitation speeds (rpm) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | S5 | S6 | | S5 | S6 | | S5 | S6 | | S5 | S6 |
| 0.5 | 0.229 | 0.168 | 2.5 | | 0.158 | 20 | 0.221 | 0.160 | 80 | 0.238 | 0.179 |
| 1.0 | 0.228 | 0.175 | 3 | 0.217 | 0.163 | 22 | 0.226 | 0.165 | 90 | 0.241 | 0.184 |
| 1.5 | 0.225 | 0.171 | 3.5 | 0.226 | 0.170 | 24 | 0.231 | 0.170 | 100 | 0.250 | 0.190 |
| 2.0 | 0.220 | 0.165 | 4 | 0.235 | 0.177 | 26 | 0.238 | 0.176 | 110 | 0.259 | 0.196 |
| 2.5 | 0.215 | 0.158 | 4.5 | 0.230 | 0.171 | 28 | 0.244 | 0.182 | 120 | 0.255 | 0.192 |
| 3.0 | 0.207 | 0.152 | 5 | 0.224 | 0.164 | 30 | 0.251 | 0.188 | 130 | 0.249 | 0.187 |
| 3.5 | 0.198 | 0.146 | 5.5 | 0.216 | 0.157 | 32 | 0.247 | 0.184 | 140 | 0.242 | 0.179 |
| 4.0 | 0.191 | 0.138 | 6 | 0.208 | 0.152 | 34 | 0.242 | 0.179 | 150 | 0.233 | 0.170 |
| 4.5 | 0.184 | 0.130 | 6.5 | 0.200 | 0.146 | 36 | 0.236 | 0.173 | | | |
| 5.0 | 0.177 | 0.124 | 7 | 0.191 | 0.139 | 38 | 0.230 | 0.165 | | | |

**Table-2. Effect of U V radiations, heat *(water bath temperature)* at 20 - 50°C on the growth of test strains *(S5 and S6 with initial O.D. 1.701, 1.062 respectively)* in growth media at pH 4 and 0.5 - 1 ml inoculum size, 30°C 110 rpm after 72 hr incubation**

| Time (minutes) | *U.V. rays* | | Various temperatures °C | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 20 | | 30 | | 40 | | 50 | |
| | S5 | S6 | S5 | S6 | S5 | S6 | S5 | S6 | S5 | S6 |
| 15 | 1.679 | 1.011 | 1.679 | 1.011 | 1.700 | 1.056 | 1.669 | 0.158 | 1.657 | 1.024 |
| 30 | 1.563 | 0.883 | 1.563 | 0.883 | 1.700 | 1.056 | 1.637 | 1.042 | 1.618 | 1.006 |
| 45 | 1.534 | 0.672 | 1.534 | 0.672 | 1.696 | 1.054 | 1.595 | 0.127 | 1.559 | 0.878 |
| 60 | 1.500 | 0.417 | 1.500 | 0.417 | 1.693 | 1.055 | 1.568 | 1.009 | 1.503 | 0.763 |

*Mean values of three replicates*

**Table-3. Effect of mercuric chloride, ferric chloride and phenol at various concentrations (0.2, 0.4 – 1.0 ml v/v) on the growth of test strains *(S5 and S6 with initial O.D. 1.701, 1.062 respectively)* in growth medium at pH 4 and 0.5 - 1 ml inoculum size, 30°C 110 rpm after 72 hr incubation**

| Concentration (% v/v) | *Mercuric chloride* | | *Ferric chloride* | | *Phenol* | |
|---|---|---|---|---|---|---|
| | S5 | S6 | S5 | S6 | S5 | S6 |
| 0.2 | 1.671 | 1.054 | 1.674 | 1.058 | 1.663 | 1.055 |
| 0.4 | 1.634 | 1.045 | 1.645 | 1.051 | 1.618 | 1.042 |
| 0.6 | 1.591 | 1.036 | 1.611 | 1.045 | 1.567 | 1.030 |
| 0.8 | 1.549 | 1.024 | 1.576 | 1.036 | 1.501 | 1.018 |
| 1.0 | 1.497 | 1.014 | 1.531 | 1.025 | 1.426 | 1.001 |

*Mean values of three replicates*

Fig. 1: Determination of growth of *Saccharomyces cerevisiae* strains S5 and S6 on M.P. broth at room temperature, 37 C at static incubator and at 37 C in orbital shaking incubator at 110 rpm.

