FERMENTATION ACTIVITY OF YEAST

(Biology of Yeast)

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By

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DEDICATED TO

MY WIFE

AND

FAMILY

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CERTIFICATE.

Certified that the research work incorporated in the thesis, entitled "Fermentation Activity of Yeast" (Biology of yeast) submitted by

SHAMRAO GANPATRAO PATIL

for the award of degree of Doctor of Philosophy, was carried out under my general supervision. The materials and help obtained from other sources has been duly acknowledged.

Shri. S. G. Patil is a senior scientist and is Incharge of National Collection of Industrial Microorganisms (NCIM), at the Division of Biochemical Sciences, NCL. He initiated the thesis project a few years ago and planned all the experiments on his own. His publications in international journals provide enough evidence for the applied potential and scope of his different ideas in the existing alcoholic fermentation technology in India.

P K Ranjekar

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I appreciate the whole hearted help of Bhaskar G. Patil (my brother) and D.V. Gokhale for helping me to carry out the experimental work, in time. I take this opportunity to thank Dr. V. Shankar for his timely suggestions.

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S.G. Patil.

THESIS ABSTRACT

The culture yeast, in the array of microorganisms, has played many vital roles in the production of solvents like ethanol and glycerol, enzymes, vitamins and single cell protein. The cultures particularly S. cerevisiae. S. uvarum and S. pombe have been used for the production of alcohol from sugars, starch and from raw materials like beet and cane molasses. This is carried out by the conventional batch fermentation method using yeast culture and thereby 5-6% (w/v) ethanol production is achieved over a period of 72 h. Continuous efforts are made to isolate a yeast culture which will give higher yield of ethanol (> 8-9 % w/v) and is viable at salt concentration (> 25%) and temperature (40-45^{o}C). However, very little success has been achieved in this respect so far.

Ethanol, an important solvent and starting compound for many other solvents, is now more valuable as an alternative source of energy in place of exhausting and expensive natural petroleum source. In developed countries, bulk of the ethanol production is carried out through a chemical route. In India, ethanol production is exclusively through batch fermentation of cane molasses which is a waste carbohydrate material from sugar factories.

Considering the importance of ethanol and the existing batch fermentation technology, a project was undertaken to improve the yield of ethanol by supplementing the fermentation medium with novel and unconventional additives such as skim milk, chitin and fungal mycelium. A totally new procedure involving the top and bottom yeast together was also attempted. The important findings from the experiments are summarized in the following paragraphs:

ISOLATION OF SALT AND HEAT RESISTANT YEAST STRAINS

To obtain yeast strains with resistance towards heat (± 40° C) and salts (25%), Yeast strains were isolated from molasses pits and from cane sugar factory by a slightly modified method. The soil samples were directly transferred to molasses medium and kept on subculturing for three days at 37°C. The cells were streaked, with required dilution on agar plates, and incubated at 30°C for 20-22 h. The tiny colonies were checked under microscope for purity and were then transferred to the molasses agar slope. Yeast strains, showing growth in molasses medium at 37°C, were picked up directly and designated as SH1 ..... SH35. When they were tested for their ethanol production activity at different temperatures (30-40°C), two strains namely SH12 and SH29

were found to be the better ones and were identified as Saccharomyces cerevisiae by the National Collection of Yeast Culture (NCYC), England. These were later deposited with National Collection of Industrial Microorganisms (NCIM), Pune, India as S. cerevisiae NCIM 3526 (SH12) and S. cerevisiae NCIM 3525 (SH29) for their maintenance and distribution.

ENHANCEMENT IN ETHANOL PRODUCTION FROM CANE MOLASSES BY SKIM MILK SUPPLEMENT

The effect of various additives on the rate of ethanol production from sugar cane molasses under batch fermentation conditions was determined by using industrially used yeast culture and isolated Saccharomyces sp. SH12. Out of the additives tested, skim milk powder (0.2%), supplement was found to markedly enhance the rate of ethanol production; about 6.0% (w/v) ethanol being formed in 48 h from cane molasses containing 13.5% fermentable sugars compared to a period of more than 72 h in its absence of the additives. This is the first report on such an effect of skim milk supplementation in cane molasses fermentation.

CHITIN SUPPLEMENT SPEEDS UP THE ETHANOL PRODUCTION IN CANE MOLASSES FERMENTATION

Different carbohydrates, mostly polysaccharides, were studied for their effect on ethanol production from cane molasses under batch fermentation conditions using the industrial yeast strains *Saccharomyces cerevisiae* NCIM 3526 and *S. uvarum* NCIM 3509. There was a marked increase in the rate of ethanol production in the presence of polysaccharides like chitin, xylan and acacia gum at 0.2% concentration. Chitin, a waste product and cheap material, produced 6.0-6.9% (w/v) ethanol after approximately 30 h at 30^{o}C from cane molasses containing 18% reducing sugars as compared to a period of 72 h in its absence. Chitin supplementation thus reduced the fermentation time and might lead to a decrease in the cost of ethanol production. It would also help to utilise more molasses with existing batch fermentation technology. Our report of using chitin to accelerate the rate of ethanol production is the first of its kind so far.

THE ACCELERATION OF ETHANOL PRODUCTION IN CANE MOLASSES FERMENTATION BY THE ADDITION OF FUNGAL MYCELIUM

The effect of fungal mycelium from different species

and waste mycelium from antibiotic industry was examined on ethanol production from cane molasses fermentation by yeast strain Saccharomyces cerevisiae NCIM 3525. Using 20% cane molasses total reducing sugars, the prepared mycelium (0.4% wet wt) or waste mycelium (0.4% dry wt.) supplement produced 7.0 - 7.6% (w/v) ethanol compared with 4.0 - 4.5% (w/v) ethanol in the control experiment after around 44 h at 30°C. Using 25% molasses total reducing sugars, waste mycelium supplement accelerated the rate of ethanol production and enhanced the yield of ethanol after 70 h from 4.9% (w/v) in control to 8.3% (w/v), a 70% more production. The use of fungal mycelium to enhance the rate of ethanol production in cane molasses batch fermentation, is the first report to our knowledge.

NOVEL SUPPLEMENTS ENHANCE THE ETHANOL PRODUCTION IN CANE MOLASSES FERMENTATION BY RECYCLING YEAST CELL

Eight alcohol producing yeast strains were screened for their sedimentation rates and it was found that S. cerevisiae NCIM 3526 (SH12) was a better flocculent strain. This strain was employed in cane molasses fermentation with yeast recycle supplemented with skim milk, chitin and fungal mycelium individually or in combination at 30°C,

using 15% fermentable sugars. On the completion of ten 16 h cycles, 20-30% more ethanol was produced in presence of these supplements and efficiency of the process was improved from 66 to 87%. The use of such novel supplements to improve the efficiency in molasses fermentation with yeast recycle, to our knowledge, is the first report.

TOP AND BOTTOM YEASTS TOGETHER ACCELERATE ETHANOL PRODUCTION IN MOLASSES FERMENTATION

Alcohol producing top and bottom yeasts were employed individually and together to asses their role in enhancing the rate of ethanol production in cane molasses fermentation at 30°C. The combination of top yeast *S. cerevisiae* NCIM 3281 and bottom yeast *S. uvarum* NCIM 3509 improved the ethanol production rate by 32.6% in batch fermentation and 25.2% in recycling yeast cell fermentation as compared to their mean value of individual ethanol production activity. The application of top and bottom yeast to accelerate ethanol production, without any additives, in cane molasses batch fermentation is the first report in the alcoholic fermentation technology.

PRODUCTION OF YEAST CELLS AND INOCULA REQUIREMENT IN ALCOHOL

BATCH FERMENTATION

Using a cheaper medium, MUMY, containing molasses sugars, urea, magnesium sulfate and yeast extract, production of yeast cells 3.5% (wet wt.) was achieved. The cost of such production was less by 70% as compared to the other medium of molasses sugars with peptone and extracts of yeast and malt, where the yield of yeast cells was 3.85% (wet wt.). The minimum inocula requirement of yeast cells of 3% and 2% (wet wt.) were sufficient in the absence and presence of chitin or fungal mycelium additives, respectively to complete the fermentation activity. Employing such sizes of inocula, 6.3-6.6% (w/v) ethanol was produced from 15% cane molasses total reducing sugars by around 24h at 30^{o}C. Use of cheaper medium for the production of yeast cells, limit on the use of yeast inoculum and reduction in the fermentation period would effectively decrease the cost of ethanol production.

All these experimental findings can be applied directly to the existing batch fermentation technology to improve the ethanol productivity.

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2. Patil, S.G. and Patil, B.G.
   Chitin supplement speeds up the ethanol production in cane molasses fermentation.
   Enzyme Microb. Technol. (1989) 11, 38-43.

3. Patil, S.G. and Patil, B.G.
   The acceleration of ethanol production activity of yeast in cane molasses fermentation by the addition of fungal mycelium.
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"Effect of glycerol and ammonium sulfate on the production of ethanol from cane molasses fermentation." Proceedings of `National Symposium on Yeast Biotechnology', held at Haryana Agricultural University, Hissar (Dec, 12-14, 1985)

Patil . S.G. and Patil B.G. (1987)

"Effect of skim milk on ethanol production using yeast cell recycling in cane molasses fermentation." National Symposium on `Yeast for Alcohol and Fat Prouduction' held at Central Food Technological Research Institute, Mysore 570 013. (Sept, 23-24, 1987)

CITATION

Patil, S.G. and Patil, B.G. (1989)

Paper entitled `Chitin supplement speeds up the ethanol production in cane molasses fermentation'. Full Abstract appeared in `News Monitor' Vol. 4 (1) 33, Publishers `Centre for Advancement of Biotechnology', Post Box No. 406, K.R. Road, Visveswarapuram, Bangalore 560 004.

COMPLIMENTS.

Dr. Gerald Reed, Editor of books on industrial fermentation, expert in the filed of brewery's industry and consultant to Universal Foods Corporation, Wisconsin, U.S.A. has acknowledged one of the papers that appeared in Enzyme Microbial Technol. 11, 38-43, (1989).

UNIVERSAL Foods CORPORATION

FERMENTATION DIVISION
RESEARCH AND DEVELOPMENT

REDSTAR

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Dec. 28, 1988

Dear Dr. Patil,

I have read your paper "Chitin supplement speeds up the ethanol production in cane molasses fermentation", Enzyme and Microbial Technology 11(1): 38-43, 1989, with great interest. Please, permit me to make a short comment.

In the wine industry it has been known for quite some time that the addition of insoluble solids (such as grape pulp or skin particles) speeds up the fermentation. The same is true for additions of diatomaceous earth and often of bentonite. The reasons have never been quite clear and have caused a good deal of speculation. Several years ago the research group in Bordeaux, France, found that some fatty acids, specifically octanoic and decanoic acid, were elaborated during the fermentation and acted as inhibitors of yeasts. Adsorption of these fatty acids on either yeast cell wall material or on activated carbon eliminated the inhibition.

Your experimental results are certainly consistent with the theory of adsorption of an inhibitor by an insoluslbe material, that is all except Sr. # 6 of Table 6 with chitin treated molasses.

With best regards,

Gerald Reed

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CHAPTER I

A GENERAL INTRODUCTION TO YEAST

1.1 HISTORY

Yeast, particularly Saccharomyces, is the most widely investigated species among all the microorganisms. Its first description has been given by Antonie Van Leeuwenhoek in the early seventeenth century using a simple microscope. Yeast, a unicellular eukaryote, has been used by man to master his life for more than 8000 years. This microorganism has got a number of firsts to its credit. For example, it was Pasteur who demonstrated in midst of nineteenth century that sugar was converted to alcohol and carbon dioxide by yeast in the absence of air, while in the presence of air little or no alcohol was formed and carbon dioxide was the only product. Likewise H. Buchner showed in 1897 that extract of yeast prepared by grinding yeast cells with sand when added to a large quantity of sugar exhibited the evolution of carbon dioxide and formation of ethanol. This accidental observation clearly indicated that the soluble enzymatic preparations of yeast were able to carry out alcoholic fermentation and it resulted in the development of modern "Biochemistry". The importance of yeast has been realized by man for its attractive contributions in the productions of industrially important

compounds like ethanol, carbon dioxide, glycerol, series of vitamins (B-complex), enzymes, single cell proteins and feed or fodder yeast. Moreover, since last century yeast has been extensively used as a tool to satisfy man's desire to uncover the basis of life.

1.2 MORPHOLOGY AND HABITAT

The cells of yeast are non motile, single and include mother cell with bud. The size of active cells varies from 4 to 8 μ in width and 5-16 μ in length. Normally, the yeast cells are round, ellipsoidal or filamentous. The filamentous strains may attain cell length even upto 30 μ. Environmental conditions do affect cell morphology and structure. A deficiency of compounds like phosphate, magnesium or biotin leads to a large and elongated cell size. The isolated strains show both smooth and rough appearance: the latter contain more lipid deposits. The chemical composition of the cell wall of the isolated baker's yeast is: carbohydrate 83%, protein 10%, lipid 3%, sterols 0.45%, ribonucleic acid 0.3% and deoxyribonucleic acid 0.04%. The carbohydrates are made up of glucan, mannan, protein (6-12%), chitin (1%), glucosamine, fatty acids, glycerides, phospholipids (0.7%) and phosphate (1.0%).

Like the other flora of microorganisms, yeast too has been distributed in many diverse environmental conditions. The most important class of yeast is found distributed in environments wherever sugars are present. Most of these yeasts do not live in free state in soil or water. The main habitat of such flora is the surface of sweet fruits, nectar or flowers (honey source) and waste sugars from industries or cane juice factory.

1.3 TAXONOMY

It was Meyen who began the yeast taxonomy in the year 1937. He was the first to show that yeast observed in bear was a living organism which reproduced by budding and stimulated the alcoholic fermentation. Yeast taxonomy comprises a very small group in the phylum Thallophyta, subphylum Eumycetes and class Ascomycetes (Lodder, 1970). Lodder, with a team of 13 taxonomists has published an up to date classification of yeast and has divided it into four major groups with 39 genera containing 349 species. According to Kregarvan Rij (1984), yeast is classified under four classes-Eumycota, Ascosporogenous, Basidosporangenous and Imperfect fungi (deuteromycetes). Yeast is covered under ascosporogenous which has two families and one of it

Saccharomycetaceae is the most useful one. The latter one has been divided into four sub-families like (1) Schizosaccharomycoideae; (2) Nadsoniodease; (3) Lipmycoideae; (4) Saccharomycoideae. The most important and industrially useful genera is covered under Saccharomycoideae, like Saccharomyces, Kluyveromyces, Pichia, Pachysolen etc. A number of strains which spoil the fermentation activity or degrade the final products are called 'wild yeast'.

1.4 REPRODUCTION

In yeast, both types of reproduction, sexual and asexual occur. Particularly asexual reproduction, a characteristics of yeast takes place with two different ways: one is budding and another is fission. The budding process is very rapid under optimum conditions of growth. It starts with a small protuberance on a cell followed by division of nucleus and cytoplasm and then full separation of cells, leading to the formation of daughter and mother cells. It has been noticed that a cell can produce 24 buds in its survival period and such process some times continues to form a short chain. In fission, nucleus and cytoplasm divide into two as in bacteria and get separated from each

other.

Sexual reproduction in yeast leads to an area of new investigation. The process is a phenomenon of three characters (a) sexual fusion; (b) nucleic fusion and (c) meiosis. The last stage of this process results in the formation of four daughter nuclei that organize into a full cell or ascospore (1-4) (haploid stage) which can also show the budding character. These cells have been identified as A and alpha or + and - strains. When two of the cells of opposite phase are fused together to become diploid, the latter can undergo reproduction by budding. The haploid phase phenomenon occurs very rarely under extreme adverse conditions in yeast.

1.5 NUTRIENT REQUIREMENT OF YEAST

The nutrient requirement of yeast is simple. Yeast can be grown easily in a defined medium or in complex medium. Like other microorganisms, yeast requires basic elements like C,H,O, N,S, Ca, Na, Mg and trace of Fe, Mn, Co, Zn, Cu, Cl. The supplement of glucose plays a dual role in yeast. It is a source of carbon and energy and provides hydrogen and oxygen. The carbon concentration and oxygen availability ratio has an effect on the production of cell mass. The

carbon source like glucose will be fermented faster under anaerobic conditions than aerobic conditions. In other words glycolysis rate is faster in absence of oxygen than in the presence of oxygen and this phenomenon is called "Pasteur effect." Repression of mitochondrial activity, in absence of oxygen, at the higher concentration of glucose (> 5%) is known as 'Crabtree effect'. These steps are important in the production of ethanol. Besides D-glucose, yeast is capable of utilising other sugars like D-galactose, mannose, fructose, melibiose, D-mannitol, glycerol sucrose and starches; and ethanol to some extent.

Yeast can utilize nitrogen, a second important element from series of organic or inorganic compounds. Although ammonium nitrate, ammonium chloride, ammonium sulfate are the better sources of nitrogen, ammonium sulfate or urea is used on industrial scale for the production of cell mass or ethanol. Amino acids are also used as nitrogen supplement by yeast. Potassium phosphate serves as the source of phosphate and potash. Growth factors like inositol, biotin, or B-complex vitamins are rarely added, because yeast requirements varies according to the species. It is important that all the nutrients of yeast are adjusted in

specific concentrations. Otherwise they will turn as inhibitors. For example, copper at 1.5 mmol l^{-1} stimulates the yeast growth while at 10 mmol l^{-1} concen-tration inhibits the yeast growth (Maiorella et al 1984).

1.6 GLUCOSE METABOLISM BY YEAST

At low level of glucose under aerobic condition, yeast will utilize glucose towards the growth and both glycolysis and citric acid cycle will be in operation. At higher concentration of glucose (more than 5%) and under anaerobic condition, yeast will convert glucose into equal amount of ethanol and carbon dioxide. According to Gay-Lussac's equation, one mole of glucose will give two moles of ethanol and two moles of carbon dioxide. The conversion of glucose to pyruvic acid takes place via metabolic route of Embeden-Mayerhof pathway. Decarboxylation of pyruvic acid leads to the production of carbon dioxide and acetaldehyde and on reduction of acetaldehyde ethanol will be produced. Total eleven enzymes are involved in this pathway and in return two moles each of ethanol, CO_2 and ATP are formed in alcoholic fermentation (Fig. 1).

$C_6H_{12}O_6$ ----> $2CH_3CH_2OH + 2CO_2 + 2$ ATP

On the completion of fermentation, Gay-Lussac has

EMBDEN MEYEROF GLYCOLYTIC PATHWAY OF ALCOHOLIC FERMENTATION

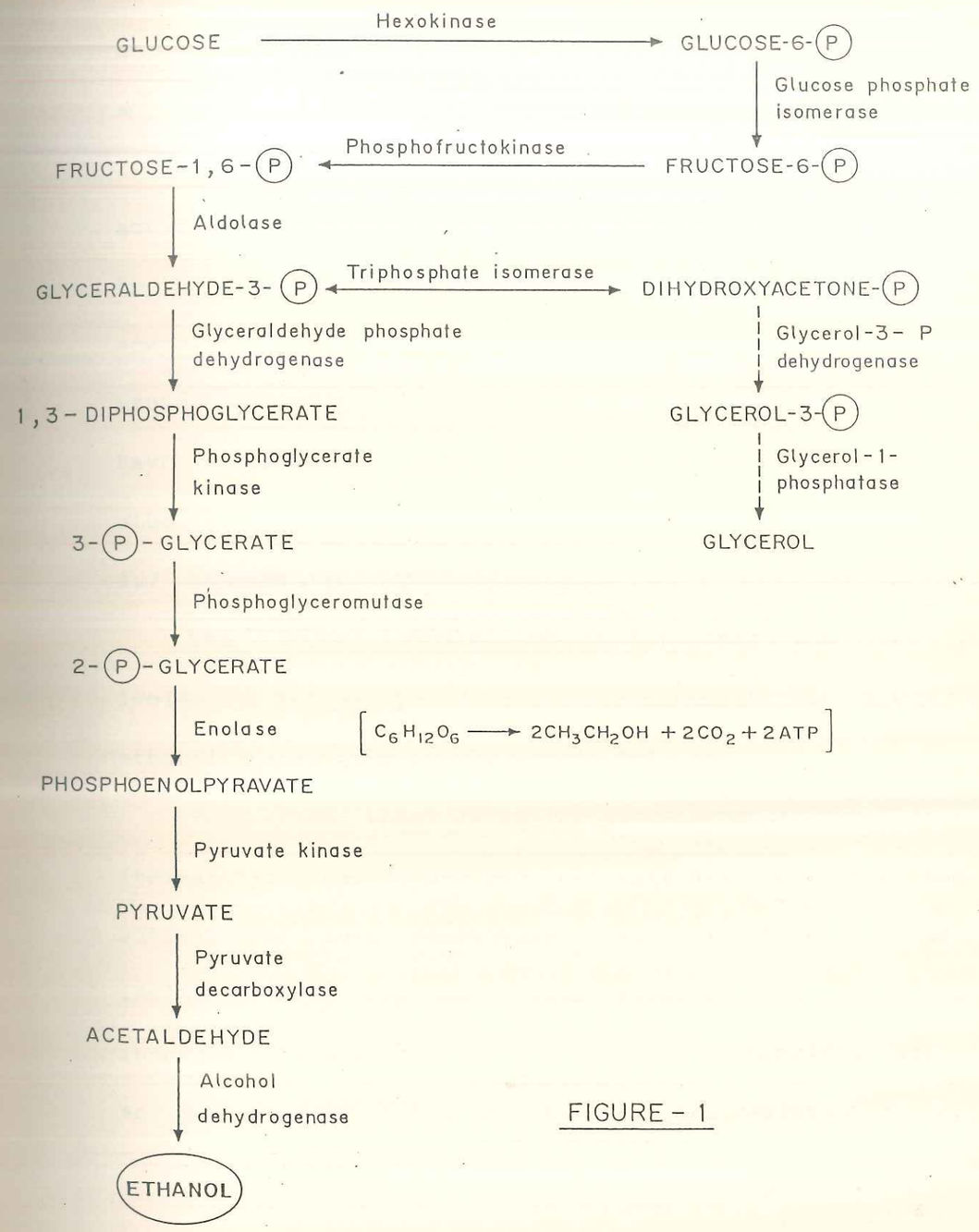


FIGURE - 1

indicated the percentage of alcohol 51.1% and CO_2 as 48.9%. Later, Pasteur expressed the yields of alcoholic fermentation under optimum conditions in the order of ethanol 48.4%, carbon dioxide 46.6%, glycerol 3.3%, succinic acid 0.6%, and other products like fuel oil, cell mass etc. 1.2%. The second important metabolite of fermentation activity is glycerol which is produced on industrial scale by the addition of $NaHCO_3$ to fermentation medium to prevent acetaldehyde formation. Here, the ratio of glycerol to ethanol is 6:1 and yield is around 25%. Maiorella et al. have shown in 1984 that high concentration of NaCl, KCl or other salts, stimulate glycerol formation.

1.7 FERMENTATION ACTIVITY

The microbiologists are continuously interested in isolating better yeast strains to get higher yield of ethanol, wine, glycerol or single cell protein.

Very often, there is an increase in temperature during fermentation period and yeast strains have to retain their activity at such temperature (+ 40^{o} C). If we succeed in isolating heat resistance yeast strains, the expenditure involved in lowering temperature during fermentation will be saved. Similarly yeast isolates that are resistant to high

concentrations of salts and sugars (> 25% of molasses); and ethanol (10-12% w/v) will help to increase the ethanol productivity. So far, it has not been possible to identify a natural yeast clubbed with all the properties of resistance to heat, salts and ethanol. Some success in this direction has been achieved by employing adaptation methods to yeast or by supplying an appropriate nutrient in the media. For example, a sake yeast, S. uvarum has been adapted for a long period to produce ethanol upto 22.4% at 15^{O} C in 35 days (Flor and Hayashida 1983). Similarly, Steinkranas et al. (1981) have reported a record yield of ethanol (25.6%) by using brewery's yeast.

In another approach, the supplementation of sterols, oleic acid and proteolipids to the fermentation medium has increased the productivity of ethanol between 18-20% as against their controls 5.5% at 20 ^{O}C over a period of 150 days in brewery fermentation (Hayashida and Ohta, 1981). In another experiment, S. uvarum 26602 and S. cerevisiae are able to reach 13 to 14 % (w/v) alcohol production as compared to S. rouxii 8383 and S. biospores 28852, 8-11% using the supplementation approach (Pierce et al. 1980).

1.8 CATEGORIES OF TOP, BOTTOM AND FLOCCULENT YEAST

In fermentation industries, yeast cultures have been described under categories like top, bottom and flocculent yeast. These are the natural yeast and are influenced under environmental conditions. The top fermenting yeasts belong to the species of S. cerevisiae and the bottom fermenting yeast to S. carlsbergensis species, now designated as S. uvarum (Lodder 1970). The top fermenting yeasts generally sporulate faster than the bottom fermenting yeasts. Normally, top fermenting yeasts are used in brewery industries while bottom yeasts are used in fermentation of molasses sugars or waste materials or carbohydrates. The yeast cultures under flocculation category are found more suitable for yeast recycling fermentation (Patil et al., 1989) or in continuous fermentation (Limtong et al., 1984). The flocculation property of yeast is determined by measuring the rate of sedimentation (Helm et al. 1953 and Patil and Patil, 1989).

The mechanisms involved in the flocculation of yeast have been studied. In one such mechanism, it has been cited that Ca^{++} is playing a vital role of cross-bridging the anionic groups on adjacent cell surface (Mill, 1964).

According to other theory, carboxyl groups on the surface layer might be responsible for the flocculent mechanism (Brian et al. 1982). The additional data has been worked out regarding phosphodiester groups involved in floc formation, using hydrogen fluoride technique (Jayatissa and Rose, 1976). The other experiments have shown that at stationary growth phase, when the density of carboxyl groups in the surface layer reaches to an optimum level, calcium mediated floc formation takes place (Beavan et al. 1979).

The categories of top and bottom yeasts have been used first time together in molasses fermentation to accelerate the rate of ethanol production (Patil and Patil, 1989).

1.9 SUBSTRATES AND ALCOHOLIC BRANDS

In India, ethanol is produced on industrial scale mainly from molasses which is a cheap, waste material from cane molasses factories. Molasses contains 50-60% fermentable sugars which are mostly sucrose, glucose and fructose. The percentage of sugars is adjusted between 15-20% by suitable dilution with water and 6-7% ethanol is obtained after the traditional yeast fermentation around 72h. Upon fractional distillation of dilute ethanol, 90-95% ethanol is obtained which is referred to as rectified spirit.

Starches from potato, rye, maize and barley; fruits like grapes, apple, cashews, cherry and sugars from cane juice or beet molasses are used for the production of potable alcohols. This is an earning industry and a lot of modifications are continuously being made to increase the yield and taste of alcohol. In these productions, the substrates are given pretreatments like acid hydrolysis or enzymatic treatments to release the hexoses for yeast fermentation.

Two types of alcoholic brands are sold in the market: one is undistilled product and the other is the distilled one. The undistilled products are sold directly or are fortified with pure alcohol to increase the alcoholic percentage. Some of the familiar names of the marketed undistilled brands are: Claret contains 7-13% alcohol, port-15-24% (fortified), Sherry 18-24% (fortified), Champagne 8-10% from apple juice and beer 3-5% from barley. The distilled alcoholic preparations are stronger in alcoholic percentages. These are whiskey (40-50%) from barley, holland (40%) from rye, rum (45-55%) from molasses and gin (40-45%) from barley. All these alcoholic products when taken internally in small quantities stimulate the human system.

In Germany, ethanol is produced by digesting cellulose-saw dust with sulfuric acid under steam pressure of 6-7 atmospheres where it is rapidly hydrolyzed to glucose. This material is neutralized with lime and is then used for yeast fermentation. In USA, alcohol is synthesized by the hydration of ethylene which is obtained from petroleum refineries while in Switzerland alcohol is manufactured by the hydration of acetylene.

Ethylalcohol, known as ethanol, alcohol or methyl carbinol with a formula CH_3CH_2OH is a colorless mobile liquid with a pleasant smell. It boils at 78.5° C and freezes at -114° C. It has a specific gravity of 0.789 at 20° C. It mixes with water in all proportions and is used for the preparation of large number of organic compounds like ethers, acetic acid, iodoform and chloroform. It is used in pharmaceutical products as well as in the preparation of perfumes, dyes and oil.

1.10 ETHANOL TOXICITY AND TOLERANCE IN YEAST

It is known that the viability of the cell, its growth rate and the ethanol production is affected above a certain concentration of ethanol in the reactor. The feedback mechanism has not been fully understood (Ingram and Bukkte,

1984, Slapack et al., 1987) have shown that ethanol denatures and inhibits the glycolytic enzymes, uncouples the oxidative phosphorylation and induces petite mutants in yeast strains. Miller et. al (1982) have shown that the ethanol concentration above 16% affect the fermentation rate and the primary site of action on the cell membrane. Brown et al (1981) have noted that growth and viability had different inhibition constants for ethanol. In view of this industries are actively working on the selection of yeast strains that are tolerant to ethanol more than 10% (w/v). Different strains show different levels of ethanol tolerance and specifically sake yeast shows a tolerance capacity upto 22.4%. Brown and Oliver (1982) have attempted to isolate yeast strains in the presence of 10% (v/v) ethanol. While Thomas et al. (1978) have improved the tolerance capacity of yeast with the addition of sterols. It is seen that the ethanol tolerance level of Saccharomyces species is in the order of sake yeasts > wine yeast> distiller's yeast> brewer's yeast. The ethanol tolerance capacity is influenced dramatically by nutritional conditions. There is not a single universally defined method to assess the ethanol tolerance of yeast because it is governed by many factors

such as temperature, salt concentrations, pH, type of substrates, substrate addition order, nutritional conditions, nitrogen source and inocula size. Likewise a number of papers have appeared to judge the intra and extra cellular ethanol percentage. A few workers claim that accumulation of ethanol in intra to extra cellular is higher while others are of opposite views. Desari et al. (1985) have designed a technique of assaying intracellular ethanol within two seconds in yeast cultures upto 8 h of age and have observed that the ethanol level never exceeds the extracellular level. They have further noticed that there is no addional intracellular accumulation of ethanol.

There are a few methods that have been used for assaying the ethanol tolerance level such as (a) suppression of yeast growth (Ranganathan & Bhat, 1958); (b) effect on fermentation activity (Hayashida et al., 1975; Yamashiro et al. 1966) (c) cell viability at different concentrations of ethanol and (d) the level of self produced ethanol (Casey et al. 1983, 1984). Based on these assays,the ethanol tolerance capacity of various strains have been reported in the following order Sacch.uvarum ATCC 26602 (13-14% w/v) Sacch. cerevisiae ATCC 26603 (8-11% w/v) and Sacch. rouxii

ATCC 8383 (8-10 w/v)

Another approach that has been adopted to increase the ethanol tolerance capacity is the addition of fatty acids, supplementation of sterols (Hossack and Rose, 1976) proteolipid (Yamashiro et al., 1966), yeast extract, ergosterol and Tween-80 (Casey et al., 1984)

1.11 YEAST STRAIN IMPROVEMENTS

There is a continuous need to improve the industrial strains of yeast for (i) better utilization of sugars; (ii) increased tolerance to alcohol, and (iii) addition of characters such as flocculence, aroma, flavour, genetic stability and fermentation rate. All these characters are mostly polygenic in nature and hence it is difficult to achieve a quick success using a single experimental approach. There are several procedures to improve the yeast strains and these include mutation, protoplast fusion, electrofusion and genetic engineering.

The protoplast fusion technique has already been used to improve the desired properties of yeast. For example fusion between two haploids, both starch fermenting yeast of S. diastaticus, has led to the production of a hybrid which has more ethanol amylase activity than the parent strains

(Sakai et al. 1986). In another effort to overcome the inhibitory effect of high sugar concentrations, a fusant from S. cerevisiae and S. mellis has shown a better fermentation efficiency (Leamann and Margalith, 1986). Likewise K, fragilis a lactose fermentor and S. cerevisiae, a high ethanol tolerant yeast were used to obtained fusants which were assimilating lactose and producing ethanol in excess of 13% v/v (Farahnak et al. 1986). In another successful experiment, fusants have been obtained from S. cerevisiae and S. diastaticus to convert dextrin and starch directly to ethanol. (Freeman 1981) Similarly fusion of S. cerevisiae a high ethanol producer flocculent with sake yeast has produced a fusant with improved osmotolerance capacity (Sakai et al., 1986). Recently, it has been shown that doubling the nutritional requirements of yeast causes an increase in the temperature tolerance of yeast upto $43^{o}C$ at which there is a complete utilization of 15% sugar to produce 6.8% (w/v) ethanol (D'Amore et al., 1989).

Adaptation of yeast strains is a more practical and simple approach of strain improvement. Additions of lipids (Ingram and Buttke, 1984) Soya flour (Damino and Wang, 1985) sterol-lipid-protein complex (Hayashida et al. 1976)

proteolipid (Ohta and Hayashida 1983) skim milk powder (Patil et al., 1986), chitin (Patil and Patil, 1989) and waste fungal mycelium (Patil and Patil, in Press) has contributed to improve the alcohol tolerance property and durability of yeast as well as yeast fermentation activity.

1.12 FERMENTATION TECHNIQUES

Fermentation techniques have substantially contributed to improve the quality and productivity of ethanol. In view of its industrial importance, the production volume of ethanol is the highest among the other solvents. The conventional process of bath fermentation although slow and inefficient is one of the oldest traditional method, that is used by the industry for the production of alcohol. In this procedure molasses or fermentable substrates are adjusted to 10-20% sugars concentration and are supplemented with nutrients and actively growing yeast cells (5-10% v/v inocula size). This is either left as such or is some times stirred mechanically, under anaerobic conditions for homogeneous mixing of yeast cells. Normally fermentation gets complete over a period of 72 h. This fermented mash is then removed for distillation and the yield of ethanol is near about 6-7% with an efficiency of 1.8-2.5l^{-1} h^{-1} (Rose

1976). A number of novel bioreactors have been fabricated in recent years. However, these have not been able to replace the existing fomenters for one reason or the other . A few types of bioreactors which will have better application to improve the yield of ethanol are described below.

1) Continuous fermentation

It is a modification of batch fermentation where the fermented mash is continuously separated leaving behind yeast cells. The advantages with this process are (1) The unit is smaller, (2) The downtime between batch fermentation cycles is eliminated;(3) The efficiency of the process is quite high as compared to batch fermentation (4) Inocula of yeast cells required every time in batch fermentation is saved. This process has been extensively applied for industrial ethanol production in USSR since 1960 and being developed in Western countries (Lyons,1984; Guidobond,1984).

2) Continuous Stirred Tank Bioreactors (CSTBR)

This is a modification of batch and continuous fermentation process. Here substrate feeding and outing devices have been incorporated into fomenter assembly and the mixing of yeast cells during fermentation is carried out

by a stirring mechanism. The feeding and outing of substrates are adjusted to get complete conversion of sugars to ethanol. By using adapted yeast strains (12 g dry wt/l^{-1}) ethanol productivity has been achieved as high as $7gl^{-1}h^{-1}$ (Cysewaski and Wilke, 1976). The only limitation of this process is that, there is a product inhibitions, after ethanol concentration of more than 7% (w/v/).

3) Cell Recycle Bioreactor.

This is another modification of batch fermentation process, where non-flocculent yeast cells released in the product stream are recovered by either centrifugation filtration or by use of microfilters and are reintroduced into the batch fermentation process or with CSTBR process. In this process, extremely high concentrations of inocula (100 g dry wt. per litre) are maintained to reach the ethanol productivity around 100 g l^{-1} h^{-1} (Ghose and Tyagi, 1979 ; Mehaia and Cheryan 1984, Nishizawa et al., 1983). For the purpose of reuse of yeast cell, capital cost involved for the centrifugation and extra labour are the disadvantages of this process. The yeast strains having better ethanol productivity can be used and have to depend upon the flocculating ability of yeast.

4) Vacuum fermentation

This technique has been developed to take care of the problem arising due to the inhibitory effect of ethanol on the fermentation activity. In vacuum fermentor (32 mm Hg at 30°C), fermentation of 50% sugar can be carried out and the ethanol yield is of the order of 82$_9$ l^{-1} h^{-1}. (Ramalingham and Finn, 1977, Cysewaki and Wike, 1977) Accumulation of toxic non volatile compounds, CO_2, absence of trace of O_2 required for cell viability are the major disadvantages with this system.

In order to overcome the disadvantages of vacuum-process, a number of attempts have been made to improve the quality of the process. This process has been clubbed with CSTBR where 40 gl^{-1} h^{-1} ethanol productivity is reached. A fully integrated-distillation process (vacuum + CSTBR + cell recycle) known as "Biostill-Process" has yielded 82 g $l^{-1}h^{-1}$ ethanol (Gouidoboni 1984, Gerlick, 1983, Maiorella et al. 1981 and Roffler et al., 1984) This technique is in operation in India at Ugar, district Belgaum (Karnataka).

1.13 ETHANOL PRODUCTION USING IMMOBILIZED YEAST CELLS

Production of ethanol from molasses using immobilized

whole cells on a suitable matrix is an active area of research in recent years. The technique is widely used for the preparation of alcohol from clarified carbohydrate so sources such as glucose, sugarcane juice etc. It has a number of advantages: (1) fermentation rate is faster; (2) yeast cells are used over a prolonged period, (3) contamination chances are greatly reduced: (4) inhibitory effects of substrate and ethanol are minimized; (5) nutrients requirements are less and (6) the capital cost is markedly reduced in recycling of immobilized yeast cells. Using immobilized yeast cells in rotor-fermentor and glucose as a substrate ethanol productivity of $27.3_g\ l^{-1}\ h^{-1}$ has been achieved (Kyung and Gerhardt, 1984 Margartis and Wilke, 1978). Likewise hallow-fibre bioreacter have been used for ethanol production and using this technique, ethanol has been produced (Inloes et al. 1983). The ethanol productivity of around $60_g l^{-1} h^{-1}$ has been recorded using saw dust as a immobilised solid support. This yield is probably the highest one in the technology (Michaux et al., 1982).

1.14 FLOCCULENT YEAST FOR ETHANOL PRODUCTION

Free flocculent yeast cells have been used for ethanol

production. Using the highly flocculent yeast strains Sacch. uvarum developed by Comberbach and Bu'Lock (1984), ethanol productivity upto $45_g\ l^{-1}\ h^{-1}$ has been recorded. The pilot scale bioreactors for flocculent yeasts have been fabricated and will be taken up on industrial scale in the near future. Entrapment of CO_2 during fermentation and inability to use molasses as such are few disadvantages of this procedure.

1.15 ALCOHOLIC FERMENTATION BY BACTERIA AND FUNGI

In bacteria, Zymomonas mobilis has been used on industrial scale for ethanol production using limited carbon sources like sucrose, cane-juice and syrups. The substrate to alcohol conversion efficiency of this culture is between 95-98 % as compared to that of yeast culture which is around 85-90 % (Doelle and Green field, 1985). The Zymomonas strains have been studied in detail with respect to their improvement for ethanol tolerance and flocculation (Rogers et al. 1982). These organisms are less efficient towards the use of fructose and are not able to utilize the other sugars like lactose, maltose, or pentoses for ethanol production. Another disadvantage of their use is the formation of undesired products such as glycerol, acetone and acetaldehyde.

The other bacteria involved in ethanol production are the *Clostridium* species. However none of these are used for ethanol production on an industrial scale. The strains are stable at high temperature (70-80°C) and hence called thermophilic organism (Zeikus *et al.*, 1979).

Fungi like *Fusarium*, *Mucor* and *Neurospora* species have shown some ethanol formation property at drastically low rate and none of these are used on industrial scale (Gong *et al.*1981, Srinivasan *et al.* 1986 and Rao*et al.*, 1983).

1.16 TOTAL ALCOHOL PRODUCTION

It is estimated that approximately 35-40 litres of alcohol are obtained from 100 kg. of molasses (Kosaric *et al.*, 1980). The main source of molasses in India are the sugar factories. The sugar production in India is around 100 lakhs of tonnes. With the appropriate use of molasses it is possible to produce 10,000 lakh litres of alcohol per year. When this will happen India will be the first country in the world for the maximum alcohol production using cane molasses. The local demand for alcohol in India is round 6000 lakh litres and the remaining quantity can possible be diverted either as a fuel in place of petrol or for export

(Business Express, 1988).

The production of alcohol is influenced by factors like economy, technology and policies of the Governments. The important point here is to level the price of alcohol with the price of crude oil, improvements in fermentation technology and firm policies of Governments. The World production of alcohol is in the order of 12,5560 lakh litres out of which Indian subcontinent contributes is only 4500 lakh litres. It is expected that the alcohol production will be around 20,5610 lakh litres by 1990 and our contribution may be of 5310 lakh litres which is less than 4% of the total production (Bremen. and Schmoltzi, 1986).

CHAPTER II

ISOLATION OF SALT AND HEAT RESISTANT YEAST STRAINS

2.1 INTRODUCTION

The yeast strains have their own importance in the array of microorganisms and have played a vital role in the production of many important products such as ethanol, glycerol, enzymes, vitamins and single cell proteins. The cultures, particularly Sacch. cerevisiae, Sacch. uvarum and Schizosacch. pombe have been used for the production of alcohol from sugars, starches and from raw materials like beet and cane molasses-sugars, pulp industry waste and cane juice. Although yeast strains have been isolated from different sources, their main habitat is surface of fruits, nectar of flowers; and waste carbohydrate product like beet, cane juice or molasses sugars. Considering the importance of yeast strains in alcoholic fermentation, attempts have been made to isolate yeast strains which will have fermentation activity at the concentration of molasses sugars of 20-25%, tolerance capacity of higher ethanol range (8-10% w/v) and stability to temperature of 40° C.

Normally isolation of microorganisms or yeast is carried out using techniques like picking up the colonies from agar plates on a suitable medium or by enrichment

method, from the samples of interest at the appropriate incubation temperature. The collected colonies are then tested for their biological performance. In the present work, a slightly modified method is described to isolate yeast strains that are resistant to ethanol salts and temperature (SH yeast strains)

2.2 ISOLATION OF SALT AND HEAT RESISTANT YEAST STRAINS (SH)

To start with, samples of soil from the area of molasses pits, and sugarcane factory were collected in the month of May (Summer period). About half a gram of this sample was directly transferred to presterilised molasses liquid medium (20 ml in boiling tube). The medium consisting of molasses sugars 10%, urea 0.25%, $MgSO_4.7H_2O$ 0.05% and yeast extract 0.2% was steam sterilised at 15 lb in^{-2} gauge for 20 minutes (pH 5.5). The inoculated tubes were kept stationary at 37° C and 40° C for two days. The tubes were checked daily for bubbling or gas formation property, and yeast strains with these characters were given transfer to the fresh medium for further enrichment. This method was repeated for three cycles by keeping other parameters unchanged.

The tubes that showed a bubbling or gas formation

property were checked under the microscope for the of microflora. The tubes with microflora were taken for single cell-pure culture isolation. The cultures were diluted with sterile water (1:100, 1:1000) and then were streaked on agar plates using the same composition of molasses medium. The agar plates were incubated at 37° C. The tiny colonies which appeared after a period of 20-22 h were transferred to the slope of molasses medium under aseptic conditions and then to liquid medium. The tubes with liquid medium were kept on a shaker (150 RPM) at 37° C. The grown cultures were checked under the microscope for their purity and morphology. The cultures which showed the character of bud formation were considered as Saccharomyces sps. and were designated as SH1...SH2...SH35. A total of 35 pure cultures were isolated with this procedure.

Employing the above modified method, a good number of SH-designated isolates of interest were collected in pure form within a period of seven days. These showed a better growth at the concentration of molasses (>10%) at stationary position as well as at 37 °C. If a normal procedure of yeast isolation is followed, at least 10 fold more colonies would have to be collected for testing their biological activity

of interest. In our modified procedure, all the extra yeast colonies that were weak at molasses concentration and at temperature of 37° C got eliminated at the initial stages of sub-culture.

2.3 MAINTENANCE OF SH YEAST STRAINS

All the isolated SH-yeast strains were maintained by the normal method of regular subculture on the slope of MUMY medium which consisted of total molasses reducing sugars 5-6%, urea 0.25%, $MgSO_4$ $7H_2$ 0.05%, yeast extract 0.2% and agar-agar 3.0%, pH 5.5 autoclaved at 10 lb/in^{-2} for 10 min. This was carried out once in two months by transferring a loop from stock cultures to a freshly prepared medium under aseptic conditions. The cultures were incubated at 30°C and 37° C separately for two days and were checked and compared with original old stock culture for purity and optimum growth. The yeast cultures were arrested at 10° C for a period of two months for longer preservation.

For long time preservation, the following procedure was adopted. The cultures were incubated to the respective temperature on the medium/slope and then were checked for their purity and sufficient growth. The slopes were overlaid

with sterile, medicinal grade paraffin oil and stored at low temperature (10-15 ^{o} C). The cultures could be revived on the same medium for their reuse. This particular procedure is having a number of advantages:

(a) The frequency of subculture is reduced.

(b) Chances of contamination are minimized.

(c) Biological activity is maintained to its optimum level.

(d) Preservation is simple and costly equipments are not required.

(e) Cultures are readily available.

(f) Risk of losing original biological activity on subculture is avoided.

2.4 ETHANOL PRODUCTION ACTIVITY OF 'SH' YEAST STRAINS

An experiment was planned to study the ethanol production activity of the SH isolates at the temperatures 30, 37 and 40^{O} C using cane molasses reducing sugars. Initially all the cultures SH1... SH 35 were transferred by collecting a loop from stock culture to inoculum medium (10 ml in boiling tube) of cane molasses total reducing sugars (5%) with urea 0.25%, magnesium sulphate 0.05% and yeast extract 0.2%, and kept on shaker (150-160 RPM) to respective

temperature for 24 h. The grown cultures were totally inoculated to fermentation medium (90 ml in 100 ml conical flask with cotton plug) consisting of total molasses reducing sugars (15%) with other additives similar to inoculum medium. All the flasks were left stationary at the respective temperature of 30, 37 and 40° C. Samples (5.0 ml) were removed after a period of 48 h for ethanol estimation.