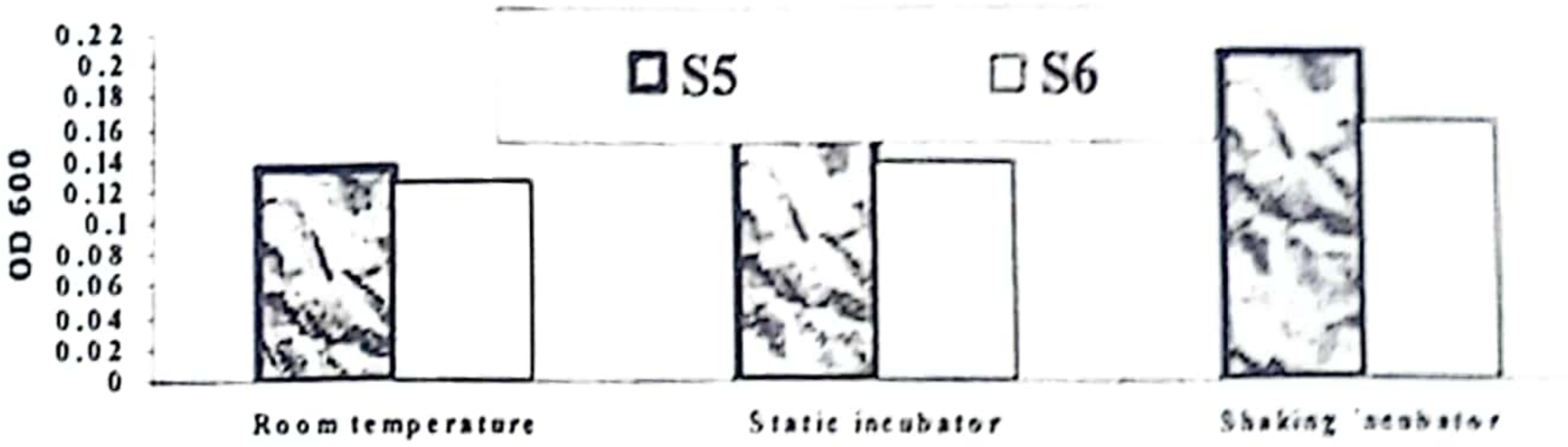



Fig.2: Comparative growth of *S.cerevisiae* strains S5 and S6 on growth media (5 and 6) respectively at stress conditions after 72 hr incubation.

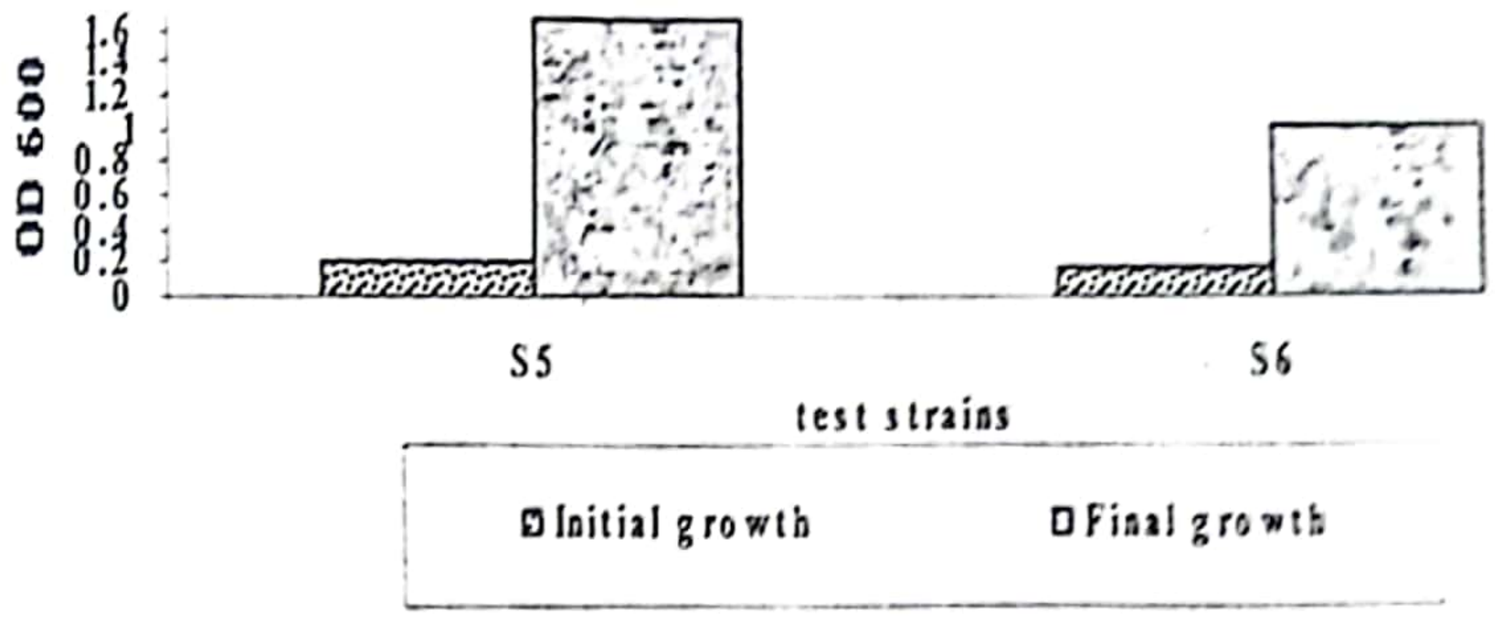