Table 1 summarizes the ethanol production activity of SH isolates at different temperatures. Out of the total SH35 isolates, 12 isolates have been tabulated here. The remaining 23 cultures were found weak with respect to ethanol production (less than 3.0% w/v). It is observed from Table 1 that the cultures SH12 and SH29 show a better ethanol production activity and have produced more than 5.0% (w/v) ethanol. The remaining cultures have shown the activity of ethanol production in the range of 3.0 - 4.5 % at 30°C.

When the cultures SH12 and SH29, were studied for their purity and morphology, they showed the characteristic of saccharomyces species. They were further used for various experiments and these data would be described in the remaining part of the thesis.

Table 1 : Ethanol Production activity of SH Isolates

Fermentation medium : Total Molasses reducing sugars 15%, + $MgSO_4 \cdot 7H_2O$-0.05% and yeast extract 0.2%, pH 5.5

Sr. No.	Culture	Ethanol % (w/v) at 48 h		
		30 °C	37 °C	40 °C
1.	SH3	4.3	3.2	2.0
2.	SH4	3.6	2.9	1.5
3.	SH7	4.2	3.5	2.0
4.	SH12 -->	5.2	4.8	4.1
5.	SH16	4.5	3.6	2.6
6.	SH17	4.1	3.3	2.1
7.	SH22	3.1	2.4	2.0
8.	SH23	3.3	2.8	1.8
9.	SH28	4.5	3.8	2.0
10.	SH29 -->	5.4	5.0	3.8
11.	SH30	4.2	3.9	3.1
12.	SH31	4.1	3.6	3.0

All the remaining SH isolates produced ethanol less than 3.0% (w/v/) hence were not recorded in this Table.

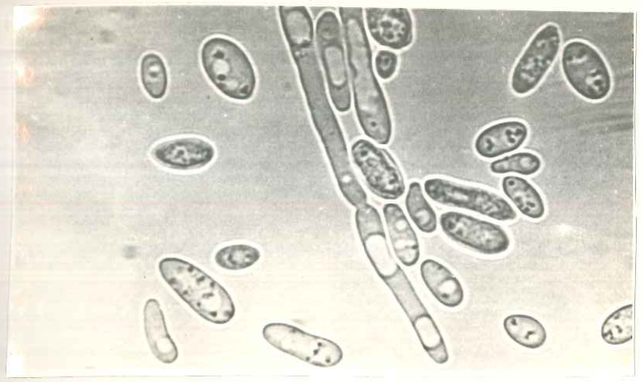
* Average value of duplicate experiments.

2.5 IDENTIFICATION AND CHARACTERIZATION OF SH12 AND SH29

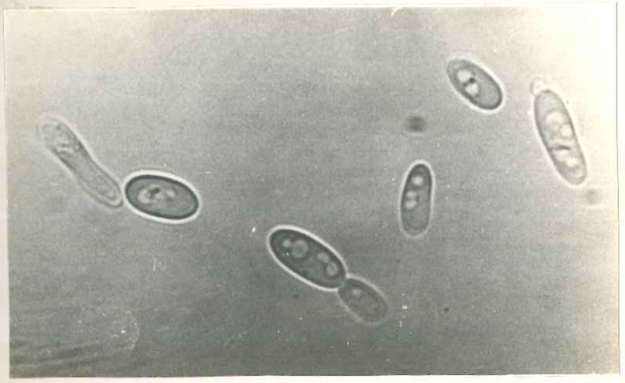
Since the two SH cultures, namely SH12 and SH29, showed a better ethanol productivity from molasses sugars (Table 1), we were specifically interested in using them for further work. However, before going ahead with these two cultures, we thought it essential to get their proper identification through an authentic organization such as `National Collection of Yeast Cultures (NCYC)', Colney Lane, Norwich NR4, 7UA, U.K.

The data and the photos (Fig. 1 and Fig 2) received from NCYC have revealed that these cultures SH12 and SH29 belong to the genus Saccharomyces cerevisiae sps. (current nomenclature) while according to the old nomenclature they belong to Saccharomyces italics and Saccharomyces capensis, respectively.

The morphology of S. cerevisiae SH 12 has been described. The cells are elongated, occurring singly (3-4) X (6-17) μ from broth growth oval to elongated occurring single (2-6) x 5-ps) μ on agar medium. They form a lumpy deposit without film or ring and give creamy, shiny smooth appearance on YM medium. Pseudomycelium is ranging from



1



2

Isolate : Saccharomyces sp. SH12

Photo 1 : Agar medium

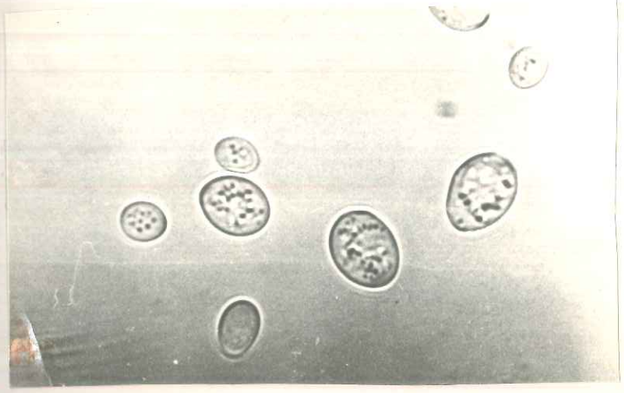
Photo 2 : Broth medium

Identified by N.C.Y.C., England as

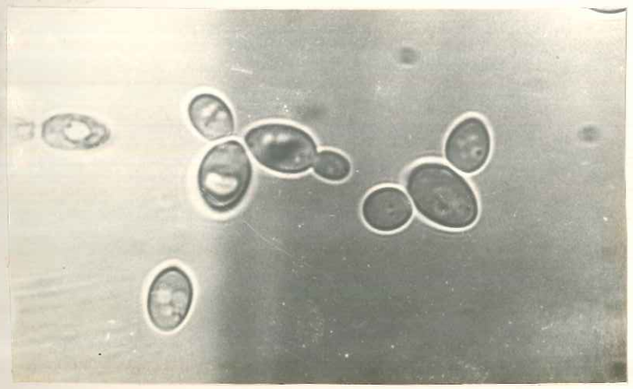
Saccharomyces cerevisiae (Current name)

Saccharomyces italicus (Old name)

(showed two morphological variants)



1



2

Isolate : Saccharomyces sp. SH29

Photo 1 : Agar medium

Photo 2 : Broth medium

Identified by N.C.Y.C., England as

Saccharomyces cerevisiae (Current name)

Saccharomyces capensis (Old name)

poorly developed to well developed site, containing ascospores at different places. Sexual spores present, ascospores round the smooth 1-4 per ascus.

The sugars like glucose, galactose, maltose, sucrose raffinose were fermented within short period, while lactose, melibiose, insulin, starch and xylose were not fermented even upto 21 days. The culture showed growth at the concentration of 10% NaCl salt and at 37 ^{o} C. It is also exhibited two morphological variants and it was found difficult to separate them. This particular finding is interesting and requires more study.

Likewise, the morphology of S. cerevisiae SH 29 revealed the following features: Shape is oval to long oval, occurring single (4-6) x (5-8) µ non-flocculent, no film or ring, slightly cream in colour, shiny smooth fairly well developed pseudomycelium. Sexual spores present. Ascospores 1-4 per ascus, observed.

The sugars like glucose, sucrose and raffinose were fermented quickly while lactose, galactose, melibiose,insulin and starch took a fairly long period. The culture showed a growth at the sodium chloride 10% concentration at 37^{o} C. Its growth did not require

vitamins.

The two cultures were later deposited with National Collection of Industrial Microorganisms (NCIM), Pune, India. as S. cerevisiae NCIM 3526 (SH12) and S. cerevisiae NCIM 3525 (SH29), respectively for their maintenance and distribution for industrial use.

2.6 GENERAL COMMENTS.

Two isolates, SH12 and SH29 out of 35 have shown effective ethanol productivity at the desired conditions of salts concentration and at elevated temperature. These were identified as cerevisiae by NCYC, England and showed a better ethanol productivity as compared to standard distillery strains S. uvarum ATCC 26602. These strains are available from NCIM, NCL, Pune-41108 Culture collection as S. cerevisiae (SH29) NCIM 3526 and S. cerevisiae (SH12) NCIM 3525 for distribution and industrial use.

CHAPTER III

ENHANCEMENT OF ETHANOL PRODUCTION FROM CANE MOLASSES BY SKIM MILK SUPPLEMENTATION.

SECTION A

SECTION A

A GENERAL PREFACE TO NOVEL ADDITIVES USED IN ALCOHOLIC FERMENTATION

INTRODUCTION

Ethanol, an important organic solvent and starting compound for many other organic solvents is a valuable alternative source of energy in place of natural petroleum sources. It is produced largely by synthetic methods, in developed countries and by the old method of yeast fermentation from waste materials like pulp, starches and cane or beet molasses in developing countries. New techniques such as vacuum fermentation (Ramlingham and Finn, 1977), rapid fermentation (Nagodawithana et al., 1976), cell recycling (Sedha et al., 1984), and whole cell immobilization (SivaRaman et al., 1982) are under investigation for ethanol production. Cane producing countries such as Brazil, the Philippines, Thailand, African countries, Mauritius and India utilize mainly cane molasses and yeast for ethanol production. In India, ethanol production is mainly by batch fermentation using cane molasses and yeast cultures.

Nutritional elements as well as growth factors play a vital role in increasing the cell mass and thereby ethanol

production. About 35 years back sufficient yeast growth was observed when ergosterol was used in a chemically defined medium under anaerobic conditions (Andereason and Stier, 1953). The proportional use of urea has shown an increased viability of yeast cells and thereby ethanol productivity (Sedha et al., 1984). Oryzenin, a major component of rice protein and proteolipid from Koji mold mycelia have been found to protect yeast and add flavours to alcoholic fermentation (Hayashida et al., 1974). Later, proteolipid of A. oryzae was analysed and it was found that unsaturated fatty acids containing phosphatidylcholine were responsible to increase the durability of yeast cells as well as ethanol production (Hayashida et al., 1976). When vegetable oils and their fractions (unsaturated and saturated fatty acids) were incorporated into molasses-sugars fermentation there was protection of yeast cells at higher temperature (40^{o}C) and increase in the yield of ethanol (Saigal and Viswanath, 1983). The ethanol tolerance of sake yeast has been increased by the addition of Tween-80, ergosterol or monoolein (Ohta and Hayashida, 1983). The combination of glycerol with ammonium sulfate brought about 15% increase in the ethanol production compared to control experiments

(Patil and Patil, 1986b)

The addition of soya flour, rich by itself for proteins, fats, vitamins and phosphate to fermentation medium has been extensively studied for ethanol productivity in batch fermentation, recycling yeast cell and immobilized system (Damiano and Wang, 1985). The incorporation of soya flour reduced the fermentation period and increased the viability of yeast cells against salt and ethanol concentrations (Viegas et al., 1985) Bajpai et al., 1988).

The other scope for the improvement of ethanol productivity is by the selection of quality yeast strains. The strains generally used in the alcoholic fermentations are Sacch. cerevisiae, Sacch. uvarum and Schizosacch pombe. Isolation of such strains which will give high yield of ethanol (8-9% w/v); and work at higher temperature (40^{o}C-45^{o}C) and at higher salts/sugar concentrations (25-30%) is a basic need of alcoholic fermentation. Techniques like mutation, hybridization or protoplast fusion are used to improve the yeast strains and these methods have tremendous scope from industrial point of view. Likewise adaptation of yeast strains to specific environmental conditions also promotes the fermentation activity.

Since industrial ethanol production in India is exclusively from sugar cane molasses by batch fermentation, studies were initiated to increase the rate of ethanol production by suitably supplementing the fermentation medium with suitable additives. During our efforts in this direction, attempts were made to speed up the rate of ethanol production by supplementing the medium with skim milk powder, carbohydrates such as acacia gum, chitin, xylan, pullulan, cellobiose, dextrin, inulin and agar, fungal mycelium prepared from various species as well as waste material designated as waste mycelium from Hindustan Antibiotic factory. Among the various supplements, skim milk chitin and waste mycelium showed a significant improvement in the rate of ethanol production in cane molasses batch fermentation process.

Skim milk-powder (0.2%) supplementation was found to enhance the rate of ethanol production and about 6.0% (W/V) ethanol was formed in 48 h from cane molasses containing 13.5% fermentable sugars as compared to a period of more than 72 h in its absence. Chitin supplementation produced 6-6.9% (W/V) ethanol after approximately 30 h 30^{o}C from cane molasses containing 18% total reducing sugars as compared to

a period of 72 h in its absence. Using 20% cane molasses total reducing sugars the prepared mycelium (0.4% wet. wt) or waste mycelium (0.4% dry wt.) supplement produced 7-7.6% (W/V) ethanol as compared with 4.0-4.5% (W/V) in the control experiment, after around 44 h at 30°C.

MATERIALS AND METHODS

CHEMICALS/REAGENTS

The chemicals and reagents used were of analytical or pure grade. They were purchased from various authentic sources such as BDH, India; Sigma, USA, E. Merck, Germany, Sarabhai Chemicals India, and Biological Products Difco, USA.

Skim milk- powder and acacia gum were purchased from local market. The whole milk of buffalo and cow was collected fresh and used directly on the same day.

The designated 'Waste mycelium' of Penicillium chrysogenum was obtained on request from Hindustan Antibiotic Limited, Pimpri, Pune 411 017, India.

YEAST AND FUNGAL CULTURES

Yeast cultures such as Sacch. cerevisiae NCIM 3095, 3107, 3157, 3176, 3528, 3524 and a few isolated

a period of 72 h in its absence. Using 20% cane molasses total reducing sugars the prepared mycelium (0.4% wet. wt) or waste mycelium (0.4% dry wt.) supplement produced 7-7.6% (W/V) ethanol as compared with 4.0-4.5% (W/V) in the control experiment, after around 44 h at 30°C.

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YEAST AND FUNGAL CULTURES

Yeast cultures such as Sacch. cerevisiae NCIM 3095, 3107, 3157, 3176, 3528, 3524 and a few isolated

cultures designated as SH12 and SH29 representing Sacch.cerevisiae NCIM 3526 and 3525 respectively, Sacch. uvarum NCIM 3455 and 3509, Sacch. cerevisiae var ellipsoideus NCIM 3281 were obtained from National Collection of Industrial Microorganisms, (NCIM), National Chemical Laboratory, Pune 411 008, India.

All these cultures were maintained on MGYP-slope (malt extract 0.3% yeast extract 0.3% peptone 0.5% glucose 2.0% and agar 2.0%) as well as on molasses slope (total molasses reducing sugars 5.0 % urea 0.25 % magnesium sulfate 0.05 % yeast extract 0.2 % and agar 3 %) pH 5.5.

The fungal cultures like Penicillium chrysogenum NCIM 737, Aspergillus oryzae NCIM 555, Sclerotium rolfsii NCIM 1084, Sporotrichum pullverulentum NCIM 1106, Aspergillus niger NCIM 573, Rhizopus nigricans NCIM 880, Neurospora sitophila NCIM 871, Fusarium tricinctum NCIM 1189 and Trichoderma reesei NCIM 1052 were also obtained from the culture collection. All these fungal cultures were maintained on PDA slopes. The latter were prepared by taking 200 gms of peeled potatoes in one litre of water and steaming them for half an hour. The water extract was collected by decantation and volume was set to one litre and

then 20 gms of glucose and 20 gms of agar were added. This mixture was melted on water bath and distributed (6-7 ml) in test tubes and sterilised at 15 lb for 20 min.

Both cultures of yeasts and fungi were sub-cultures to fresh medium, once in two months to their respective medium, by transferring a loop from old stock under aseptic conditions. These tubes were incubated for their optimum growth at room temperature for 3-4 days for yeast cultures and 8-10 days for fungal cultures. The growth of these cultures was arrested at 10-12^{o}C after assessing their purity.

COMPOSITION AND CLARIFICATION OF MOLASSES

The sugarcane molasses was obtained from local sugar factory. It contained total reducing sugars in the range of 50-60 % other salts 10-15 %. The composition of final molasses is : water 18.5 %-19.5 % fermentable sugars as reducing sugars 50-55 %, ash 14.5 % gums including starch 3.35 %, starch 0.45 %, waxes 0.91 %, total nitrogen 0.48 %, silica (SiO_2) 0.33 %, phosphates (P_2O_5) 0.91 %, potash (K_2O) 3.92 %, Ca (CaO) 1.68 % and magnesium (MgO) 0.89 % (Barnes 1974). The composition of molasses depends upon the

season, period of crops and collection; and soil.

The molasses used was clarified and adjusted to a required percentage of reducing sugars before it was taken for experimental purposes. Sodium hydroxide or hydrochloric acid was used for the adjustment of pH to 5.0 or 5.5. The diluted and pH adjusted molasses liquid was steamed for 30 min and left at room temperature for 24 h. The precipitated solid materials (mostly $CaSO_4$) were removed by decantation. The basal media for growth or fermentation were compounded with urea 0.25 % and magnesium sulfate 0.05 % and yeast extract 0.2 %.The latter was deleted in specific experiment.

ESTIMATION OF REDUCING SUGARS

The total molasses reducing sugars were estimated by dinitrosalicylic acid (DNSA) method (Fishcer and Stein, 1961). During preparation of DNSA reagent 20 gms 3,5-dinitrosalicylic acid was dissolved in approximately 400 ml distilled water. A solution of NaOH (32 gms in 300 ml distilled water) was added drop wise with continuous stirring (if required this preparation was heated gently on water bath) to obtain a clear solution. Potassium sodium tartarate (600 gms) was added in small portion and a clear

solution was obtained on heating. The total volume was made to 2 litre, the solution was filtered and kept in dark at room temperature.

For estimation of molasses reducing sugars, the samples were diluted with water (in the range of 1:10, 1:20 or more to have the sugar range in between 1-5 %). A mixture containing 5 ml of total molasses reducing sugars + water 3.5 ml + HCl 10 % 1.0 ml. This was boiled on a water bath for 10 min to hydrolyze mainly sucrose. Later the hydrolyzed sample (0.2 ml) was mixed with 0.5 ml of water and 1 ml of DNSA reagent. This mixture was again boiled for 5 min. cooled and then diluted with water (10 ml) to make the final volume upto 12 ml. The optical density was read at 540 nm. The reading of 0.3 OD corresponds to 500 µgms of reducing sugars. The sensitivity of this method was from 100 µgms to 500 µgms of reducing sugars.

ESTIMATION OF ETHANOL

Ethanol formed in alcoholic fermentation was estimated with the help of a colorimetric method using cerric ammonium nitrate procedure (Reid and Truelove 1952). The reagent was

prepared by dissolving 20 gms of pure cerric ammonium nitrate in 100 ml of standardised 4 N nitric acid. The solution, if necessary, was filtered through whatman paper to get clarity.

A sample of 5.0 ml of fermented mash with the addition of 15 ml water for alcohol estimation was taken in a closed system for distillation. Upon distillation, a clear solution of 5.0 ml was collected. The distilled 0.5 ml sample was diluted with water (3.5 ml) and mixed with the reagent of cerric ammonium nitrate (1.0 ml). The solution was mixed thoroughly and read at 486 nm immediately. A standard run with pure alcohol (ethyl alcohol in the range of 0-4.0%) was also carried out, every time. The samples to be estimated were calculated conveniently with the standard run for estimation of (W/V) ethanol.

EQUIPMENTS USED

For the production of yeast cells, a `Control Environment Incubator' shaker of New Brunswick Scientific, Edison, N.J., USA and Indian make `Enmvee' shakers were used.

The turbidity measurements of grown cells were carried

out in a model of Klett Summerson .- photoelectric colorimeter, Klett MFG, Co., USA was used.

To collect the desired phase of grown-cultures, either supernatant or precipitate a centrifuge of `Damon IEC', B-20A A centrifuge USA make was used.

The colorimetric estimation of reducing sugars or ethanol content from the samples was measured with the help of 'Shimadzu' - double beam spectrophotometer, uv -210 A, USA make.

For autoclaving the glassware materials, the media and reagents a service of 35 years old 'Castle'-autoclave of USA make was taken. The temperature control incubators of reputed firms of India were used for fermentation experiments or for growth of cultures.

REPRODUCIBILITY

All the experiments were repeated at least two times. The percentage error was found to be not more than 5 %, in all the cases.

SECTION B

3.1 INTRODUCTION

Nutritional requirements as well as growth factors play a vital role in increasing the cell mass of yeast, and thereby production of ethanol. Studies were initiated to increase the rate of ethanol production by suitably supplementing the fermentation medium. Supplements like glycerol, lactose, ergosterol, β-carotene, casein and complex material like skim milk (powder) or whole milk of cow or buffalo were used to judge their effect on alcoholic fermentation. The milk (liquid/whole) on average contain water (90.5%) and solids (9.5%). The latter is made up of lactose (5.05%), nitrogenous materials (3.6%) and fat (0.1%). In this communication, a significant increase in the rate of ethanol production by the addition of skim milk during cane molasses fermentation is reported.

3.2 MATERIALS AND METHODS

The fermentation medium was supplemented separately with yeast extract 0.2%, skim milk 0.2%, glycerol 0.5%, ergosterol 0.0015%, B-carotene 0.05%, lactose 0.2%, whole milk 2.5 ml/100 ml and casein, 0.2%. All the media (pH 4.8) were sterilised by autoclaving at 15 lbs/in^2 for 15 min.

One of the isolates designated as Sacch. cerevisiae (SH12) NCIM. 3526 was obtained from molasses pits by plating out on molasses agar plate at 37° C. Along with this culture, stock cultures obtained from NCIM were used in these experiments.

3.3 RESULTS

EFFECT OF SKIM MILK AND DIFFERENT SUPPLEMENTS ON ALCOHOLIC FERMENTATION

Throughout the experiments, 10% inoculum (v/v) was used. Inocula were prepared stepwise by transferring a loop from grown slants to 10.0 ml sterile 5% molasses sugars medium containing urea and yeast extract and kept on a rotary shaker (150 RPM) for 24 h at 37°C. The fermentation media (90 ml in 100 ml conical flask) with 15% cane molasses sugars and urea were supplemented separately with yeast extract, skim milk, glycerol, lactose, ergosterol, B-carotene, casein or whole milk of buffalo or cow. A control experiment without the addition of any supplement was also carried out under identical conditions. Samples were removed for ethanol estimation at intervals.

FERMENTATION BY INDUSTRIAL CULTURES IN PRESENCE OF SKIM MILK

Initially the performance of industrially used yeast cultures S.cerevisiae NCIM 3085, 3159, 3176 and S.uvarum NCIM 3455 obtained from the National Collection of Industrial Microorganisms (NCIM), Pune, India and an isolate Saccharomyces sp SH 12 was assessed in the presence of (i) Cane molasses containing 15% total sugars supplemented with .25% urea, (ii) The molasses containing 15% sugars + 0.25% urea + 0.2% yeast extract and (iii) The molasses containing 15% sugars + 0.25% urea + 0.2% skim milk. All the fermentations were carried out at 37^{o}C and ethanol was estimated after 48 h.