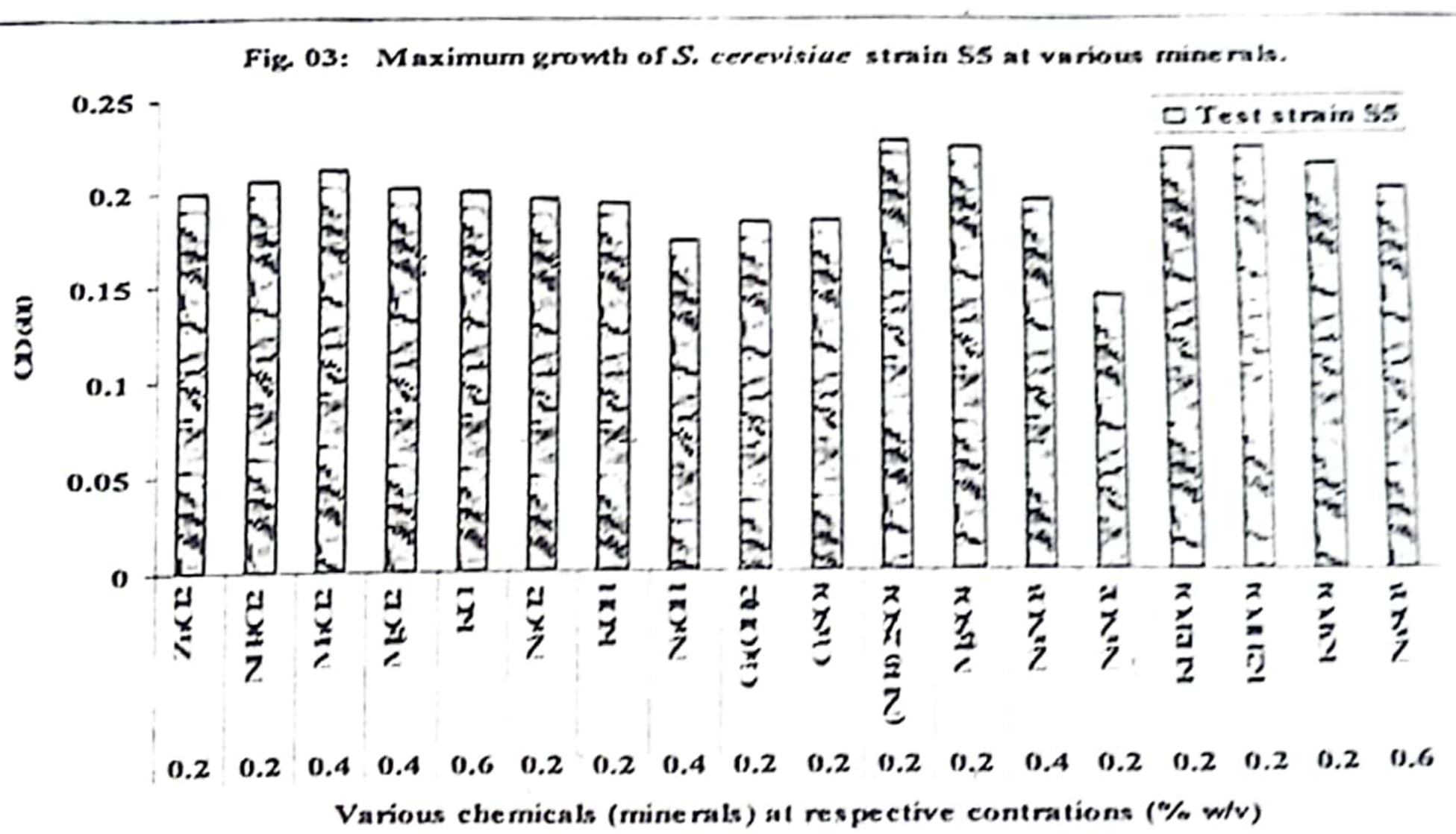



Fig. 03: Maximum growth of *S. cerevisiae* strain S5 at various minerals.



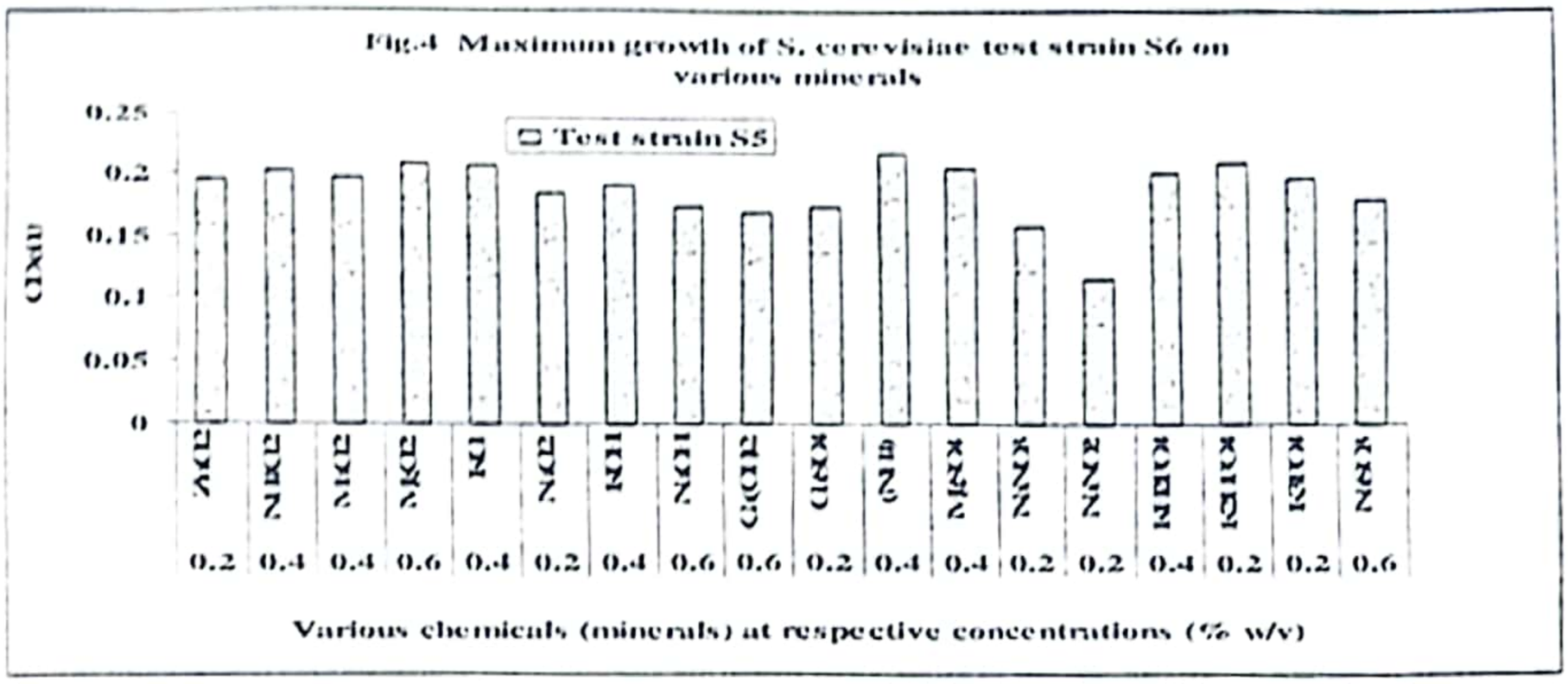



Fig.4 Maximum growth of S. cerevisiae test strain S6 on various minerals

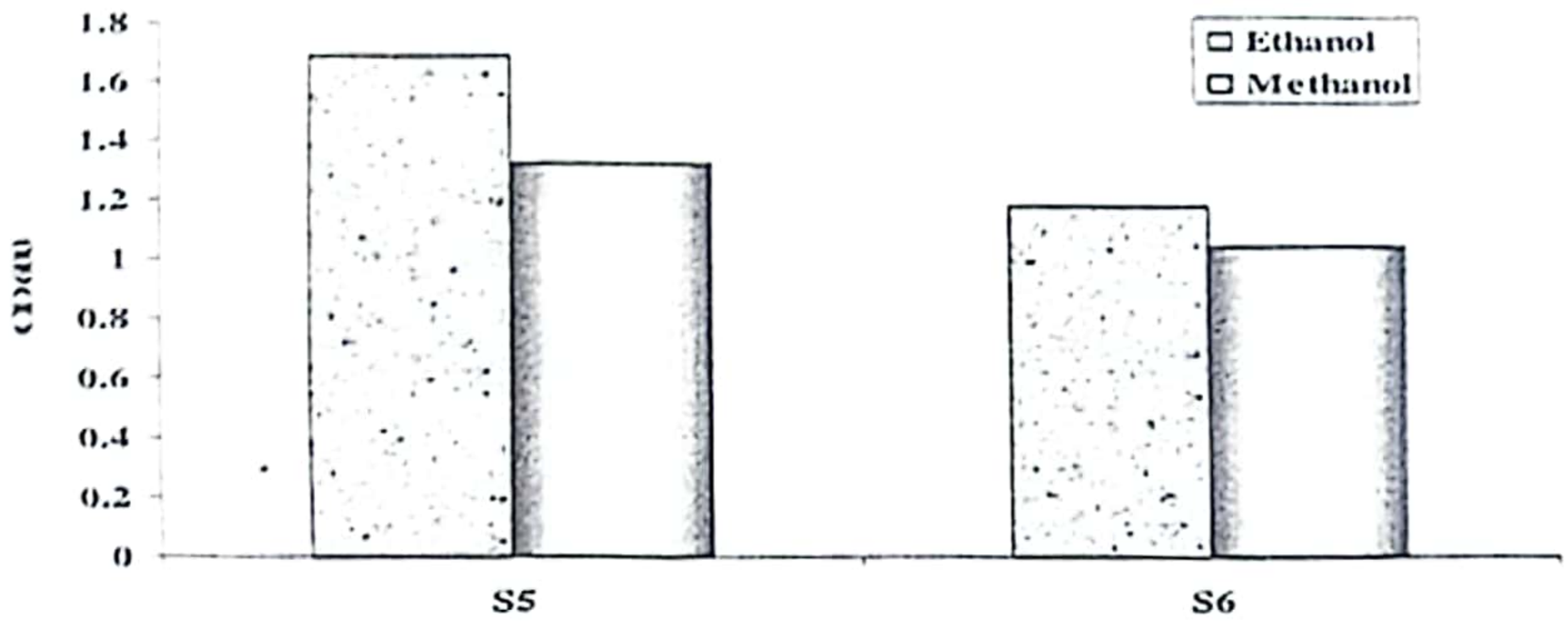



Fig. 5 Comparasion of ethanol and methanol tolerance of *S. cerevisiae* strains S5 and S6 at various concentrations

*S. cerevisiae* test strains S5 and S6

## 4. Discussions

Globally, biofuel technology has emerged and being used as substitute of fuel oil by yeast from waste as a source of energy (Bawa and Yoshiyuki, 1992; Moritz and Sheldon, 1996; Noor and Hameed, 1998, 1999). The essence of the present study is to enhance the growth of *Saccharomyces cerevisiae* strains S5 and S6 using different growth requirements including the minerals (feed-I), sugars, amino acids and vitamins (feed-II) after treating under stress conditions for ethanol fermentation by shake flask method.

Drying of baker's yeast causes rupture of cytoplasmic membrane and brings about changes in the composition of the nucleic acids, proteins, lipids, carbohydrates. The re-hydration provides the moisture level for the growth and metabolic activity (Alpas *et al.*, 1996).

Activation of yeast strains in malt medium supports the higher cellular mass and enzymatic activity. Sub culturing of lyophilized cultures and synthetically prepared modified MYPGA in which peptone was replaced with Yeast Nitrogen Base i.e. Malt extract Yeast extract Yeast Nitrogen Base Agar (MYNGA). From these media, MYNGA was found a better medium because it contains yeast nitrogen base, which includes ammonium sulphate, asparagines, minerals, vitamins, trace