In the second set of experiments, fermentation of cane molasses was carried at the two different temperature of 30^{o}C and 37^{o}C using two strains namely S. uvarum 3455 and Saccharomyces sp. SH 12. Inocula were grown at the respective temperatures, transferred to 15% cane molasses total sugars in the presence of yeast extract or skim milk as described earlier for fermentation. Aliquots were removed after 20 h, 30 h, 48 h and 74 h for ethanol estimation.

To check the cell mass increment in the fermentation an

experiment was carried out using 15% molasses sugar at 30oC. The yeast cells were harvested by centrifugation after a fermentation period of 10 h and 72 h. The cells were washed with an equal volume of water. The packed cells were dried at 80°C (24 h) to a constant dry weight.

To determine specific ethanol productivities, cells of S. uvarum 3455 were grown on a rotary shaker (150 RPM) at 30°C or 37°C for 22 h in 500 ml molasses medium containing 20% reducing sugar and supplemented with urea and yeast extract. The cells were harvested by centrifugation. Fermentation was carried out at 30°C or 37°C in 150 ml conical flasks by transferring 3.0 g wet weight of packed cells pregrown at the corresponding temperature and added to 100 ml of 20% molasses total sugars supplemented with yeast extract or skim milk. Samples (5.0 ml) were removed for ethanol estimation at 4 h, 6 h, 10 h and 26 h.

The fermentation of cane molasses by the isolate Saccharomyces sp. SH12 was first studied at 37°C in the presence of different supplements such as yeast extract, skim milk, glycerol, lactose, ergosterol, β-carotene, casein and whole milk of buffalo and cow (Table 1). Ethanol production after 48 h was maximum in the presence of skim

Table 1: Effect of different supplements on cane molasses fermentation at 37°C. Culture inoculated: Saccharomyces sp. SH12. Molasses sugars 15% + 0.25% (fermentable sugar 13.5%). Percentage increase in alcohol was estimated by comparing with the control. Results are average values of duplicate experiments.

Supplements added	Quantity (%)	Ethanol (%, w/v)	Enhancement level (%)
Control (no supplement)	-	5.05	-
Yeast extract	0.2	5.52	10.5
Skim milk	0.2	6.21	23.0
Glycerol	0.5	5.72	13.2
Lactose	0.2	5.62	11.2
Ergosterol	0.0015	5.38	6.5
β-Carotene	0.05	5.80	14.8
Casein	0.2	5.10	1.0
Whole milk (buffalo)	2.5 (v/v)	6.10	20.8
Whole milk (cow)	2.5 (v/v)	5.80	14.8

milk as compared with other supplements and the control experiment. Skim milk was therefore, used in the subsequent experiment.

The fermentation performance of industrially used yeast cultures and of isolated Saccharomyces sp. SH12 was studied in the presence of yeast extract and skim milk.

From Figs.1,2 and Table 2, it can be seen that ethanol is produced more rapidly in the presence of skim milk than with yeast extract or control by all the industrial strains of yeast. The culture S. uvarum 3455 and isolate Saccharomyces sp. SH12 showed better ethanol production compared with the other strains.

Table 3 summarizes the results obtained during cane molasses fermentation in the presence of yeast extract and of skim milk by S. uvarum 3455 and Saccharomyces sp. SH12 at different time intervals and at the two different temperatures of 30°C and 37°C. A control experiment was also run at each temperature under similar conditions. Ethanol production was almost complete by 48 h in the presence of skim milk as against 74 h required in the control experiment as well as with yeast extract.

The cell mass increases during fermentation under

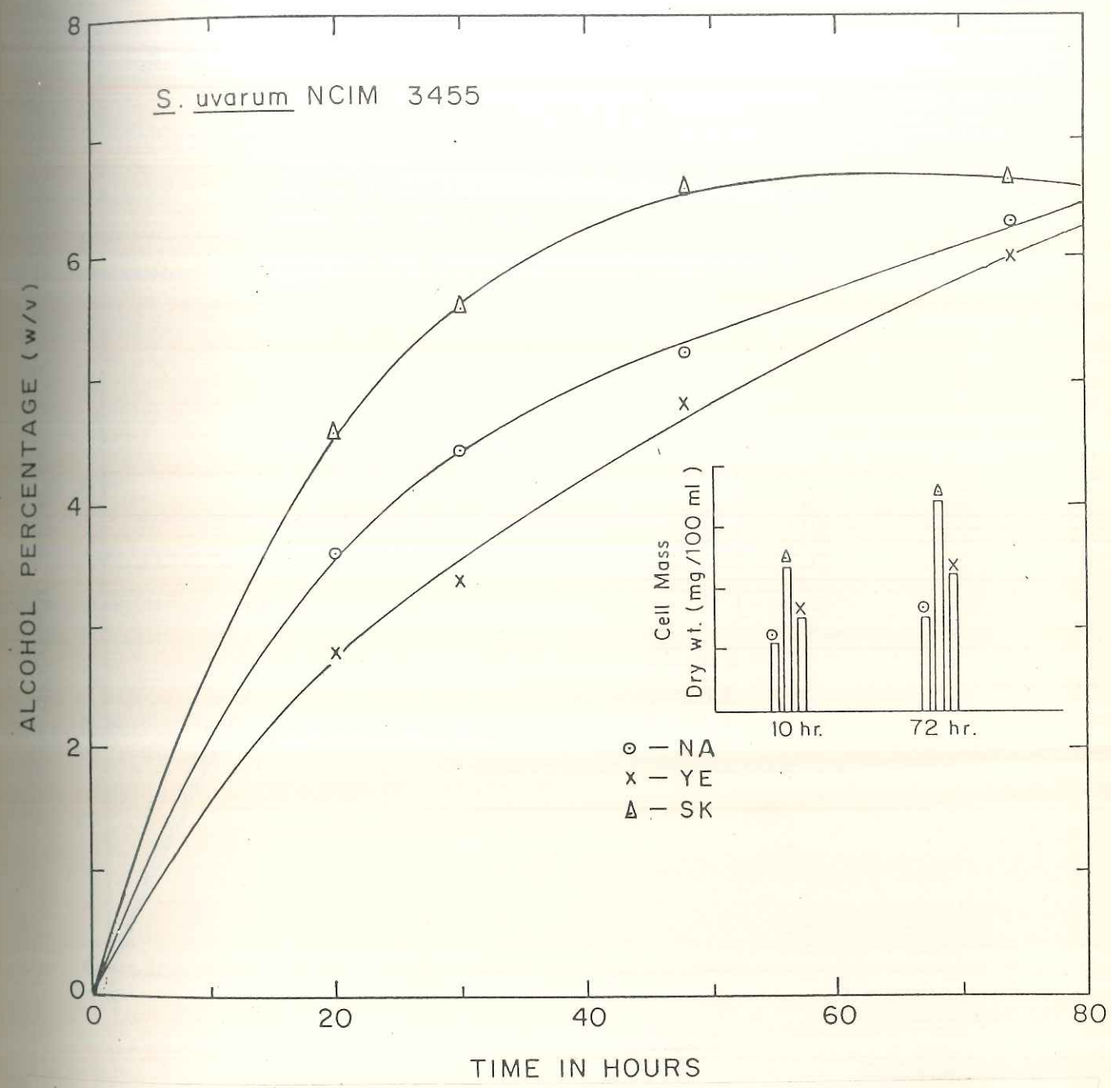


FIGURE 1

Ethanol production in the presence of skim milk and yeast extract at 37°C

Molasses total reducing sugars 15% + urea 0.25%, Sacch. uvarum NCIM 3455
SK = Skim milk; YE = Yeast extract, NA = Control

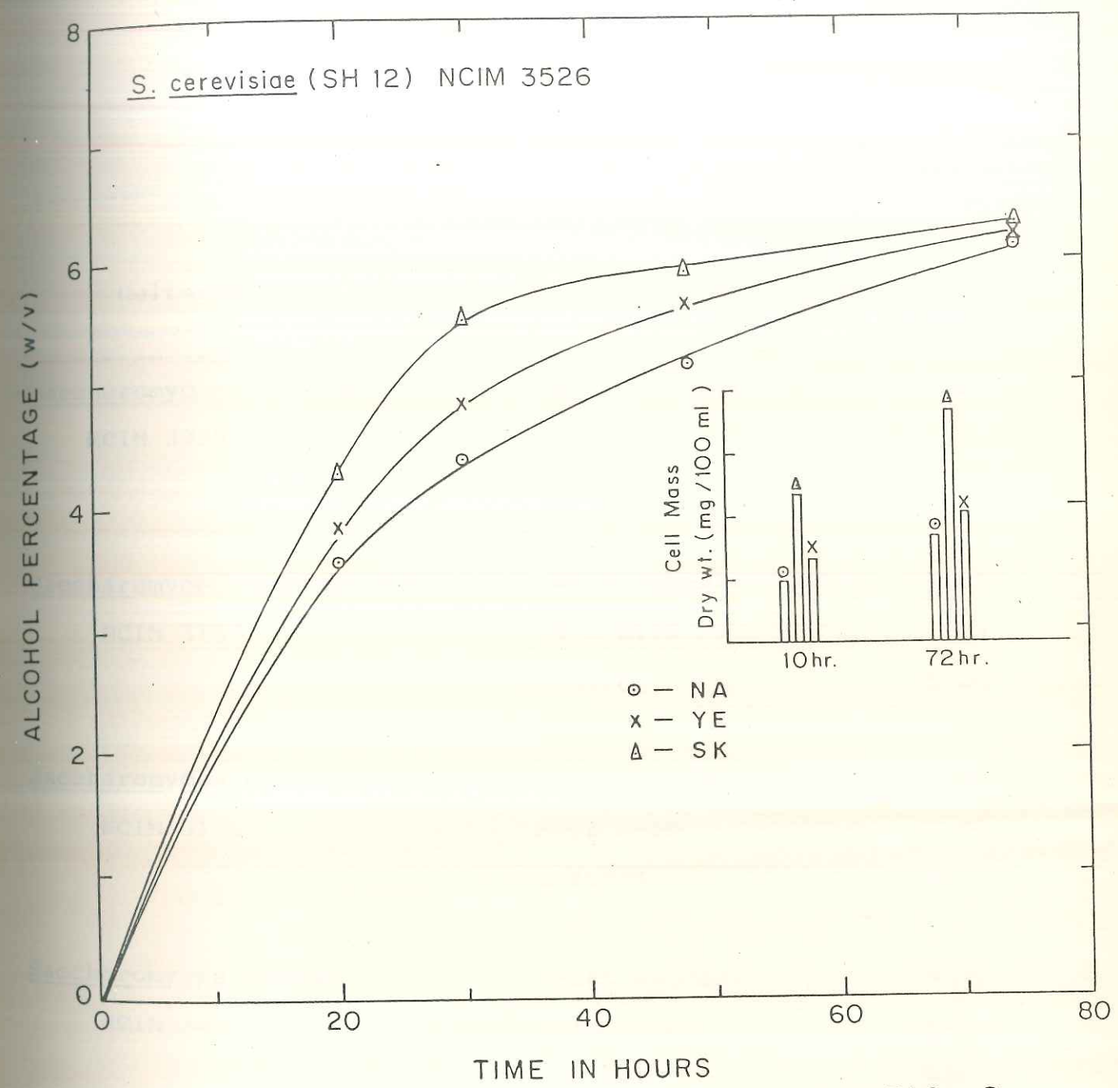


FIG. 2

Ethanol production in the presence of skim milk and yeast extract, at 37°C

Molasses total reducing sugars 15% + urea 0.25%, Sacch. cerevisiae (SH12), NCIM 3526, SK = Skim milk; YE = Yeast extract; NA = Control

Table 2 : Effect of skim milk on industrial yeast cultures in cane molasses fermentation at 37 C. Molasses 15% + urea 0.25% (fermentable sugars 13.5%). Control was without supplement. Results are average values of duplicate experiments.

Cultures inoculated	Supplement added (0.2%)	Ethanol after 48 h (% w/v)
Saccharomyces cervisiae NCIM 3095	Yeast extract	4.45
	Skim milk	5.87
	Control	3.80
Saccharomyces cervisiae NCIM 3157	Yeast extract	5.72
	Skim milk	6.00
	Control	5.40
Saccharomyces cervisiae NCIM 3176	Yeast extract	5.60
	Skim milk	6.05
	Control	5.32
Saccharomyces uvarum NCIM 3455	Yeast extract	5.47
	Skim milk	6.25
	Control	5.12
Saccharomyces sp, SH12	Yeast extract	5.60
	Skim milk	6.12
	Control	5.22

TABLE 3: CANE MOLASSES FERMENTATION AT TWO DIFFERENT TEMPERATURES IN THE PRESENCE OF SKIM MILK, MOLASSES SUGAR 15% + UREA 0.25% (FERMENTABLE SUGARS 13.5%). CONTROL WAS WITHOUT SUPPLEMENT. RESULTS ARE AVERAGE VALUES OF DUPLICATE EXPERIMENTS

Culture inoculated	Temperature (°C)	Supplement added (0.2%)	Alcohol (%, w/v) at 20 h	30 h	48 h	74 h	Ethanol increment at 30°C (%)	Time for almost complete ethanol production (h)
Saccharomyces SH12	30	Yeast extract	3.82	4.85	5.66	6.22	29.0	74
	30	Skim milk	4.29	5.55	5.95	6.20	-	48
	30	Control	3.55	4.30	5.15	6.11		74
	37	Yeast extract	3.33	4.25	5.53	6.20	28.1	74
	37	Skim milk	4.36	5.15	6.10	6.20	-	48
	37	Control	3.00	4.02	5.20	5.85		74
Saccharomyces uvarum NCIM 3455	30	Yeast extract	2.53	3.40	4.80	6.00	-	74
	30	Skim milk	4.60	5.85	6.60	6.71	31.4	48
	30	Control	3.60	4.45	5.20	6.30	-	74
	37	Yeast extract	2.77	3.82	4.91	5.91	-	74
	37	Skim milk	4.29	5.63	6.47	6.11	30.0	48
	37	Control	3.00	4.33	5.20	5.80		74

different conditions with S. uvarum and Saccharomyces sp. SH12 are shown in Fig 1 and 2 insets respectively. The dry weights of cells at the two periods of fermentation of 10 h and 72 h indicate that the cell mass increase is significantly higher in the presence of skim milk compared to the control without supplementation and to that with yeast extract addition.

To determine the specific ethanol productivity, an experiment was carried out in the presence of S. uvarum 3455 (3.0 g wet weight cells/100 ml) and 20% molasses sugar (to ensure enough substrate concentration for fermentation). As seen in Table 4, the addition of skim milk results in an enhancement in the initial specific ethanol productivity with both at 30°C and 37°C,temperatures compared with control and yeast extract addition.

3.4 DISCUSSION

It can be concluded that supplementation of a cane molasses medium containing 13.5% fermentable sugars with (0.2%) skim milk significantly enhances the rate of ethanol production and the process is virtually complete by 48 h compared to 74 h with yeast extract supplementation alone and in the control without any supplement.

TABLE 4 CANE MOLASSES FERMENTATION TO STUDY THE SPECIFIC ETHANOL PRODUCTIVITY IN THE PRESENCE OF SKIM MILK. CULTURE INOCULATED WAS SACCHAROMYCES UVARUM NCIM 3455. INOCULUM USED WAS 3.0 G WET WT/100 ML. MOLASSES SUGAR 20% + UREA 0.25% (FERMENTABLE SUGAR 18%). CONTROL WAS WITHOUT SUPPLEMENT. RESULTS ARE AVERAGE VALUES OF DUPLICATE EXPERIMENTS

Supplement added (0.2%)	Temperature (°C)	Alcohol (%, w/v) at				Specific ethanol productivity at Q_p^4 h ($g g^{-1} h^{-1}$)
		4 h	6 h	10 h	26 h	
Yeast extract	30	2.02	3.01	4.70	7.92	0.58
Skim milk	30	2.65	3.30	5.41	8.55	0.77
Control	30	1.94	2.76	4.21	7.47	0.56
Yeast extract	37	2.00	2.81	3.95	6.63	0.58
Skim milk	37	2.53	3.69	5.17	7.97	0.73
Control	37	2.33	2.90	3.90	7.00	0.70

The high content of potassium, calcium salts and of silica etc. in cane molasses together with a deficiency in nutritional requirements is probably responsible for the lowered rate of ethanol production in the control experiment. Supplementation with yeast extract (0.2%) resulted in some enhancement both of growth of cells and ethanol production rate. Skim milk supplementation, however, resulted in further improvement both in growth and ethanol production rate by 48 h fermentation. Whether the action of skim milk can be attributed to better cell mass production or whether it offers a protection effect against the salts present in cane molasses is being investigated.

CHAPTER IV

CHITIN SUPPLEMENTATION SPEEDS UP THE ETHANOL PRODUCTION IN CANE MOLASSES FERMENTATION.

4.1 INTRODUCTION

In the previous chapter, I have described the use of skim milk (powder) to enhance the ethanol production in cane molasses fermentation. During my continued efforts in this direction, attempts were made to speed up the rate of ethanol production by supplementation (0.2%) with carbohydrates such as acacia gum, chitin, xylan, pullulan, cellobiose, dextrin, inulin and agar. For comparison, yeast extract and skim milk powder were used separately and in combination. Two yeast strains. Sacch. cerevisiae, NCIM 3526 and Sacch uvarum NCIM 3509, were employed for all the fermentation reactions. Among the carbohydrate supplements that were used, Chitin, a waste product and cheap material, was the most effective supplement in accelerating the rate of ethanol production and hence was studied in detail. Chitin is made up of N- acetylated glucosamine and is processed from discarded shell of prawns, lobsters, shrimps, other crustaceans and major exoskeleton of insects.

Secondly chitin is one of the components of fungi and yeast cultures. In yeast, chitin is localised as a ring around the bud sear and the enzyme chitin synthetase is located out side the cytoplasmic membrane. When yeast cells

are treated with Chitinase, only the bud scar-ring is found to be eliminated without affecting the integrity of the yeast cell. (Cabib and Bowers, 1971, and Keller and Cabib 1971). Chitin has now emerged as effective wonder drug to arrest haemorrhage and to speed up wound healing process. It is also used as a water purifier. The chitin production plant with a capacity of 3000 kg per annum has been set up in Kerala State recently.

By supplementing chitin (0.2% or less) in the fermentation medium, 6.0 - 6.9 % ethanol was formed after about 30 h at 30°C from cane molasses containing 18% total reducing sugars, as compared to a period of more than 72 h in the absence of any supplement.

4.2 MATERIALS AND METHODS

The inoculum medium consisting of total molasses reducing sugars 5.0 %, urea 0.25 %, $MgSO_4 . 7H_2O$ 0.05 % and yeast extract 0.2 % (pH 5.5) was autoclaved at 1.02 bar (15 lb./in^2 gauge) for 20 min. The required inoculum was built up by transferring a loop from the grown stock culture to a 10.0 ml of inoculum medium in boiling tube and then to a required volume (in conical flasks) and kept on a rotary shaker (150 RPM) for 24 h at 30° or 37°C. In all the

experiments, 10 % homogeneous inoculum was used.

The fermentation was carried out with molasses containing 16 or 18 % reducing sugars and at pH 5.0. The fermentation medium (90 ml in 100 ml conical flasks) supplemented with urea and $MgSO_4.7H_2O$ as a described above was added separately with yeast extract, skim mil, acacia gum, chitin, pullulan, xylan, dextrin, inulin, agar or cellobiose in the range of 0.1 to 0.2 %. All these media were autoclaved at 15 lb./ in^{-2} gauge for 20 min.

The effects of various supplements on ethanol production rate, during cane molasses fermentation were studied by estimation of ethanol after suitable periods at both $30^{o}C$ and $37^{o}C$.

4.3 RESULTS

EFFECT OF DIFFERENT CARBOHYDRATE SUPPLEMENTS IN MOLASSES FERMENTATION

To study the effect of natural polysaccharides in cane molasses fermentation at $37^{o}C$, an experiment was conducted by taking 90 ml of fermentation medium in 100 ml conical flasks, having 16 % reducing sugars with urea and $MgSO_4 7H_2O$. All the supplements like yeast extract, skim

milk, chitin, acacia-gum, xylan, pullulan, inulin, cellobiose, dextrin and agar were added separately to a final concentration of 0.2 %. After adjusting the pH to 5.0, the media were sterilised at 15 lb./in^2 gauge for 20 min and were then inoculated with 10 % homogeneous inocula of Sacch. uvarum NCIM 3509 and Sacch. cerevisiae NCIM 35 26, pregrown at 37^{o}C on a rotary shaker 150 RPM) for 24 h. A control experiment was also run without addition of any supplement. Samples were removed for ethanol estimation from stationary fermentation flasks kept at 37^{o}C after a period of 36 h.

As seen in Table 1, ethanol formation rate is improved in the presence of chitin, acacia gum, xylan and dextrin, while other carbohydrate supplements like cellobiose, agar, inulin, pullulan show either a moderate or no improvement in ethanol fermentation by both the yeast cultures used. The maximum rate of ethanol formation (around 50%) is observed at 37^{o}C after a period of 36 h in the presence of chitin as against 72 in the control experiment without any supplement.

EFFECT OF CHITIN ON INDUSTRIALLY USED CULTURES

Several yeast cultures such as Sacch. cerevisiae NCIM 3107, 3107, 3186, 3287, 3523, 3526, Sacch. cerevisiae var.

TABLE 1 : EFFECT OF DIFFERENT CARBOHYDRATE SUPPLEMENTS IN MOLASSES FERMENTATION

Molasses reducing sugars 16% + urea + $MgSO_4.7H_2O$

Temperature 37°C, pH 5.0

Sr. No.	Supplements added 0.2%	Ethanol % (w/v) after 36 h	% increment in ethanol
	Culture inoculated: Sacch. uvarum NCIM 3509		
1.	Control	3.74	-
2.	Yeast extract	3.50	-
3.	Skim milk (powder)	4.83	29
4.	Chitin	5.60	50
5.	Acacia gum	4.68	25
6.	Xylan	4.50	20
7.	Pullulan	3.10	-
8.	Inulin	3.31	-
9.	Cellobiose	3.90	-
10.	Dextrin	4.40	17
11.	Agar	3.60	-
	Culture inoculated: Sacch. cerevisiae NCIM 3526		
1.	Control	3.45	-
2.	Yeast extract	3.52	-
3.	Skim milk (Powder)	4.29	24
4.	Chitin	5.38	55
5.	Acacia gum	4.21	22
6.	Xylan	4.20	21
7.	Pullulan	3.78	-
8.	Inulin	3.55	-
9.	Cellobiose	3.58	-
10.	Dextrin	4.05	17
11.	Agar	3.30	-

Control: Without supplement

Percentage increase in ethanol was estimated by comparison with control

Results are average values of duplicate experiments

Less than 10% increase in ethanol production is not recorded.

ellipsoideus NCIM 3281 and S. uvarum NCIM 3509 were screened to assess their performance for ethanol production rate in presence of (A) cane molasses reducing sugars 16 % + urea + $MgSO_4 7H_2O$, as control (B) same as A + yeast extract 0.2 % as an inoculum control, (C) same as A + chitin 0.2 %. All the cultures mentioned above were inoculated and fermentations were carried out under similar conditions and at the same temperature of 37°C. Ethanol was estimated after a period of 36 h.