elements and amino acids provides a good nitrogen sources as well as other growth factors for carbon assimilation and fermentation (Walker, 1997; Edgerton, 2001). The activated cultures were grown in basic medium e.g. ME broth Age and size of inocula were observed after four hour incubation, which revealed 72 hr age of test strains. This is due to the fact that the growth of the starter reaches its log phase, having faster metabolic activity and the optimized volume of inocula of active yeast culture competes more for available nutrients. Addition of various inorganic compounds in optimized concentration range could result the enhanced growth and have the stimulatory effects on ethanol production

during fermentation process. Growth rate and fermentation rate are not inversely proportional. This is due to the fact that the starter culture requires the specific growth rate, time, nutrients and other miscellaneous parameters for their enzymatic activity. Our observations pertaining to the size of inocula are in agreement with the results of Choudhary *et al.*, (1986), Panday and Agarwal (1993), Noor and Hameed (1997).

According to Stanford and Anslow (1996) the hydrogen ions such as protons are very significant for both intracellular and extracellular pH which has a dramatic influence on growth and metabolism of yeast cells. The effects of various pH ranges are due to the weak acids, which dissipate plasma membrane proton gradients and depressing cell pH when they dissociate into ions in the yeast cytoplasm. On the other hand, medium chain fatty acid causes rapid cell death by disruption of cell membrane integrity during growth.