From Table 2, it can be observed that ethanol production rate is more for all the cultures used, in presence of chitin than simply with yeast extract supplement or in the control experiment. The two standard cultures S.cerevisiae NCIM 3526 and S. uvarum NCIM 3509 were used in the following experimental tests.

SYNERGISTIC EFFECT OF SUPPLEMENTS ON ETHANOL PRODUCTION

To study the synergistic effect of various supplements a few combinations of supplements were used during these experiments and the concentration of each sugars in fermentation media was kept at 16 % (pH 5.0). The

Table 2

Effect of chitin on industrially used cultures
Molasses reducing sugars 16 % + urea + $MgSO_4 . 7H_2O$
Temperature 37°C pH 5.0

Cultures used	Supplement added 0.2%	Ethanol after 36 h (W/V)	% increase in ethanol
Sacch. cerevisiae NCIM 3107	Control	3.12	-
	Yeast extract	3.00	-
	Chitin	5.20	66
Sacch. cerevisiae NCIM 3186	Control	3.23	-
	Yeast extract	3.20	-
	Chitin	5.00	54
Sacch. cerevisiae Var ellipsoideus NCIM 3281	Control	2.65	-
	Yeast extract	2.45	-
	Chitin	5.45	105
Sacch. cerevisiae NCIM 3287	Control	2.70	-
	Yeast extract	2.50	-
	Chitin	5.35	106
Sacch. cerevisiae NCIM 3523	Control	2.18	-
	Yeast extract	2.20	-
	Chitin	5.30	143
Sacch. cerevisiae NCIM 3526	Control	3.40	-
	Yeast extract	3.20	-
	Chitin	5.40	58
Sacch. uvarum NCIM 3509	Control	3.65	-
	Yeast extract	3.78	-
	Chitin	5.52	50

Control : Without any supplement.
Average values of duplicate experiments.

Table 2

Effect of chitin on industrially used cultures
Molasses reducing sugars 16 % + urea + $MgSO_4 . 7H_2O$
Temperature 37°C pH 5.0

Cultures used	Supplement added 0.2%	Ethanol after 36 h (W/V)	% increase in ethanol
Sacch. cerevisiae NCIM 3107	Control	3.12	-
	Yeast extract	3.00	-
	Chitin	5.20	66
Sacch. cerevisiae NCIM 3186	Control	3.23	-
	Yeast extract	3.20	-
	Chitin	5.00	54
Sacch. cerevisiae Var ellipsoideus NCIM 3281	Control	2.65	-
	Yeast extract	2.45	-
	Chitin	5.45	105
Sacch. cerevisiae NCIM 3287	Control	2.70	-
	Yeast extract	2.50	-
	Chitin	5.35	106
Sacch. cerevisiae NCIM 3523	Control	2.18	-
	Yeast extract	2.20	-
	Chitin	5.30	143
Sacch. cerevisiae NCIM 3526	Control	3.40	-
	Yeast extract	3.20	-
	Chitin	5.40	58
Sacch. uvarum NCIM 3509	Control	3.65	-
	Yeast extract	3.78	-
	Chitin	5.52	50

Control : Without any supplement.
Average values of duplicate experiments.

fermentation of all these combinations were carried out by inoculating S. cerevisiae NCIM 3526 and incubating at 37°C. Samples were removed for ethanol estimation after a period of 30 h.

It is clear from Table 3 that the ethanol production rate has improved most in the presence of (i) yeast extract + chitin (ii) Skim milk + chitin (iii) acacia gum + chitin and (iv) acacia gum with skim milk. It is interesting to note that the ethanol production was much as 79 % more for the combination of skim milk and chitin. Also it is demonstrated that even 0.1 % chitin supplement showed improvement in ethanol production compared with 0.2 % of the other supplement alone. The other combination of yeast extract with pullulan or xylan and skim milk with pullulan or xylan were not so effective as that of chitin in improving ethanol production.

PROGRESSIVE RATE OF ETHANOL PRODUCTION IN PRESENCE OF CHITIN.

The next experiment was planned to determine the ethanol production rate in presence of chitin at 30°C in total molasses reducing sugars 20 % (fermentable sugars 18%). Here instead of 90 ml fermentation media, 450 ml of media were

Table 3

Synergistic effect of supplements in molasses fermentation
Culture inoculated S. cerevisiae NCIM 3526
Molasses reducing sugars 16 % + urea + $MgSO_4 . 7H_2O$
Temperature 37°C pH 5.0

Sr. No.	Supplement	% added	Ethanol after 30 h	Increment in ethanol %
1.	Control	-	3.10	-
2.	Yeast extract	0.2	3.00	-
3.	Skim milk	0.2	4.65	50
4.	Acacia gum	0.2	4.20	35
5.	Chitin	0.2	5.00	61
6.	Pullulan	0.2	3.31	-
7.	Xylan	0.2	4.00	29
8.	Yeast extract + Acacia gum	0.1 each	5.01	61
9.	Yeast extract + chitin	0.1 "	5.53	78
10.	Yeast extract + pullulan	0.1 "	3.40	-
11.	Yeast extract + Xylan	0.1 "	3.70.	19
12.	Skim milk + Acacia gum	0.1 "	5.46	76
13.	Skim milk + chitin	0.1 "	5.55	79
14.	Skim milk + Pullulan	0.1 "	3.90	21
15.	Skim milk + Xylan	0.1 "	4.43	42
16.	Acacia gum + chitin	0.1 "	5.38	73

Control : Without supplement.
Average values of duplicate experiments.

taken in 500 ml conical flasks and three sets of flasks were prepared, (A) total molasses reducing sugars 20% + urea + $MgSO_4.7H_2O$, (B) same as A + yeast extract 0.2 %, (C) same as A + chitin 0.2 %. Inocula of cultures S. uvarum NCIM 3509 and S. cerevisiae NCIM 3526 were prepared on a rotary shaker (150 RPM) at 30°C for 24 h and 50 ml inocula were transferred to fermentation media. All these flasks of fermentation were incubated at 30°C. Aliquots (5 ml), in duplicate, were removed at intervals of 20, 30, 50, 60, 70 and 80 h for ethanol estimation.

From Table 4 and Fig 1 and 2, it can be observed that ethanol production rate is considerably higher in the presence of chitin compared to that with yeast extract addition and the control without any addition. Moreover, it is clear that ethanol formation is nearly complete after around 30 h (6-6.9 % W/V) while it takes more than 70 h to reach the same level of ethanol formation in the control or with yeast extract addition.

CELL MASS DATA

Cell mass formation was determined during an experiment using total molasses reducing sugar 15 %

Table 4

Progressive rate of ethanol production in presence of chitin
Culture inoculated S.cerevisiae NCIM 3526
S. uvarum NCIM 3509
Total molasses sugars 20% + urea + $MgSO_4.7H_2O$
Temperature 30°C pH 5.0

Sr. No.	Supplement added 0.2%	Ethanol % (W/V) at different hours 20	30	50	60	70	80
	Culture used S. cerevisiae NCIM 3526						
1.	Control	3.65	5.30	6.55	6.94	7.09	7.25
2.	Yeast extract	4.00	6.08	6.65	7.13	7.20	7.40
3.	Chitin	5.94	6.90	7.20	7.30	7.35	7.45
	Culture used S. uvarum NCIM 3509						
1.	Control	3.35	4.48	5.39	5.80	6.48	7.12
2.	Yeast extract	3.10	3.50	4.91	5.20	6.16	7.18
3.	Chitin	5.75	6.09	6.80	7.05	7.10	7.30

Control : Without supplement.
Average values of experiments.

FIGURE 1

Ethanol production in the presence of chitin, yeast extract and control without supplement, at 30°C (pH 5.0).
Culture S. uvarum NCIM 3509
Fermentable sugars 18% + urea 0.25% + $MgSO_4.7H_2O$ 0.05%.

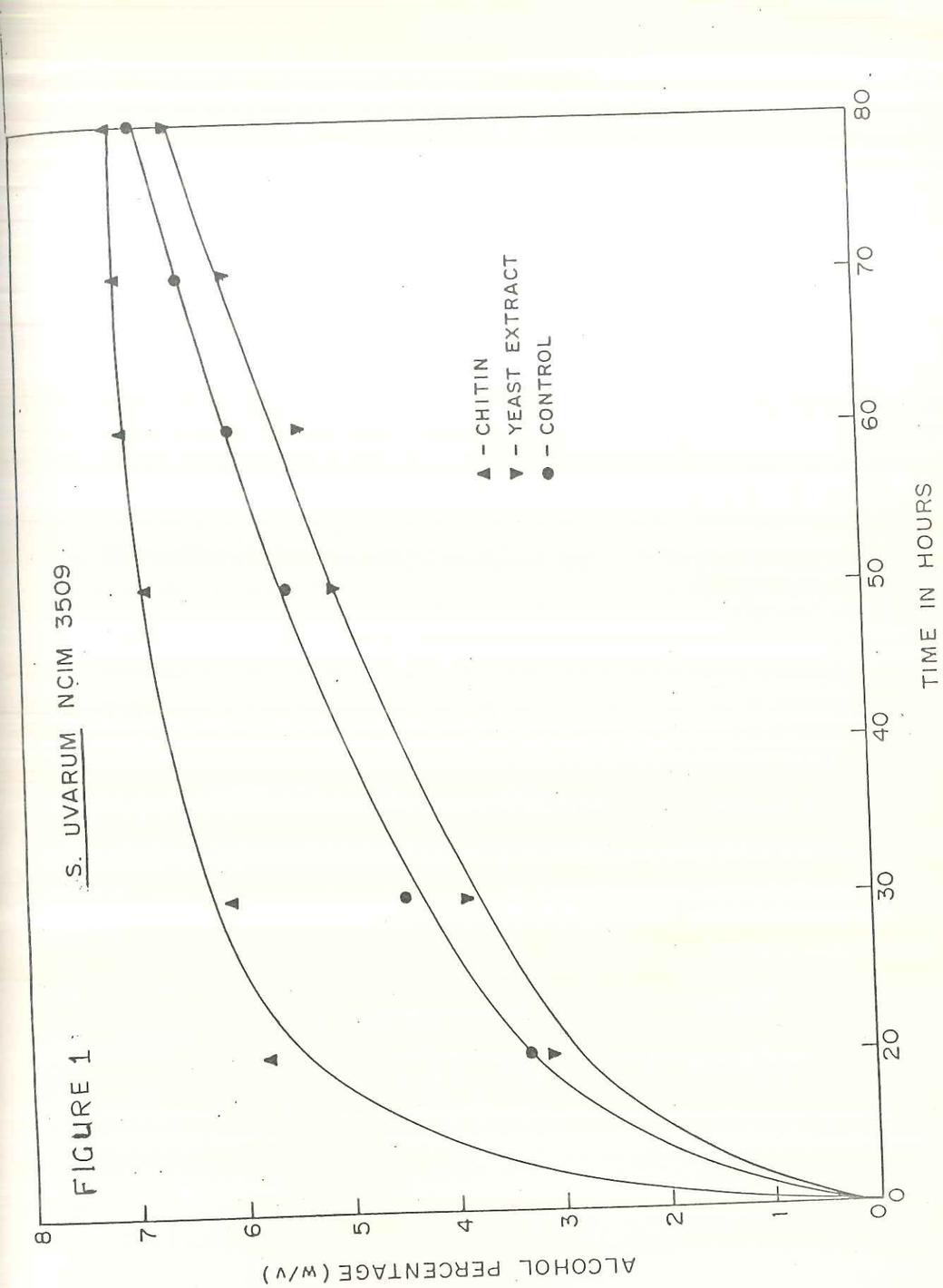
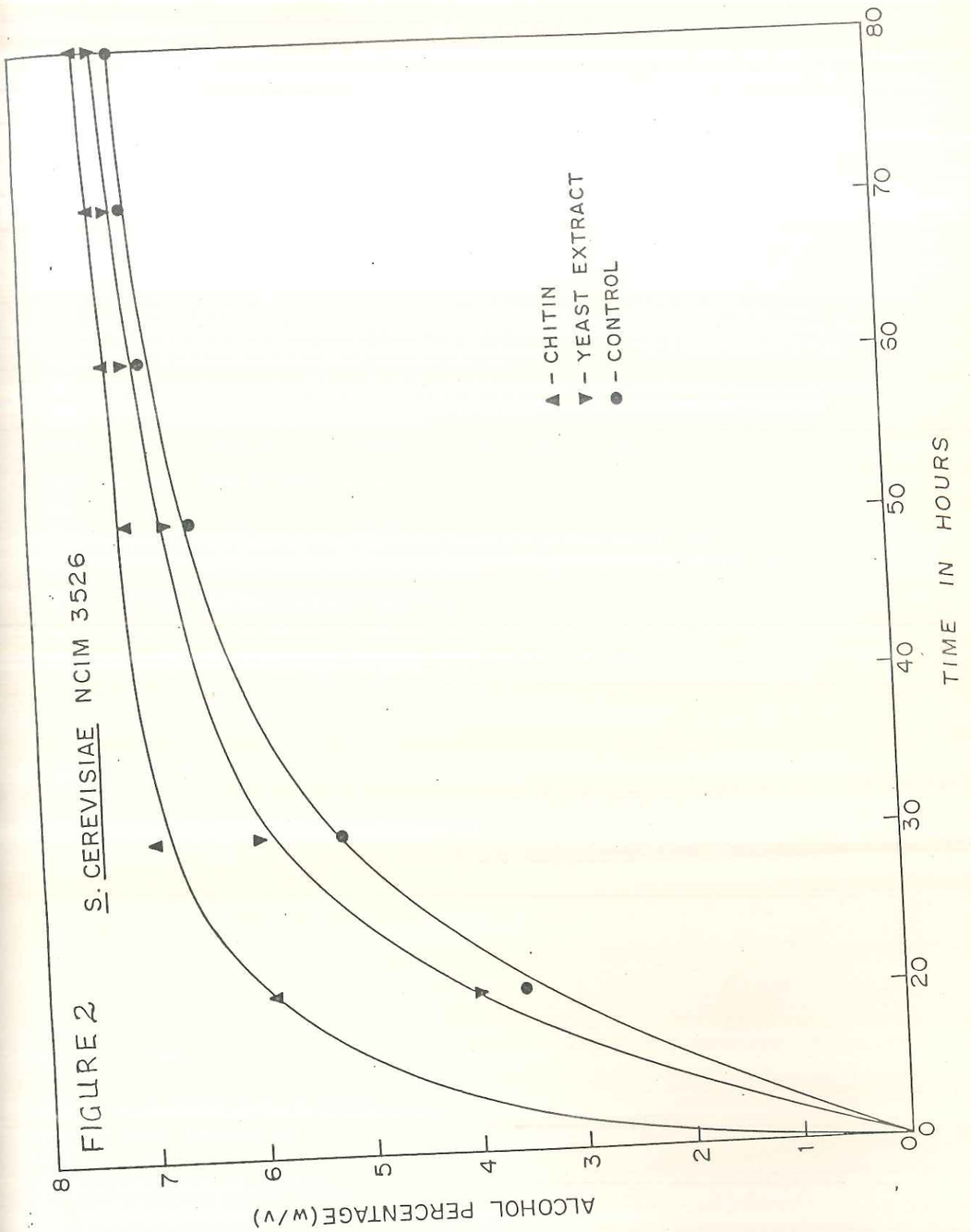


FIGURE 2

Ethanol production in the presence of chitin, yeast extract and control without supplement, at 30°C (pH 5.0).
Culture S. cerevisiae NCIM 3526
Fermentable sugars 18% + urea 0.25% + $MgSO_4.7H_2O$ 0.05%.



containing urea $MgSO_4.8H_2O$ as the basal medium and with addition of yeast extract and chitin separately in a total fermentation medium volume of 100 ml. The preparation of the inocula and the incubation of these fermentation flasks were carried at 37°C as described earlier, inoculating with both the cultures of S. cerevisiae NCIM 3526 and S. uvarum NCIM 3509. The yeast cells were harvested by centrifugation after a period of 72 h and were thoroughly washed with water twice and recentrifuged. The packed cells were dried at 80°C (24 h) to a constant dry weight. The correct cell mass in the presence of chitin fermentation was determined by subtracting the weight of added chitin from the total dry weight.

Table 5 indicates that there is an increase in cell mass in the fermentation medium supplemented with chitin as compared to that in control experiment and even with yeast extract supplement.

ROLE OF CHITIN IN MOLASSES FERMENTATION

To investigate the role of chitin in enhancing the rate of alcohol fermentation, one more fermentation experiment

Table 5

Cell Mass data in cane molasses fermentation
Total molasses sugars 15 % + urea + $MgSO_4 . 7H_2O$
Temperature 37°C pH 5.0

Sr. No.	Supplement added 0.2 %	Yeast cell mass mg/ 100 ml.	% increment in cell mass.
	Inoculated culture S. cerevisiae NCIM 3526		
1.	Control	430	-
2.	Yeast extract	426	-
3.	Chitin	635	47.6
	Inoculated culture S. uvarum NCIM 3509		
1.	Control	415	-
2.	Yeast extract	380	-
3.	Chitin	610	46.9

Control : Without any supplement.
Average values of duplicate experiments.

was conducted by inoculating S. cerevisiae NCIM 3526 and S. uvarum NCIM 3509 in media with different types of treated chitin and supernatant. To start with, chitin (1:10 W/V) was given a mild treatment of hydrolysis in water at pH 5.0 and then autoclaved at 10 lb./in^2 gauge for 20 min. After filtration, the volume was made up to 10 ml (chitin extract) and the filtered chitin was dried at 50^{o}C to a constant weight (dried chitin). Filtrate chitin and dried chitin were added as supplements at the level of 2.0 ml/100 ml and 0.2% respectively. The fermentation medium of 200 ml containing 16% fermentable sugars + urea + $MgSO_4.7H_2O$ and chitin 0.2 % was autoclaved under the same conditions and two flasks were prepared by collecting 90 ml supernate and by decantation (chitin treated molasses) in one and 90 ml molasses along with chitin (residual molasses) in the other. Seven sets of duplicate flasks were prepared for fermentation, (A) fermentable sugars 16 % + urea + $MgSO_4.7H_2O$, (B) same as A + yeast extract 0.2 % (C) same as A + chitin 0.2 %, (D) same as A + chitin extract 2 ml/100 ml, (E) same as A + dried chitin 0.2 %, (F) same as A but chitin treated molasses, (G) same as A but with residual molasses. These flasks were inoculated using yeast strains S. uvarum and S. cerevisiae

as described earlier, at 30oC and the ethanol content was estimated after a period of 30 h.

It can be observed from Table 6 that the addition of chitin extract with water do not improve the rate of ethanol production while dried chitin improves ethanol formation. In the next set of chitin treated molasses as well as left molasses, fermentation of ethanol formation is improved compared to control and yeast extract supplement.

4.4 DISCUSSION

The supplementation of chitin (0.2 %) in cane molasses fermentation containing 16 % reducing sugars showed an enhanced rate of ethanol production. It is further observed that ethanol increased by 50 % more as compared to that in control without any supplement or with the yeast extract supplement, at 37^{o}C after a period of 36 h. From the other experiment a few important industrially used yeast cultures have exhibited increased rates of ethanol production in presence of chitin and a few cultures show 100-140 % more ethanol at 36 h and at 37^{o}C. The synergistic effect of chitin with skim milk, yeast extract and acacia gum had shown considerable improvement in ethanol production rate (79 % more) as compared to the rate of control, when these

Table 6

Role of chitin in molasses fermentation.
Culture inoculated S. cerevisiae NCIM 3526
S. uvarum NCIM 3509
Molasses reducing sugars 16 % + urea + $MgSO_4 . 7H_2O$
Temperature 30°C pH 5.0

Sr. No.	Supplement	Added %	Ethanol % after 24 h	% Increment in ethanol
	Inoculated culture S. cerevisiae NCIM 3526			
1.	Control	-	2.90	-
2.	Yeast extract	0.2	2.70	-
3.	Chitin	0.2	5.42	86
4.	Chitin extract	2.0 ml/ 100 ml	2.88	-
5.	Dried chitin	0.2	5.30	82
6.	Chitin treated molasses	-	5.00	72
7.	Residual molasses	-	5.80	100
	Inoculated culture S. uvarum NCIM 3509			
1.	Control	-	3.00	-
2.	Yeast extract	0.2	3.10	-
3.	Chitin	0.2	5.55	85
4.	Chitin extract	2.0 ml/ 100 ml	3.15	-
5.	Dried chitin	0.2	5.38	79
6.	Chitin treated molasses	-	5.00	66
7.	Residual molasses	-	5.60	86

Control : Without any supplement.
Average values of duplicate experiments.

were supplemented individually at 37°C. Further ethanol production was virtually complete around 30 h at 30°C in 18 % reducing sugars in presence of chitin as compared to the control. Cell mass too was increased in the presence of chitin. The mild hydrolysis chitin extract in water did not enhance ethanol production, while dried chitin enhanced the rate of ethanol formation and chitin treated molasses supernatant showed ethanol production rate similar to chitin supplemented fermentation.

The unbalanced nutritional quality and presence of inhibitory substances in cane molasses fermentation may be the reasons for reducing the rate of ethanol production. Supplementation with yeast extract or skim milk powder favoured speeding up ethanol production. Chitin, a cell mass component of yeast (Reed and Peppler, 1973) supplementation further improved the rate of ethanol production as well as cell mass formation. Our data on the addition of chitin extract in water show that chitin is probably not contributing as nutritional factor needed by yeast culture. There is yet another possibility of partial hydrolysis of the molasses at the time of autoclaving and thereby resulting in the possible release of some nutritional

factors. Chitin treated molasses supernatant also ferments molasses sugars faster with the ethanol production similar to chitin supplementation. This may be due to removal of inhibitory compounds by chitin, which speeds up ethanol production. By this method of chitin supplementation, the fermentation time will be reduced by upto approaching one third and thereby the cost of the ethanol production might be expected to be reduced (other factors being equal) and secondly more molasses will be utilised in the existing batch fermentation technology. All these factors are being studied. Attempts will be made to scale up these experiments and to implement the fermentation at the industrial level.

---------- CHAPTER V ----------

THE ACCELERATION OF ETHANOL PRODUCTION OF YEAST IN CANE MOLASSES FERMENTATION BY THE ADDITION OF FUNGAL MYCELIUM.

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5.1 INTRODUCTION

Growth factors such as ergosterol, fatty acid, yeast extract, Tween-80 and soya flour have increased the ethanol production. In the previous two chapters skim milk-powder and chitin have been shown to improve the rate of ethanol production in cane molasses fermentation. It is known that the yield of ethanol is increased by the addition of Aspergillus oryzae - proteolipid in brewery fermentation. The chemical composition of proteolipid represents phospholipid, protein, fatty acids, phosphatidylcholine, sphingolipid and ash. The fatty acids contains palmitic, oleic and linoleic acids. Among these factors phosphatidylcholine has been reported to promote the growth and activities of yeast cells (Hayashida et al 1976)

In the present chapter the use of fungal mycelia has been described as a supplement in cane molasses fermentation to improve the yield of ethanol. Fungal mycelia of Penicillium chrysogenum NCIM 737, Aspergillus oryzae NCIM 1106, Aspergillus niger NCIM 573, Rhizophus nigricans NCIM 880, Neurospora sitophila NCIM 871, Fusarium tricinctum NCIM 1189 and Trichoderma reesei NCIM 1052 were prepared in this laboratory and waste mycelium of P. chrysogenum was

obtained from Hindustan Antibiotics, Pimpri, Pune. By supplementing the prepared fungal mycelium (0.4 % wet wt.) or waste mycelium (0.4 % dry wt.) in fermentation medium, 7.0-7.6 % (W/V) ethanol was formed in control experiments without any supplement. The effects of the concentration of molasses sugars, glucose fermentation and inocula requirements in presence of mycelia are also reported.

5.2 MATERIALS AND METHODS

The inoculum medium `MUMY' consisting of total molasses reducing sugars 5.0 %, urea 0.25 %, $MgSO_4 . 7H_2O$ 0.05 % and yeast extract 0.2 % (pH 5.5) was autoclaved at 15lb./in^{-2} for 15 min. The required inocula were built up by transferring a loop from the well grown yeast stock slope to 10 ml inoculum medium in a boiling tube and then to the required volume in conical flasks and kept on a shaker (150 RPM) for 24 h at 30^{o}C or 37^{o}C. homogeneous inoculum of 10 % (V/V) ca. 400 mg/10 ml wet wt. of yeast cells was used.

The mycelia from various fungal cultures, were prepared in the growth medium by taking two to three loops from cultures pregrown for 10 days at 30^{o}C on PDA slopes. The growth medium consisted of malt extract 0.3 %, glucose 4.0 %

and peptone 0.3 %, (100 ml in 500 ml conical flasks) pH 6.0, autoclaved at 10 lb/in^{-2} for 15 min. The inoculated media were kept on a shaker (150 RPM) at 30^{o}C for 6-7 days. The mycelium was collected by filtering through a clean muslin cloth and then washed twice by resuspending in distilled water. It was then pressed under Whatman filter paper to remove adhering water and stored at -10^{o}C. Wet mycelium (0.4 %) was added to molasses or glucose fermentation medium before autoclaving.

The fermentation media consisting of total molasses sugars in the range of 10-30 % with urea 0.25 % and $MgSO_4 \cdot 7H_2O$. 0.05 % were considered as 'control' without any supplement. The same media supplemented with yeast extract 0.2 % were taken as 'Inoculum control' separately. Prepared fungal mycelium from various species 0.4% dry wt. (ca. 0.14% dry wt.), waste mycelium of P. chrysogenum of industry (designated as 'waste mycelium') 0.4 % dry wt., dried at 50^{o}C to a constant weight, chitin 0.2 %, and skim milk powder 0.2 % were added separately to known concentrations of media. All these media with (pH 5.0) were autoclaved at 10 lb./in^{-2} for 15 min.

The defined glucose fermentation medium consisted of glucose 20 %,$(NH4)_2 SO_4$ 0.1 %,urea 0.2 %,KH_2PO_4 0.2%, $CaCl_2$

$2H_2O$ 0.015 % and yeast extract 0.2 %.The same medium composition was used for inoculum medium only by reducing the glucose to 4.0 % from 20 %. The conditions of autoclaving, inocula preparation and fermentation were the same as described earlier.

The autoclaved mycelium and its filtrate were prepared by taking mycelium (2.5 g wet wt.) in 10 ml distilled water and autoclaving at 10 lb./in^{-2} for 10 min. After cooling, these preparations were filtered through muslin cloth and filtrate volume was made up to 10 ml with water (designated as 'Filtrate PC' and 'Filtrate WM', prepared from P. chrysogenum and waste mycelium, respectively). The remaining mycelium was pressed under filter paper to remove water (designated as 'Autoclaved PC' and 'Autoclaved WM', from the species). Filtrates of these mycelia (1 ml/100 ml) and autoclaved mycelia (0.4 % wet wt.) were added to fermentation media to study their effects on ethanol production.

The total fermentation volume in most of the experiments was 100 ml which included 10 % inoculum in each flask.

5.3 RESULTS

EFFECT OF FUNGAL MYCELIUM ON ETHANOL PRODUCTION

The effect of fungal mycelium in cane molasses fermentation was examined using 90 ml of 20 % total reducing sugars in 100 ml conical flask. The prepared mycelium (0.4 % wet wt.) of various species and waste mycelium 0.4 % dry wt. were added to bring the total volume 100 ml including 10 % inoculum. Samples (5 ml) were collected for ethanol estimation from stationary flasks after 44 and 66 h.

Table 1 shows that all the flasks containing mycelium exhibit significantly higher rates of ethanol formation as compared to the control experiment after fermentation periods of 44 h and 66 h. Ethanol production in the presence of mycelium is higher by 30 to 65 % at 30^{o}C and 30 to 110 % at 37^{o}C compared with the control experiment. The fermentation was almost complete after 44 h in the presence of mycelia from the species of Penicillum. Trichoderma, Aspergillus, Sporotrichum and Rhizophus but takes about 66 h in the presence of mycelia from the remaining species.

WASTE MYCELIUM REQUIREMENT FOR ETHANOL PRODUCTION

The fermentation medium with 20% molasses sugar was

Table 1

Effect of fungal mycelium on ethanol production. Total molasses reducing sugars 20 % + Urea + $MgSO_4 . 7H_2O$ Culture inoculated S. cerevisiae NCIM 3525, Supplements mycelium 0.4 % wet wt. pH 5.0. Yeast extract supplement 0.2 %

Sr. No.	Supplement from fungal species	Ethanol % (W/V) ** 44 h	% increase	66 h	% increase
	Temperature 30°C				
1.	Control *	4.6	-	5.7	-
2.	Yeast extract	4.2	-	5.8	-
3.	P.chrysogenum 737	7.6	65	7.7	35
4.	A.oryzae 555	7.0	52	7.7	35
5.	S.rolfsii 1084	6.2	30	6.8	20
6.	S.pullverulentum 1106	7.5	63	7.8	36
7.	A.niger 573	7.0	52	7.5	31
8.	R.nigricans 880	7.3	59	7.4	30
9.	N.sitophila 871	5.7	24	7.0	22
10.	T.reesei 1052	7.6	65	7.7	35
11.	F.tricinctum 1189	6.8	47	7.8	36
12.	Waste mycelium	6.6	43	6.9	21
	Temperature 37°C				
1.	Control *	2.7	-	3.9	-
2.	Yeast extract	3.0	-	4.9	-
3.	P.chrysogenum 737	5.3	96	6.8	74
4.	A.oryzae 555	5.5	103	6.6	69
5.	S.rolfsii 1084	3.5	30	4.8	23
6.	S.pullverulentum 1106	5.5	103	7.0	79
7.	A.niger 573	4.9	81	6.3	61
8.	R.nigricans 880	5.8	114	6.8	74
9.	N.sitophila 871	4.4	63	6.0	54
10.	T.reesei 1052	5.6	107	7.2	84
11.	F.tricinctum 1189	3.5	30	5.3	35
12.	Waste mycelium	5.3	96	6.7	72

* Control without supplement. ** Average values of duplicate experiments.

supplemented with waste mycelium at the rates of 0.2% and 0.4% wet wt. and 0.1, 0.2, 0.4, and 0.6% dry wt. respectively. Samples for ethanol estimation were removed after 68 h at 30°C.

Table 2 shows that waste mycelium (0.4 % dry wt.) produces greater ethanol formation than other supplements and rates. There is very little increase in ethanol formation in the presence of 0.6 % mycelium. Hence waste mycelium 0.4 % dry wt. supplement is used in all the subsequent experiments.

EFFECT OF WASTE MYCELIUM IN GLUCOSE FERMENTATION

Waste mycelium in the ranges of 0.25, 0.5, and 0.75 % as wet wt. and 0.2, 0.4, and 0.6 % as dry wt. was added to 20 % glucose fermentation medium. Samples were removed for ethanol estimation after 44 h and 72 h.

Table 3 shows that the ethanol production is also faster in the presence of waste mycelium in the fermentation of the defined glucose medium. As with molasses, a minimum of 0.4 % dry wt. mycelium is required for maximum production of ethanol. The fermentation reaches 9.1 % (W/V) ethanol formation, which is 65 % more than in the control experiment.

Table 2

Waste mycelium requirement on ethanol production. Total molasses reducing sugars 20 % + Urea $MgSO_4 \cdot 7H_2O$. Temperature 30°C pH 5.0.

Culture inoculated S. cerevisiae NCIM 3525.

Sr. No.	Supplement	% added type	Ethanol** % (W/V) after 68 h	% increment in ethanol
1.	Control *	-	4.8	-
2.	Yeast extract	0.2	5.6	-
3.	Waste mycelium	0.4 wet wt.	6.3	29
4.	Waste mycelium	0.1 dry wt.	6.0	25
5.	Waste mycelium	0.2 dry wt.	7.0	46
6.	Waste mycelium	0.4 dry wt.	7.3	52
7.	Waste mycelium	0.6 dry wt.	7.4	54

* Control without supplement.
** Average values of duplicate experiments.

Table 3

Effect of waste mycelium in glucose fermentation. Glucose 20 % + $(NH_4)_2SO_4$ + Urea + KH_2PO_4 + $CaCl_2$ + $MgSO_4 \cdot 7H_2O$ and yeast extract.

Temperature 30°C pH 5.0

Culture inoculated S.cerevisiae NCIM 3525

Sr. No.	Supplement	% added type	Ethanol % (W/V) ** at 44 h	at 70 h	Ethanol % increase at 70 h
1.	Control *	-	3.6	5.5	-
2.	Yeast extract	0.2	4.1	6.2	-
3.	Waste mycelium	0.25 wet wt	4.0	6.5	18
4.	Waste mycelium	0.5 wet wt	4.4	7.9	43
5.	Waste mycelium	0.75 wet wt	4.4	8.2	49
6.	Waste mycelium	0.2 dry wt	5.0	8.6	56
7.	Waste mycelium	0.4 dry wt	5.9	9.0	63
8.	Waste mycelium	0.6 dry wt	6.0	9.1	65

* Control without supplement.

** Average values of duplicate experiments.

EFFECT OF WASTE MYCELIUM ON INDUSTRIALLY USED YEAST CULTURES

Industrially used yeast strains such as Sacch. cerevisiae NCIM 3287, 3523, 3525 and 3526, acch. cerevisiae var. ellipsoideus NCIM 3281 and Sacch.uvarum NCIM 3455 were screened for their ethanol production activity from molasses medium in the presence of waste mycelium. The cultures were inoculated into 20 % fermentation medium with and without waste mycelium supplement. Samples were removed for ethanol analysis after fermentation at 30°C for 60 h.

Table 4 shows that the ethanol production activity of all the industrially used cultures is enhanced in the presence of waste mycelium rather than that in the unsupplemented control.

EFFECT OF INOCULA SIZE ON ETHANOL PRODUCTION IN PRESENCE OF MYCELIUM

Two inocula concentrations were examined. The required inocula were pregrown in the inocula medium 'MUMY' at 30°C. Inoculum A (10 % V/V) contained ca. 410 mg/10 ml biomass of S.cerevisiae NCIM 3525. Particular inoculum B was prepared by centrifuging (at 5000 RPM for 10 min) 20 ml

Table 4

Effect of waste mycelium on industrially used yeast cultures. Total molasses reducing sugars 20 % + Urea + $MgSO_4 . 7H_2O$.
Temperature 30°C pH 5.0

Sr. No.	Culture inoculated	Waste mycelium % supplement dry wt.	Ethanol % (W/V) ** at 60 h
1.*	S. cerevisiae 3525	-	5.0
2.	S. cerevisiae 3525	0.4	7.2
3.*	S. uvarum 3455	-	4.2
4.	S. uvarum 3455	0.4	7.1
5.*	S. cerevisiae var ellipsoideus 3281	-	3.5
6.	S. cerevisiae var ellipsoideus 3281	0.4	7.1
7.*	S. cerevisiae 3287	-	5.8
8.	S. cerevisiae 3287	0.4	7.3
9.*	S. cerevisiae 3523	-	5.6
10.	S. cerevisiae 3523	0.4	7.0
11.*	S. cerevisiae 3526	-	5.1
12.	S. cerevisiae 3526	0.4	7.0

* Control without supplement.
** Average values of duplicate experiments.

of culture. The supernatant (10 ml) was removed and discarded while the remaining 10 ml with biomass was used as inoculum B. The inocula A and B were then transferred to (A) Fermentation medium of 20 % molasses sugars which served as a control, (B) the same as A with yeast extract 0.2 % which served as inoculum control. (C) the same as A with mycelium supplement 0.4 % wet wt. of 3 different species, separately. The ethanol concentration was determined at 22, 48 and 70 h.

Table 5 shows that the inocula having double biomass has improved ethanol production at 22 h in all the flasks. At 48 h, the inocula sizes have no significant effect on the ethanol production in the presence of mycelium. At 70 h, the control flask having double biomass inocula showed about 15 % more ethanol formation as compared to the normal inoculum control flask and has reached the ethanol production level (ca. 7.5 % W/V) equal to the other flasks having fungal mycelium supplement. The use of 10 % (V/V) which contained ca. 400 mg/10 ml biomass produces satisfactory levels of ethanol production, in accordance with the findings of Strehaiano (1983).

RATE OF ETHANOL AND BIOMASS PRODUCTION IN PRESENCE OF FUNGAL MYCELIUM

Table 5

Effect of inocula size on ethanol production in the presence of fungal mycelium.

Total molasses reducing sugars 20 % + Urea + $MgSO_4$ $7H_2O$.
Temperature 30°C pH 5.0
Culture inoculated *S. cerevisiae* 3525
Mycelium supplement 0.4 % wet wt. Yeast extract 0.2 %

Sr. No.	Supplement	Inocula size	Ethanol % (W/V) at ** 22 h	48 h	70 h
1.	Control *	A	2.5	4.8	6.4
2.	Control *	B	3.2	5.2	7.5
3.	Yeast extract	A	3.1	5.6	7.4
4.	Yeast extract	B	4.1	6.9	7.6
5.	*P. chrysogenum* 737	A	4.7	7.0	7.6
6.	*P. chrysogenum* 737	B	5.8	7.4	7.7
7.	*F. tricinctum* 1189	A	4.1	7.5	7.5
8.	*F. tricinctum* 1189	B	4.4	7.4	7.5
9.	*T.reesei* 1052	A	4.3	7.5	7.6
10.	*T. reesei* 1052	B	5.8	7.6	7.8

A : 10 ml homogeneous inocula contain 410 mg/wet wt. cells
B : 10 ml homogeneous inocula contain 835 mg/wet wt. cells
* Control without supplement.
** Average values of duplicate experiments.

Ethanol production rate and biomass formation data were determined in the presence of fungal mycelium in total molasses sugars of 22 %, sufficient to extend runs upto 80 h. The fermentation media 450 ml were taken in 500 ml conical flasks and 50 ml inocula were used. Three sets of media were prepared (A) fermentation medium as control, (B) the same as A + yeast extract 0.2 % as inoculum control, (C) the same as A + prepared fungal mycelium of P. chrysogenum. After 20 h fermentation under stationary conditions at 30°C, aliquots were removed every 10 h for ethanol estimation. The yeast cells were harvested after 80 h by centrifugation and washed twice with distilled water at 5000 RPM for 10 min. The biomass yields were corrected for the presence of the mycelium by subtracting the weight of the added mycelium.

Table 6 and Figure 1 show that in the presence of mycelium, the ethanol production rate is significantly high throughout as compared to the control or to the inoculum control. The yield of ethanol increase is effectively linear with time and reaches 8-8.5 % (W/V) at 80 h in the presence of mycelium. Biomass increased by more than 40 % in the presence of mycelium as compared to the control. This increment appears to be responsible for the improvement in

Table 6

ate of ethanol and biomass production in the presence of fungal mycelium.
olasses reducing sugars 22 % urea + $MgSO_4 \cdot 7H_2O$
otal fermentation volume 500 ml in each flask
emperature 30°C pH 5.0
Culture inoculated S. cerevisiae NCIM 3525

Sr. No.	Supplement (%)	Ethanol % (W/V) at hour **							Biomass wet wt after 80 h g/100
		20	30	40	50	60	70	80	
1.	Control *	2.0	2.4	3.1	3.5	4.2	4.9	6.1	1.25
2.	Yeast extract (0.2)	2.2	2.7	3.6	4.2	5.1	5.3	6.6	1.35
3.	P. chrysogenum (0.4)	3.0	3.6	5.7	7.2	7.6	8.0	8.7	1.77

* Control without supplements.
** Average values of duplicate experiments.

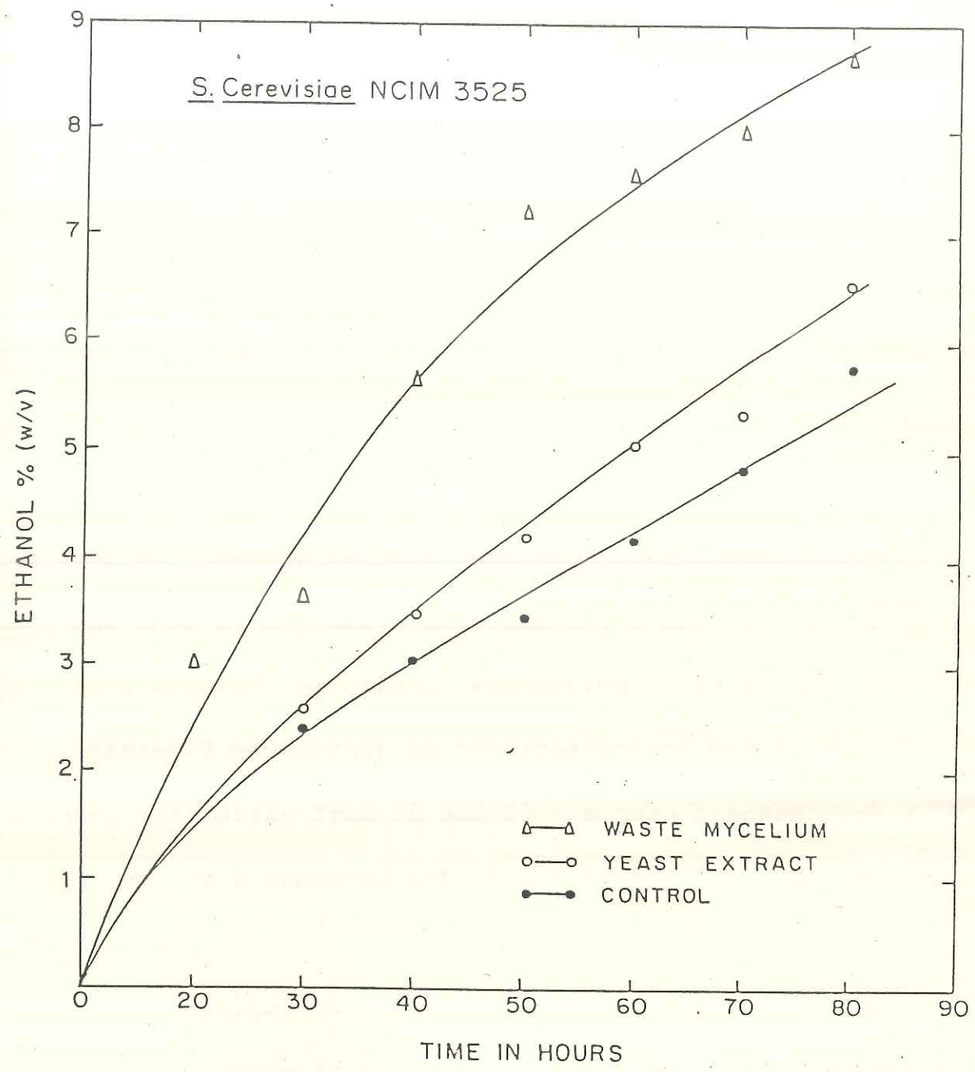


FIGURE 1
Rate of ethanol production in presence of fungal mycelium, yeast extract and control without additives 30°C, Sacch. cerevisiae (SH29) NCIM 3525.

the rate of ethanol production. The mycelium may be releasing important nutritional factors protecting the yeast cells against the greater total solids concentration and against the higher ethanol accumulation.

INFLUENCE OF WASTE MYCELIUM ON THE CONCENTRATIONS OF CANE MOLASSES SUGARS

The effect of mycelium on ethanol production from concentrations of 10,15,20,25 and 30 % molasses sugars was observed in the presence of urea (0.25 %) and magnesium sulfate (0.05 %). Triplicate sets were prepared including (A) fermentation media of different sugars' concentration serving as controls (B) the same as A but inoculum controls. (C) the same as A + waste mycelium 0.4 % dry wt. Samples (5 ml) were removed for ethanol estimation at 20,40 and 70 h.

Table 7 shows that in the presence of waste mycelium, ethanol formation from 20 and 25 % sugars reaches 8-8.3 % (W/V) at 70 h compared with 5-6.5 % (W/V) in the control (Figure 2).