Oxygen is used for respiratory metabolism, temperature is important for inoculum development, maintenance of fermenter productivity in industrial ethanol production (Noor *et al.*, 2003). An initial increase at 37°C showed increase in growth but the final cell mass concentration attained very low (Sedha *et al.*, 1984, Williams and Munnecke, 1993). In present study, the maximum growth of yeast strains was observed at 30°C when incubated for 72 hours, which shows same pattern as reported by Tabera *et al.*, (1985), Elahi *et al.*, (1989), Enhanced growth of test strains S5 and S6 was observed when incubated in orbital shaking incubator adjusted at 110 rpm. This is because agitation speed provides oxygen supply, which activates the mitochondria for cyclic metabolism and also providing uniform distribution of nutrients. Agitations and temperatures beyond the optima results in cellular damage Choudhary *et al.*, (1986).

During the optimization studies of growth in various chemicals, it was observed that the maximum growth occurred in between 0.2 - 0.6 % (w/v) of ME broth. Concentration of minerals beyond the optima is toxic for the growth of yeast (Walker and Maynard, 1997; Gadd and Laurence, 1996; Avery *et al.*, 1996). Phosphorus is essential for all yeasts which are present in nucleic acid and phospholipids. The major part of sulfur is inorganic orthophosphate (Aiking and Tempest, 1976; Theobald *et al.*, 1996[a]) which acts as a substrate and effectors for many enzymes, including those involved in energy transduction depending upon the levels that fluctuate according to the mode of sugar catabolism (Theobald *et al.*, 1996[b]).

It is reported that chloride transport occurs via a proton-chloride or sodium chloride symport mechanism which may be involved in yeast cell water content (Andre, 1995). Divalent cations in yeast cells act primarily as enzyme cofactors. $Mg^{2+}$ ions transport mechanism is involved in regulating free $Mg^{2+}$ ion in the yeast cytosol (Beeler *et al.*, 1997). Manganese is essential for yeast growth and metabolism in trace concentrations, which may accumulate in vacuole and act as an intracellular regulator for key enzymes (Kihn *et al.*, 1988; Auling, 1994). $Ca^{++}$ ions have been linked to the cell cycle regulation and implicated in the transition from lag to exponential phase in batch cultures of *S. cerevisiae* (You att, 1993; Loukin and Kung, 1995). Potassium and magnesium are regarded as macro elements required to establish the main metallic cationic environment in the yeast cell. Copper and iron act as cofactors in several enzymes including the redox pigments of the respiratory chain (Joslyn, 1941; Walker, 1997). These undergo unique chemistry due to ability to adopt distinct redox states, either oxidized (Cu-II) or in the reduced (Cu-I) state (Maria *et al.*, 1999). It is involved in a variety of biochemical processes, such as cytochrome oxidase, Cu, Zn, superoxide mutase, lysyl oxidase and dopamine-monooxygenase and also play a critical role in assimilation (David and Fred, 1999). They require traces of $Zn^{2+}$ that is essential for structure-function of the enzymes during metabolism for example, the important terminal step enzymes in yeast alcoholic fermentation namely alcohol dehydrogenase is a zinc-metalloenzyme $Zn^{2+}$ uptake is time, temperature and concentration dependent and saturable (Fuhrmann and Rothstein, 1968; Mowll and Gadd, 1983, White and Gadd, 1987). Sulphates are transported for assimilation into sulphur-containing amino acids like methionine and peptides like glutathione but no singular sulphate transporter gene has been identified in yeasts at present (Andre, 1995). Sulphur is required in the form of a variety of sulphur compounds e.g. for the biosynthesis of sulphur

contaning amino acids. In *S. cerevisiae* (in most of the microorganisms and in plants) the formation of sulfite occurs through reductive sulfate assimilation which is the route of methionine and cysteine biosynthesis (Walker, 1997; Thomass and Surdin-Kerjan, 1997). Sulphur prevents enzymatic and non-enzymatic browning reactions and in free form it prevents microbial growth of undesirable microorganisms, which allows the yeast growth in diverse form when provided in liquid form under optimal physical growth conditions for required time (Morris and Haugh, 1956; Walker and O'Neill, 1990; Gimeno *et al.*, 1992; Tudor and Board, 1993; Thomas, 1993).