The presence of waste mycelium increases the rate of ethanol production in all the range of sugar concentrations compared to their respective controls.In the presence of waste mycelium, fermentation from 10 % sugars is almost complete

Table 7

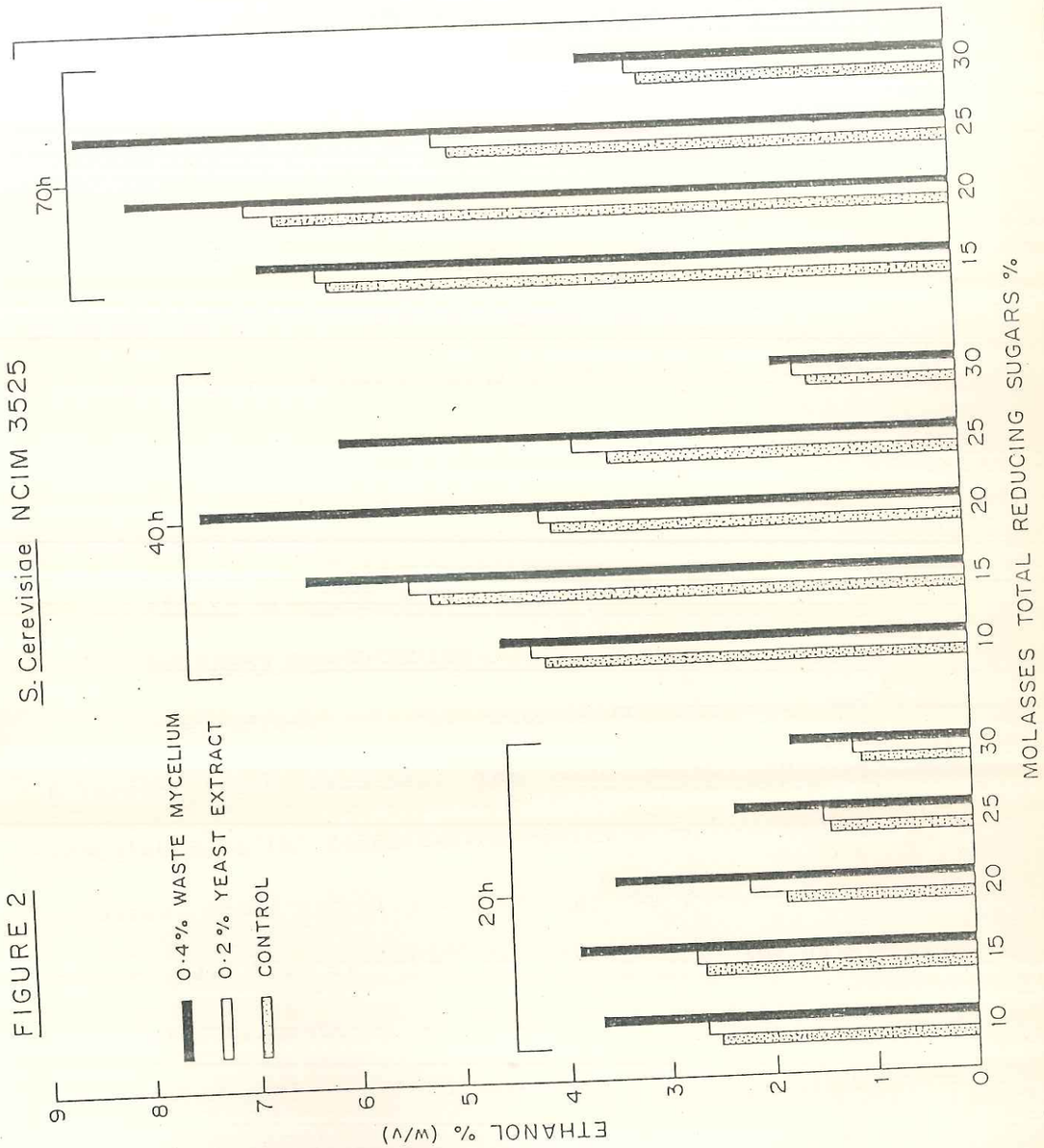
Influence of mycelium on the concentrations of cane molasses sugars.
To all concentrations of molasses sugars urea, 0.25 % ;
$MgSO_4 . 7H_2O$, 0.05 % were added.
Temperature 30°C pH 5.0
Culture inoculated S. cerevisiae NCIM 3525
Waste mycelium supplement 0.4 % dry wt. Yeast extract 0.2 %

Sr. No.	Concentration of molasses sugars %	Supplement added	Ethanol % (W/V) ** at 20 h	40 h	70 h
1.	10	Control *	2.5	4.4	4.6
2.	10	Yeast extract	2.7	4.5	4.7
3.	10	Waste mycelium	3.6	4.8	4.8
4.	15	Control *	2.6	5.2	6.1
5.	15	Yeast extract	2.7	5.4	6.3
6.	15	Waste mycelium	3.7	6.3	6.8
7.	20	Control *	1.9	4.0	6.5
8.	20	Yeast extract	2.3	4.2	6.8
9.	20	Waste mycelium	3.4	7.5	8.0
10.	25	Control *	1.3	3.3	4.9
11.	25	Yeast extract	1.4	3.5	5.0
12.	25	Waste mycelium	2.3	5.8	8.3
13.	30	Control *	1.2	1.8	3.0
14.	30	Yeast extract	1.1	1.9	3.0
15.	30	Waste mycelium	1.7	2.0	3.7

* Control without supplements.
** Average values of duplicate experiments.

FIGURE 2

Influence of waste mycelium on the concentrations of cane molasses sugars.
S.cerevisiae NCIM 3525 pH 5.0
To all the concentrations of molasses sugars, urea 0.25%, $MgSO_4.7H_2O$ 0.05% were added.
To this medium , yeast extract 0.2% and waste mycelium 0.4% dry wt. were supplemented separately.



before 40 h and by about 40 h from 15 % sugars. Ethanol production from 30 % molasses sugars is very low in all cases but a slight improvement is detected in the presence of mycelium. In the control experiment, the extent of conversion of sugars to ethanol effectively decreases as the concentrations of molasses sugars increases. In the presence of mycelium the formation of ethanol increases with increase in the concentrations of sugars until the 25 % level is exceeded.

EFFECT OF AUTOCLAVED MYCELIUM AND ITS FILTRATE IN MOLASSES FERMENTATION

The effect of the autoclaved mycelium and its filtrate were examined. The same yeast culture was inoculated to (A) fermentation medium a control having 20 % molasses total reducing sugars, (B) the same as A + yeast extract 0.2 % as inoculum control, (C) the same as A + 1.0 ml/100ml `Filtrate PC' or `Fitrate WM, (D) the same as A + `Autoclaved PC' or `Autoclaved WM'. After incubation at 30^{o}C, samples were removed for ethanol analysis at 40 and 80 h.

Table 8 shows that the addition of normal mycelium increases ethanol production as expected. The addition of autoclaved mycelia enhances ethanol production less than the addition of normal mycelia. However, the addition of autoclaved filtrates of the mycelia actively inhibits ethanol production which is less in the range of 30 -35 % than the control, at 40 h. Thus, mycelium separately autoclaved or together with the fermentation medium enhances ethanol production while filtrate is inhibitory.

EFFECT OF FUNGAL MYCELIUM IN THE COMBINATION WITH OTHER SUPPLEMENTS IN MOLASSES FERMENTATION

We have earlier reported that skim milk powder and chitin or polysaccharides supplements increase ethanol production in cane molasses fermentation. The effect of these supplements in combination with fungal mycelium was examined. The fermentation was carried out using the same yeast culture and the same percentage of molasses sugars. The fermentation flasks were prepared as before in respect of fermentation medium, control and inoculum control, with the addition of skim milk powder, chitin, mycelium from *P. chrysogenum* and combinations of these. Fermentation was carried out at 30° C and samples were removed after 48 and 72 h.

Table 8.

Effect of autoclaved mycelium and its filtrate in molasses fermentation.
Total molasses reducing sugars 20 %, + Urea + $MgSO_4 . 7H_2O$.
Temperature 30°C, pH 5.0.

Culture inoculated *S. cerevisiae* NCIM 3525

Sr. No.	Supplement	% added	Ethanol % at 40 h (W/V)	Ethanol % at 80 h (W/V)	At 40 h Increment	At 40 h Inhibition
1.	Control *	-	4.0	6.3	-	-
2.	Yeast extract	0.2	4.3	6.5	7.5	-
3.	P.chrysogenum 737	0.4 wet wt	7.0	7.8	75.0	-
4.	Waste mycelium	0.4 dry wt	6.8	7.2	70.0	-
5.	`Filtrate PC'	1.0 ml	2.6	5.0	-	35
6.	`Filtrate WM'	1.0 ml	2.8	-	-	30
7.	`Autoclaved PC'	0.4 wet wt	5.9	7.0	47.5	-
8.	`Autoclaved WM'	0.4 wet wt	5.8	7.1	45.0	-

* Control without supplements.
** Average values of duplicate experiments.

Table 9 shows that skim milk powder or chitin supplement enhances the rate of ethanol production. The combination of mycelium with skim milk powder or chitin slightly improves ethanol production and these fermentations are almost complete at 48 h unlike the control or inoculum control.

5.4 DISCUSSION

A substantial increase was found in the rate of ethanol production in cane molasses fermentation with the addition of fungal mycelium. The latter produces 7-7.6 % (W/V) ethanol from 20 % total molasses reducing sugars, at 44 h at 30°C , as against 4-4.5 % (W/V) ethanol in the unsupplemented control. In distillery practice on an average, 6.0-6.5 % ethanol is recovered from 15 -18 % cane molasses sugars. In these present experiments 8.3% (W/V) ethanol is produced in the presence of mycelium as against 4.9 % (W/V) ethanol in control from the increased concentration of total molasses sugars of 25 % at 70 h at 30°C. The effect of mycelium was similarly demonstrated in the defined medium of 20 % glucose where 9.1 % (W/V) ethanol formation was recorded at 70 h. The yeast strain used in

Table 9

Effect of fungal mycelium in combination with other supplements in molasses fermentation.

Molasses sugar 20 % + Urea + $MgSO_4 . 7H_2O$

Temperature 30°C pH 5.0.

Culture inoculated S. cerevisiae NCIM 3525

Sr. No.	Supplements	% added	Ethanol % (W/V) 48 h	72 h
1.	Control *	-	4.9	6.5
2.	Yeast extract	0.2	5.3	7.2
3.	Skim milk powder	0.2	6.4	7.2
4.	Chitin	0.2	7.4	7.7
5.	P.chrysogenum 737	0.4 wet wt	6.9	7.6
6.	P.chrysogenum 737 + Skim milk	0.4 " " 0.2	7.25	7.6
7.	P.chrysogenum 737 + Chitin	0.4 " " 0.2	7.8	7.9

* Control without supplement.
** Average values of duplicate experiments.

these experiments was limited by temperature, ethanol and sugars concentration. By selecting a suitable strain, tolerant of temperature, salts and ethanol, a better yield of ethanol in presence of fungal mycelium is possible from any type of molasses fermentation.

The slight variations observed in the amount of ethanol production in the presence of fungal mycelium from different species may be due to differences in their cell well composition (Barticki-Gracia,1968). The filtrate of autoclaved mycelium was shown to inhibit ethanol production. This may be due to the presence of fatty acids which might have been released during autoclaving. It has been reported that the unsaturated fatty acid (Hayashida et al,1976) components of proteolipids inhibit cell growth and thereby reduce ethanol production. Based on the above findings, it is proposed that the factors responsible for upgrading the fermentation reaction may be derived from the cell wall rather than from the cell contents.

It is recorded that A.oryzae-proteolipid is responsible for increasing the ethanol concentration and for fermentation concentrated wort in brewery yeast fermentation. A similar observation was noticed in this

work of cane molasses fermentation. The effect of Aspergillus species can be enhanced by coimmobilising [13] with Zymomonas mobilis for the production of ethanol from starch products (Tanaka et al,1986). The present data show that as many as ten different fungal species mycelium supplement, accelerate the ethanol production rate in cane molasses fermentation. Use of waste mycelium, having a negligible cost has accelerated the rate of ethanol production, has conferred stability on yeast in concentrated molasses and has increased the ethanol concentration under batch fermentation conditions. The waste mycelium of P. chrysogenum may carry a traces of antibiotic,which will help indirectly in the removal of bacterial contamination during molasses fermentation.

Application of the present findings to existing batch fermentation technology should considerable reduce the cost of ethanol production. Such developments are now in progress. The use of waste mycelium in the production of ethanol from waste molasses holds promise for the future.

---------- CHAPTER VI ----------

NOVEL SUPPLEMENTS ENHANCE THE ETHANOL PRODUCTION IN CANE MOLASSES FERMENTATION BY RECYCLING YEAST CELLS.

==========

6.1 INTRODUCTION

To improve the yield of ethanol, techniques like vacuum, rapid and continuous fermentation or immobilization of yeast cells have been widely used. The use of effective low cost supplements in conventional alcohol fermentation is a suitable approach to improve the yield of alcohol. In the present report an attempt has been made to assess the effect on ethanol production of skim milk (Patil et al., 1986), chitin (Patil and Patil, 1989), and fungal mycelium (Patil and Patil, 1989, communicated), individually or in combinated, using recycling yeast cell and the yeast culture S. cerevisiae NCIM 3526, in cane molasses fermentation.

6.2 MATERIALS AND METHODS

The yeast cultures S. cerevisiae NCIM 3281,3287,3524,3524,3525 and 3526 and S. uvarum NCIM 3455 and 3509 were obtained from National Collection of Industrial Microorganisms (NCIM), a section of this Laboratory and maintained on the slope of MGYP (malt extract 0.3 %, glucose 2 %, yeast extract 0.3 % peptone 0.5 % and agar 2.0 %). The fermentable sugars were adjusted to 5 or 15 % with water and used after steaming for half an hour and

solids were removed by decantation. The yeast cell mass pregrown in MMYP inoculum medium (molasses sugars 5 %,malt extract 0.3 %, yeast extract 0.3 % and peptone 0.5 %)for 24 h on the shaker (150 RPM) at 30°C (pH 5.5) was harvested and washed with water on centrifugation. The inoculum of cultures (wet wt 3.0 g/100ml) was transferred to fermentation medium containing fermentable sugars 15 %, urea 0.25 % and $MgSO_4.7H_2O$, 0.05 %. The supplements were added separately or in combinations to the fermentation medium in the range of 0.2 to 0.4 %. All these media were steam sterilized by autoclaving at 121°C for 15 min.

6.3 RESULTS

DETERMINATION OF SEDIMENTATION RATE OF YEAST CULTURES

In order to select a better flocculent yeast, eight alcohol producing yeast strains were screened for their sedimentation rate. The cultures were grown in MGYP medium for 24 h and on harvest were taken for measuring the decrease in Klett reading of standard cell suspension in 0.9% sodium chloride over a period of 5 h (at 650 nm). The percentage of sedimentation rate was expressed by the

following formula:

$$\% \text{ SR} = \frac{\text{Total drop in Klett reading X 100}}{\text{Klett reading at 0 hour.}}$$

From such observations, culture 3526 shows the highest degree of sedimentation rate (96.2 % at 5 h) while 3281 exhibits the lowest rate (82.8 % at 5 h).

RECYCLING OF YEAST CELL IN PRESENCE OF DIFFERENT SUPPLEMENTS AND THEIR COMBINATIONS IN CANE MOLASSES FERMENTATION

Fermentation experiments were carried using strain 3526, as a recycling yeast strain with addition of different supplements. A sufficient inocula of flocculent strain NCIM 3526 was prepared in inoculum medium (MMYP) by growing it is the usual manner. The cells were harvested and washed with water and then 3g wet wt./100 ml of cells mass was transferred to the seven sets of cane molasses fermentation medium. These sets were made up of (A) fermentable sugars 15% + urea 0.25% + $MgSO_4$ $7H_2O$, 0.05%; (B) medium A + skim milk 0.2%; (C) medium A + chitin 0.2%; (D) medium A + fungal mycelium 0.4%; (E) medium A + skim milk 0.2% + chitin 0.2%; (F) medium A + skim milk 0.2% + fungal mycelium 0.4; (G)

Table 1:

DETERMINATION OF SEDIMENTATION RATE OF YEAST CULTURES

Growth medium: MGYP, Temperature: 30°C, Klett reading at 650 nm, at periodic hour

Strains	Klett reading at hours 0	1	2	3	4	5	Drop in reading at 5 h	% SR*
3281	525	450	390	300	140	90	435	82.8
3287	530	430	350	130	80	35	495	93.3
3455	530	390	125	70	70	30	495	93.3
3509	530	320	195	75	45	30	500	94.3
3523	523	440	400	340	170	75	450	85.7
3524	520	430	375	265	115	70	450	86.5
3525	535	410	225	85	45	40	495	92.5
3526	530	310	185	65	409	40	510	96.2

Average values of duplicate experiments. S.cerevisiae NCIM 3281, 3523, 2534, 3525 and 3526. S. cerevisiae NCIM 3455 and 3509

SR * = Sedimentation Rate.

medium A + chitin 0.2% + fungal mycelium 0.4%. The fermentation flasks were incubated in duplicate at 30°C. The fermented mash was removed every 16 h by decantation, leaving behind sedimented cells and then refilled with respective fresh medium to the same volume cells and then (100 ml) of fermentation. These flasks closed with cotton plug were gently shaken for mixing cells with the fermentation medium and again left at the same temperature. The process was repeated for ten cycles and each time ethanol was estimated from the fermented mash. After the 10th cycle, the cell mass was determined to judge the losses in the fermented mash.

Table 2 depicts the effect of various supplements on the rate of ethanol production in cane molasses fermentation by recycling yeast cell. It is clear that more ethanol is formed in presence of supplements like skim milk (58.8g), chitin (60.8g) fungal mycelium (60.1g) or their combinations (63.2-65.3g) as compared to control without supplements (50.1g) from 150g of fermentable sugars. From the values, it can be seen that the improved rate of ethanol production is approximately constant in the presence of supplements, throughout the ten cycles. It is further observed that

TABLE 2 : RECYCLING OF YEAST CELL IN PRESENCE OF DIFFERENT SUPPLEMENTS AND THEIR COMBINATIONS

Cane molasses fermentable sugars 15% + urea 0.25% + $MgSO_4.7H_2O$ 0.05%

Culture inoculated S. cerevisiae NCIM 3526, Inocula 3 g wet wt./100 ml

Fermentation volume 100 ml, pH 5.0; Temperature 30°C ; Fermentation duration 16 h

Supplement added %	CYCLES 1	2	3	4	5	6	7	8	9	10	Total ethanol %	Increase %	Efficiency %	*Cell mass mg
	Ethanol estimated % (w/v)													
CT	4.3	5.5	5.3	5.4	5.5	5.1	5.5	4.2	4.5	4.8	50.1	-	66.8	680
SK	4.6	6.0	6.1	6.2	6.1	6.2	6.2	6.3	5.5	5.6	58.8	17.3	78.4	560
CH	5.5	5.8	6.2	6.3	6.4	6.3	6.2	6.1	5.5	6.3	60.6	20.9	80.8	470
FM	5.3	6.0	6.3	6.1	6.5	6.2	6.1	5.5	5.6	6.5	60.1	20.0	80.1	440
SK + CH	5.5	6.0	6.1	7.0	6.9	6.7	6.8	5.9	6.0	6.3	63.2	26.1	84.2	490
SK + FM	5.4	6.3	6.9	7.0	7.1	6.7	6.6	6.2	6.3	6.4	65.0	29.7	86.6	470
CH + FM	5.5	6.3	6.9	7.2	7.3	6.8	6.9	5.9	6.3	6.2	65.3	30.3	87.0	425

Average values of duplicate experiments

CT - Control; SK - Skim milk, 0.2%; CH - Chitin, 0.2%; FM - Fungal mycelium, 0.4%

*Cell mass - Wet wt. from 10th fermented mash

individual supplements show 17-20% and in combination 26-30% more ethanol, moreover the efficiency of ethanol production is round 84-87%, as against 66% in the absence of supplements. The cell mass data indicates that supplemented fermented mashes have generated slightly less cell mass as compared to the controls.

6.4 DISCUSSION

We have earlier reported the usefulness of novel supplements such as skim milk, chitin and fungal mycelium in accelerating the ethanol formation in molasses batch fermentation. Alcohol fermentation, using recycling yeast cell, has a number of advantages over batch fermentation and in fact is closer to the standard industrial procedure used in many new installations. We, therefore, wanted to know whether the effects of supplements reported in batch fermentation were also observed in the recycling yeast cell fermentation. From our data summarized in Table 2, it is clear that both the yield and efficiency of ethanol formation are very substantially improved. The exact role of these supplements in enhancing the rate of ethanol fermentation is not clear at this stage . We have earlier reported skim milk and chitin may provide specific

nutritional requirements to yeast cell and thereby increase the yield of ethanol. Fungal mycelium on the other hand may be a good source of proteolipid reported by Hayashida et al. (1976), responsible to increase ethanol yield. The supplements used are very cheap and fungal mycelium in particular is a waste material. The use of such supplements, therefore, not only reduces the cost of ethanol production but also has a direct application to existing fermentation technology. Experiments are in progress to scale up the procedure and then to implement it at the Industrial level.

CHAPTER VII

TOP AND BOTTOM YEASTS TOGETHER ACCELERATE ETHANOL PRODUCTION IN MOLASSES FERMENTATION.

7.1 INTRODUCTION

In the previous chapter, top and bottom yeasts have been characterized with respect to their sedimentation rate. In the present work, an attempt has been made to use top yeast and bottom yeast together to assess their effects on ethanol production during batch and recycling yeast cell fermentation. The top yeast S. cerevisiae var. ellipsoideus NCIM 3281 and bottom S. uvarum NCIM 3509 jointly showed an increase rate of ethanol production by 32.6% in batch and 25.2% in recycling yeast cell fermentation, as compared to their mean value of individual ethanol production level at 30°C. The use of top and bottom yeast together in molasses fermentation, to our knowledge, is the first report.

7.2 MATERIALS AND METHODS

The yeast culture S. cerevisiae, var. ellipsoideus NCIM 3281, a wine producer, with a poor sedimentation rate (82.8% at 5 h) is considered a top strain and S. uvarum, NCIM 3509, distillery strain having better sedimentation rate (94.5 % at 5 h) considered a bottom strain (Patil et al., 1988). The inoculum was prepared in growth medium'

MMYP, which contained malt extract 0.3 % molasses sugars 5%, yeast extract 0.3 % and peptone 0.5 %. Fermentation medium was made up of fermentation molasses sugars 15% or 20% with urea 0.25 % and $MgSO_4 . 7H_2O$ 0.05 %. All these media were sterilized at 15 lb. in^{-2} for 20 min.

7.3 RESULTS

BATCH FERMENTATION WITH TOP AND BOTTOM YEASTS

Batch fermentation experiments were conducted by inoculating top and bottom yeast separately and together using cane molasses sugars at 30 ^{o}C, to evaluate ethanol production rate. Sufficient inocula of top 3281 and bottom 3509 of individual cultures were prepared in growth medium MMYP under normal conditions. The required inocula V/V (approximately 400 mg/ 100 ml) were used directly. While cell mass inocula were collected by harvesting on centrifugation. Two sets of triplicate flasks of fermentation media were prepared, set (A) total molasses reducing sugars 15% (W/V) with urea and magnesium sulfate and set (B) total molasses reducing sugars 20% (W/V) with urea and magnesium sulfate. The set (A) flasks one and two had separate inoculum of 10% (V/V) of top and bottom yeasts

respectively while the third flask had inoculum of 5% (V/V) of each.To judge the ethanol productivity, two flasks of set (B) were inoculated with wet weight, 1 g/100 ml of individual top and bottom yeast and third flask with half the inocula of each. All these sets, in duplicate, were incubated at 30°C and samples for ethanol detection were removed after 48 h from set A and 24 h from set B.

It is observed from Table 1 (set A), that a combination of top and bottom yeast increases the ethanol yield by 32.6 % as compared to the mean value of ethanol production obtained from the two cultures separately. The value of ethanol production of 6.1% (W/V), further indicates that it is nearly completed and efficiency has reached to 90% by 48 h, in presence of these two mixed cultures. In set B, 8.6 % (W/V) ethanol is produced within 24 h when 0.5 g wet wt. of these yeasts were used together as compared to individual mean production around 6.7% (W/V). The yield was increased by 27.4%, from 20% molasses sugars and fermentation was found to be nearly completed.

RATE OF ETHANOL PRODUCTION BY TOP AND BOTTOM YEAST

COMBINATION

Two strains 3281 and 3509 were employed in total molasses sugars 20% with malt extract 0.3%, peptone 0.5% and yeast extract 0.3% (pH 5.0), and fermentation was run at 30°C. Sufficient inocula of both cultures were prepared in the inoculum medium MMYP. These cultures were inoculated individually using 10 % (V/V) and half of the inocula of each as combination to fermentation medium. These sets were fermented stationary at 30°C and samples (5 ml) were removed for ethanol estimation every 12 h.