Growth media (5 and 6) were prepared by addition of minerals in optimized concentrations along with sugars, amino acids and vitamins, which supported the growth of test strains S5 and S6 respectively. Growth under stressed conditions revealed that various physical and chemical agents, radiations are responsible to inhibit the yeast growth when they are exposed to various durations and concentrations beyond the optima respectively. During the observations of thermotolerance, it was reported that temperature beyond optima shows the greater cellular damage i.e. cell viability declines because the yeasts are unable to regulate their internal temperature and the high temperatures provided to them resulting the disruption of hydrogen bonding and hydrophobic interactions leading to general denaturation of protein and nucleic acids (Coote *et al.*, 1991, Shah and Hameed, 2004).

Yeast growing quickly in glucose rich medium is more sensitive to heat and other stresses compared to stationary phase cells. This may be due to the fact that glucose carriers act as the sensors of glucose availability, which rapidly adjust their growth and metabolism to changing environmental conditions (Lagunas, 1993). Sub-lethal heat shock treatment of yeast leads to the induction of synthesis of a specific set of highly conserved heat-shock proteins known (Ruis and Schuller, 1995; Walker, 1997). Mild water stress in yeasts occurs during osmostress caused either by hyperosmotic shock or by hypoosmotic shock (Piper, 1995; Halsworth 1998) producing dramatic changes in membrane structural arrangements and permeability properties (Walker, 1997).

*Saccharomyces cerevisiae* strain S5 and S6 showed insignificant decrease in the growth when examined under stressed growth conditions and a complete decline stage after the specific time period and concentration respectively. This is due to the activation of transcriptional control elements (STREs) in *S. cerevisiae*. The high osmolarity glycerol (Hog1) gene product encodes a mitogen-activated protein kinase and the pathway starts with the activation of plasma membrane bound receptor proteins which act as osmosensors. MAPK cascades transducer signals trigger by heat shock and UV radiations etc. Certain stress metabolites play role as stress-protectant molecule including osmotolerant, antidesicant, chemical detoxicant, cryoprotectant and thermoprotectant (Hino *et al.*, 1990; De Virgillo *et al.*, 1994). Radiations cause DNA damage in *S. cerevisiae*. UV causes dimerization, nicks and other lesions. These cause intrachromosomal recombination in cell cycle-dependent manner and can inhibit certain membrane functions by affecting membrane lipid phase transitions and by interacting with photosensitive chromogenic molecules within the cell (Galli and Shiestl, 1995; Walker, 1997).

Chemical stresses in yeast cells may cause cellular damages during growth and metabolism due to acetaldehyde and ethanol stress produced during growth and fermentation. Low ethanol concentrations are inhibitory to yeast growth and cell division, but higher concentrations can be lethal beyond the concentration 20% v/v. Watson and Cavicchioli (1983) reported that, exposure to ethanol causes the increased membrane fluidity and decreased membrane integrity. Ethanol and methanol toxicity is reduced to the increase of fatty acyl chain length, unsaturated fatty acids and sterols in the cell membrane, membrane lipids, palmitic acid and cholesterol, phosphatidylserine, ergosterol or campesterol, linoleic acid, yeast hull which supports enhanced growth, viability and nutrient uptake. Higher concentration of ethanol destroys enzymes and kills the yeast cells in the medium due to the accumulation of dissolved toxic products, which slows down and stops the growth of test strains. Methanol on the other hand did not allow the test strains to grow beyond the concentration 4-6 % (v/v) in the fermentation medium. This is because the test strains are not the methanol utilizing yeasts and are unable to metabolize by an oxygen-

dependent oxidase to formaldehyde, which is then converted into dihydroxyacetone by DAH synthase enzyme. Our results are well acquainted with Minier and Goma, (1982). Magnesium ($Mg^{2+}$) is a known substance that protects yeast from ethanol stress by reducing its fermentative activity (Dombek and Ingram, 1986; Birch and Walker, 1996; Ciesarova *et al.*, 1996) and increasing the proton and anion permeability caused by ethanol (Petrov and Okorokov, 1990).

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