Table 2 and Figure 1 indicate that the bottom yeast has a better ethanol production activity as compared to top yeast. The rate of ethanol production is not only improved but is found to increase in a linear way throughout the fermentation period, in presence of combined inocula as compared to their indivdual ethanol production activity. hese data support the effect of binary cultures in accelerating the rate of ethanol production.

TOP AND BOTTOM YEASTS IN RECYCLING YEAST CELL FERMENTATION

Top and bottom yeasts were again used in recycling

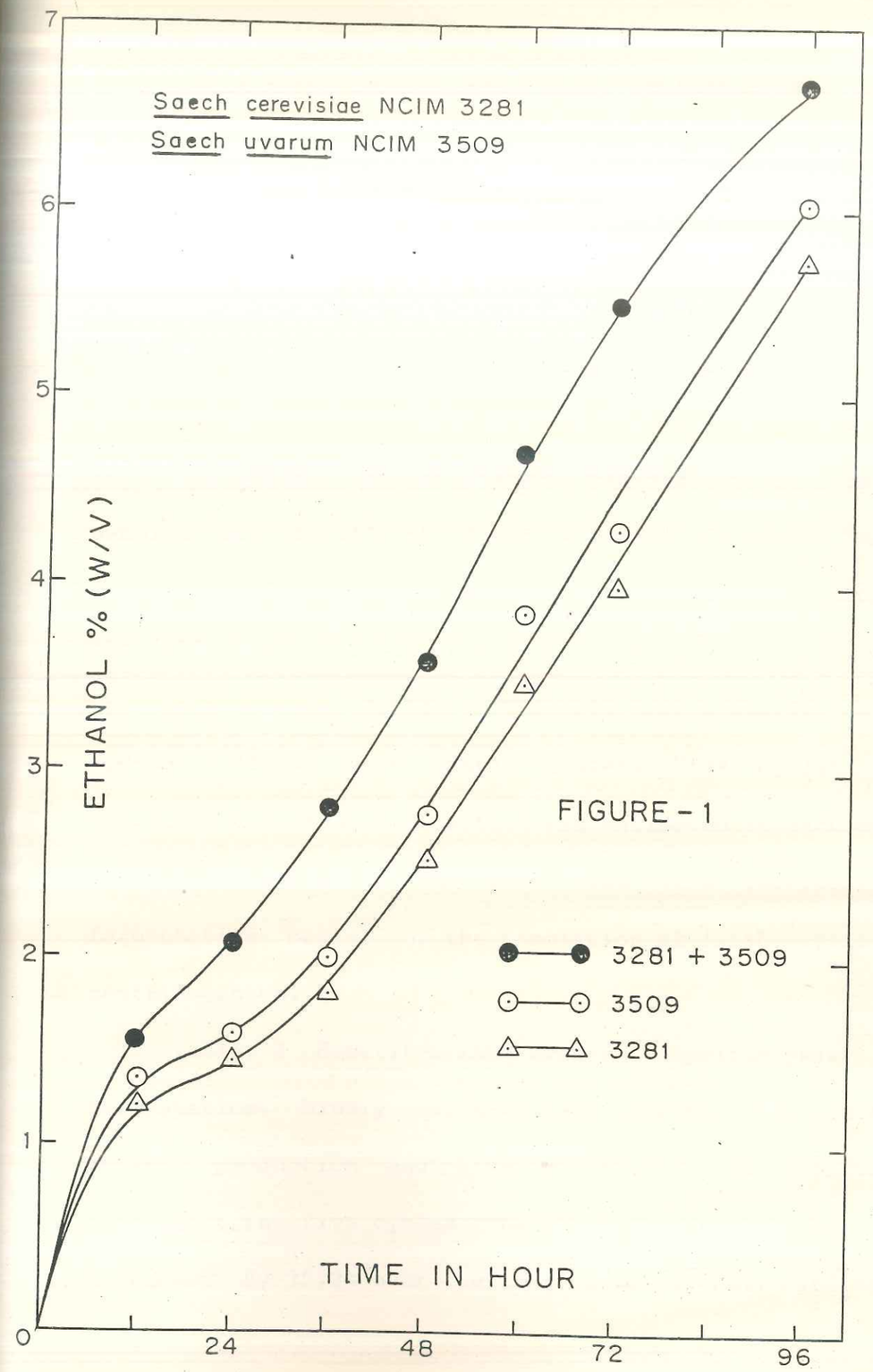

Saech cerevisiae NCIM 3281
Saech uvarum NCIM 3509
ETHANOL % (W/V)
TIME IN HOUR
0
1
2
3
4
5
6
7
24
48
72
96
3281 + 3509
3509
3281

FIGURE - 1

yeast cell fermentation. The two yeasts were grown in the growth medium MMYP and cells were harvested by centrifugation. The wet wt. inocula of top and bottom strains were inoculated separately and in combination to fermentation medium (100 ml in 150 ml conical flask). The fermentation medium consisted of fermentable sugar 15 % with urea and magnesium sulfate. All the flasks were incubated at 30°C and ethanol estimations were carried out by removing samples periodically at 12 and 24 h. The fermented mashes were removed every 24 h by decantation without disturbing the cell mass and fresh fermentation media were refilled to the same volume. To mix up the cell mass properly in the medium, flasks were gently shaken. Five cycles were completed and ethanol formation was estimated at each cycle. The wet weights of the cell mass were recorded from fermentation media, on the completion of fifth cycle, on centrifugation.

Table 3 describes that even in recycling yeast cell fermentation, binary cultures have improved the rate of ethanol production and have maintained the improvement throughout the five cycles. The ethanol production has been increased by 25.2% over the mean value of their individual

TABLE 3 : TOP AND BOTTOM YEASTS IN RECYCLING OF YEAST CELL FERMENTATION

Fermentable molasses sugars 15% + urea 0.25% + $MgSO_4.7H_2O$ 0.05%

Culture inoculated S. cerevisiae NCIM 3281 and S. uvarum NCIM 3509

Temperature 30°C, Fermentation Volume 100 ml., pH 5.0

Sr. No.	Culture used	Inocula Wet wt. %	Ethanol estimated % (W/V) I 12	I 24	II 12	II 24	III 12	III 24	IV 12	IV 24	IV 12	IV 24	% Increase@	Cell mass* g
1.	3281	1	3.5	4.8	4.0	5.8	4.2	6.0	4.7	6.2	4.8	6.3	-	4.85
2.	3509	1	3.7	5.2	4.2	6.1	4.7	6.1	5.0	6.3	5.1	6.4	-	5.45
3.	3251 + 3509	0.5 + 0.5	4.2	6.1	5.5	6.4	6.1	6.5	6.2	6.6	6.2	6.6	25.2	7.00
4.	3281	2	4.7	5.3	5.0	6.1	5.3	6.2	6.1	6.6	6.0	6.3	-	6.85
5.	3509	2	4.5	5.5	5.3	6.1	5.4	6.4	5.4	6.4	6.1	6.5	-	6.7
6.	3281 + 3509	1 + 1	5.7	6.4	5.7	6.5	6.3	6.8	5.5	6.4	6.4	6.6	5.8	8.65

Average values of duplicate experiments

@ % increase = percent increment at 12 h of fifth cycle

* Cell mass = Cell Mass data from fermentation medium of fifth cycle

ethanol production level, estimated at the middle stage of fermentations, (12 h) of the fifth cycle. The table further indicates that wet weight of binary cultures (0.5% each) was sufficient to a fermentable sugar of 15%. Using such ratios, the fermentation nearly got completed around 12 h from the third cycle onward. It is also noticed that cell mass has increased with binary inocula as compared to individual inocula in their respective fermentation media.

7.4 DISCUSSION

Our experiments have clearly shown that top and bottom yeast together have improved the rate of ethanol production in both fermentations of batch and recycling yeast cell. The binary cultures have not only improved the rate of ethanol formation but have also maintained it in the recycling yeast cell fermentation. Ruding and Hough, (1959) have employed a series of binary mixtures of selected yeast (top and bottom) in continuous beer fermentation but failed to produce promising results. Since then, few attempts were made to exploit the potentiality of top and bottom yeast, mixture of known characteristic cultures in cane molasses fermentation. The binary cultures comprising a top yeast (wine producer) and bottom yeast (distillery strain)

together appear to be more effective in converting molasses sugars to ethanol in fermentation media and show thereby an accelerated rate of ethanol production. The fermentation flasks with combined inocula showed greater cell mass and this might be another contribution to the improvement. The top yeast S. cerevisiae var. ellipsoideus NCIM 3281 and bottom S. uvarum NCIM 3509 jointly showed an increased rate of ethanol production by 32.6% in batch and 25.2% in recycling yeast cell fermentation as compared to thier mean value of individual ethanol production level at 30° C. These findings have several important implications. For example, the rate of ethanol production is markedly improved without any specific supplements. These will have a direct application to the existing and traditional fermentation technology or to various new technology of fermentations. Finally it will reduce the cost of ethanol production by saving fermentation time and labour. Attempts are being made to scale up the experiments to industrial level.

CHAPTER VIII

PRODUCTION OF YEAST CELLS AND INOCULA REQUIREMENT IN ALCOHOL BATCH FERMENTATION.

8.1 INTRODUCTION

The production of yeast cells, a basic need for various purposes and in yeast fermentation is carried out by using various media, reported by a number of worker (Sharma et al, 1980, Varma et al, 1983 and Novak et al, 1981). However, no comparative account of these media is available. In the present work, 12 sets of media were prepared and were used for the production of cell mass. A comparative account of these media helped us to select a cheaper medium for this purpose. For example, the medium consisting of molasses sugars, urea, magnesium sulfate and yeast extract produced 3.5 % (wet wt) yeast cells while the other media consisting of molasses sugars, peptone and extracts of malt and yeast produced 3.85 % (wet/wt) of yeast cells.

Different inocula sizes (Strehaiano et al, 1983 and Vega et al, 1987) have been used by research workers for ethanol production as well as for the production of yeast cells. Different sizes of inocula are also used to study the inhibitory effects of added ethanol or produced ethanol and it has been shown that increase in inocula size results in the decrease of inhibition. Inocula sizes ranging from 0.05 to 60 % (V/V) were used to study the fermentation activity

by Strehaiano. A number of factors like type of substrates and their percentage, salts' concentration, pH, additives and temperature are involved in the search of the exact requirement of inoculum. The inoculum requirement in yeast fermentation has not been defined and has also not been fully studied. Considering these requirements, the experimental conditions were formulated like total molasses reducing sugars 15 % (around 13.5 % fermentable sugars are normally used by industries), temperature 30^{o}C, pH 5.5 and fermentation period 24 h in cane molasses batch fermentation, with and without supplements.

In the present work, besides the production of yeast cells, attempt has been made to find out the exact requirement of inoculum size in cane molasses fermentation. Inocula of yeast in the range of 1 to 6 % wet wt were used in the fermentation medium containing total molasses reducing sugars (15 %). It was observed that 3 % and 2 % wet wt inocula brought out the completion of fermentation activity in absence and in presence of additives, respectively, around 24 h at 30^{o}C. The cheaper medium used for yeast production and minimum inocula requirement for alcohol production, reported here may reduce the cost of

ethanol production.

8.2 MATERIALS AND METHODS

Molasses was obtained from a local factory; and its clarification and dilution was carried according to the methods described earlier. The required inoculum for the production of yeast cells was prepared in the growth medium (A) Glucose 4.0 %, KH_2PO_4 0.5 %,$(NH_4)_2SO_4$ 0.2 %, $MgSO_4.7H_2O$ 0.04 %; and yeast extract 0.1 %. The inoculum required for alcohol fermentation was prepared in seed medium MUMY (B) molasses sugars 5 %, urea 0.25 %, $MgSO_4$. 7 H_2 O 0.05 % and yeast extract 0.2 %. All the media were steam sterilized at 15lb./in^{-2} (1.027 bar) for 20 min.

8.3 RESULTS

PRODUCTION OF YEAST CELLS IN VARIOUS MEDIA

The culture Sacch. uvarum NCIM 3509 was used for the production of yeast cells from various formulated media. This culture was grown initially by collecting a loop from the slope to the growth medium (A) and sufficient inocula was built up at 30^{o}C by keeping on shaker (150 RPM) for 24 h. The homogeneous inocula of 10 % (V/V) were transferred to

the media having total molasses reducing sugars in the range of 2 to 6 % with urea 0.25 %, magnesium sulfate 0.05 % and yeast extract 0.2 %. Likewise other seven sets of media with constant total molasses sugars (5 %) and compounded with various salts' percentages or biological ingredients, as indicated in Table 1, were inoculated. The duplicate sets of these media (total production media 100 ml in 500 ml conical flasks) were kept on shaker (150 RPM) at 30°C for 24 h. The yeast cells were harvested and washed with water on centrifugation and their average wet weights were recorded.

Table 1 indicates that total molasses reducing sugars (5 %) are adequate for optimum yeast cells production (3.5% wet wt) in the first five sets.The highest yeast cells production of (3.85 %) is from the medium No.8 where biological ingredients have been compounded. in the presence of skim milk, the yield of yeast cells reaches to 3.7 %. The other media containing inorganic nitrogen source and salts have produced yeast cells in the range of 3.25 % to 3.5 %. The medium MUMY (medium No.4), formulated with salts like urea, magnesium sulfate and yeast extract, gives a yield of 3.5 %, which is found to be superior as compared to the other sets of sugars, but is lower by 10 % as compared to

Table 1 PRODUCTION OF YEAST CELLS FROM VARIOUS MEDIA

Culture added Sacch. uvarum NCIM 3509. Temperature 30° C. pH 5.5
Inoculum used 10 % (V/V) , Total production medium - 100 ml

No. of Media	1	2	3	4	5	6	7	8	9	10	11	12
(g/100 ml)												
TMRS	2	3	4	5	6	5	5	5	5	5	5	5
Urea	0.25	0.25	0.25	0.25	0.25	0.25	0.25	-	-	0.3	0.5	-
$MgSO_4.7H_2O$	0.05	0.05	0.05	0.05	0.05	0.05	0.05	-	-	-	-	0.04
NaH_2PO_4	-	-	-	-	-	-	-	-	-	0.15	-	-
KH_2PO_4	-	-	-	-	-	-	-	-	-	-	-	0.5
$(NH_4)_2SO_4$	-	-	-	-	-	-	-	-	-	-	-	0.2
YE	0.2	0.2	0.2	0.2	0.2	-	-	0.3	-	-	-	0.1
SK	-	-	-	-	-	-	0.2	-	0.3	-	-	-
ME	-	-	-	-	-	-	-	0.3	0.3	-	-	-
PT	-	-	-	-	-	-	-	0.5	0.5	-	-	-
Wet Wt. of Yeast cells g/100 ml	2.35	2.55	2.85	3.50	3.35	3.25	3.35	3.85	3.70	3.25	3.40	3.45

Average values of duplicate experiments. TMRS : Total Molasses Reducing Sugars
YE : Yeast Extract, SK : Skim Milk, ME : Malt Extract and PT : Peptone

the medium with biological ingredients.

INOCULA REQUIREMENT OF YEAST CELLS IN BATCH FERMENTATION

The inoculum of Sacch. uvarum NCIM 3509 inoculum was built up by growing it in the molasses medium MUMY and cells were collected on centrifugation. The wet weight inocula in the range of 1 to 6 % (approximately 1 g wet wt equivalent to 260 -270 mg dry wt) were transferred to fermentation medium consisting of total molasses reducing sugars 15 % + urea 0.25 % + $MgSO_4.7H_2O$ 0.05 %. The duplicate inoculated flasks (total volume 100 ml in 150 ml conical flask with cotton plug), after proper mixing of the inocula, were left stationary at $30^{o}C$. The samples (5 ml) were removed at 12 h and 24 h for ethanol estimation.

It is observed from Table 2 that stepwise increment of inocula size increases the yield of ethanol as detected at the middle stage of fermentation (12 h). At 6 % inocula size,the yield of ethanol is recorded maximum (6.2 %) having 6 % inoculum size. The yield of ethanol after 24 h of fermentation is in the range of 6.3 % - 6.6 % with inocula size of 3 % and onward with the fermentation

Table 2.

INOCULA REQUIREMENT OF YEAST CELLS IN BATCH FERMENTATION

Culture added - S. uvarum NCIM 3509.

Total Molasses Reducing Sugars 15 % + Urea 0.25 % + $MgSO_4 . 7H_2O$ 0.05 %.

Fermentation volume 100 ml pH 5.0; Temperature 30°C.

Sl. No.	Inocula g/100 ml (wet wt)	ETHANOL % (W/V) 12 h	24 h	Increament in inocula percentage	Increament in ethanol percentage
1.	1	2.9	5.1	-	-
2.	2	3.9	5.7	100	13.7
3.	3	4.8	6.3	50	6.9
4.	4	5.2	6.5	33.3	3.1
5.	5	5.3	6.6	25	-
6.	6	6.2	6.6	20	-

Average values of duplicate experiments.

activity reaching towards its completion. By doubling the inocula size (1 % to 2 %), the yield of ethanol is improved by 13.7% (5.1 to 5.8 %) while further increasing it by 50 % (2 to 3%) the ethanol yield is more by 6.9 % (5.8 to 6.3 %). With a further increment of inocula size by 33.3 % (3% to 4%), the yield of ethanol is raised by 3.1 % (6.3 to 6.5 % W/V). The ethanol increment with further higher sizes of inocula is found to be negligible, as it reaches the completion stage of fermentation, at 24 h at 30° C from 15% total molasses reducing sugars.

INOCULA REQUIREMENT OF YEAST CELLS IN PRESENCE OF SUPPLEMENTS IN BATCH FERMENTATION

The cells of Sacch.uvarum NCIM 3509 were harvested as in the earlier experiments and 1 % to 2 % wet wt were inoculated to total 100 ml fermentation medium in 150 ml conical flasks. The sets of these fermentation medium were composed of:

(1) total molasses reducing sugars 15 % + urea 0.25% + $MgSO_4.7H_2O$ 0.05 % as control

(2) same as 1 + yeast extract 0.2 % inoculum control

(3) same as 1 + skim milk (powder) 0.2 %

(4) same as 1 + chitin 0.2 %

(5) same as 1 + fungal mycelium 0.4 %.

All these sets of fermentation flasks were left stationary at 30°C and 5.0 ml samples were removed for ethanol detection after 5 h, 20 h and 25 h.

It can be seen from Table 3 that the rate of ethanol formation is improved in the flasks with 2 % inocula of yeast cells as compared to the flasks with 1% inocula in all the five sets of fermentation. The yeast extract supplemented fermentation medium has shown a slightly lower production of ethanol as compared to the control. The ethanol formation rate is improved in all the flasks having supplements like skim milk, chitin and fungal mycelium, as compared to the respective inocula size without supplements. It is further noticed that the ethanol formation is complete in those flasks having 2 % inocula and supplements like skim milk, chitin and fungal mycelium , as compared to the control flasks without supplements.

Table 3

INOCULA REQUIREMENT OF YEAST CELLS IN PRESENCE OF SUPPLEMENTS IN BATCH FERMENTATION.

Culture added S. uvarum NCIM 3509

Total Molasses reducing sugars 15 % + Urea 0.25 % + $MgSO_4 . 7H_2O$ 0.05 %

Fermentation volume 100 ml, pH 5.0, Temperature 30°C

Sl. No.	Inocula g/100 ml (wet wt)	Supplements *	% added	ETHANOL % (W/V) at 10 h	20 h	25 h
1.	1	-	-	2.20	4.30	5.20
2.	2	-	-	3.00	4.85	5.80
3	1	YE	0.2	2.35	4.20	4.85
4.	2	YE	0.2	3.90	4.65	5.45
5.	1	SK	0.2	2.75	4.75	5.40
6.	2	SK	0.2	4.15	5.30	6.20
7.	1	CH	0.2	3.30	5.60	6.20
8.	2	CH	0.2	4.50	5.65	6.60
9.	1	FM	0.4	3.35	5.70	6.40
10.	2	FM	0.4	4.30	5.75	6.55

Average values of duplicate experiments. * YE : Yeast Extract; SK : Skim Milk; CH: chitin; FM : Waste Fungal Mycelium.

8.4 DISCUSSIONS

It is observed from our experiments that the medium MUMY containing molasses sugars 5 % with urea, magnesium sulfate and yeast extract produces 3.5 % (wet wt) yeast cells. Normally molasses sugars contains elements like sodium, calcium, potassium, zinc, iron, magnesium, copper and traces of phosphate and important vitamins like biotin which are essential for the growth of microorganisms like yeast and hence molasses sugars are better for yeast cells production. The yeast cells production is 3.85 % with the medium having nutrients like malt extract, yeast extract and peptone in molasses sugars which is higher by 10 % as compared to the MUMY medium. However, the cost of this medium seems to be more by 70 %. Hence, it is worth to use the medium MUMY for the production of yeast cells, avoiding costlier ingredients like peptone and extract of yeast and malt.

Our experiments have shown that 3 % wet wt inocula of yeast is fairly sufficient to complete the fermentation activity of 15 % total molasses reducing sugars using S. uvarum NCIM3509 in the absence of supplement, while 2 % wet wt inocula is adequate in presence of supplements like skim

milk, chitin or waste fungal mycelium. Normally 15 % molasses sugars are used by distilleries to recover around 6.0 - 6.5 % (W/V) ethanol.

The pitching of heavy inocula from the earlier fermentation batch is practiced by a few industries to suppress the contamination and to get quick production of ethanol. Using such method, the risk of transferring a bulk quantity of wild yeast growth may not be ruled out. By this procedure, ethanol production rate is increased the initial stage, but these may not be a real requirement and the addition of extra yeast cells will be a waste. The extra yeast cells will utilize sugars for their own growth and hence indirectly there is a loss in the production of ethanol. It is observed under fermentation or anaerobic condition that yeast cell mass shows increment which will complete the total fermentation activity at the holding time of mash earlier to distillation. The use of fresh inocula of limited yeast cells to the batch fermentation will be more suitable to get the expected rate of ethanol production.

From our experiments, it is clear that MUMY is a simple and cheap medium which has produced a satisfactory yield of yeast cells and wet wt inoculum of 2 % are sufficient in

presence of supplements like chitin, skim milk or fungal mycelium, to complete the fermentation activity around 24 h at 30°C. All these findings have a number of applications

(1) Simple and cheaper medium can be used for the production of yeast cells.

(2) Saving of yeast cells by keeping a limit on the use of inocula size.

(3) Applying fresh inocula, having a better viability to keep desirable fermentation activity is possible.

(4) Indirectly,the cost of ethanol production is markedly reduced.

(5) Fermentation time is reduced.

We propose to undertake to scale up, using our present data in order to assess its commercial potential in alcohol industry.

ACHIEVEMENTS

1. Total 35 yeast strains were isolated from molasses pits to have better activity of ethanol production using concentrated molasses sugars and at elevated temperature. Two strains identified as Sacch. cerevisiae have shown the desired activity of ethanol production at the expected conditions.

2. A series of novel and new additives were studied to increase the rate and productivity of ethanol in cane molasses batch fermentation. Out of these supplements skim milk-powder, chitin and waste mycelium or their combination have markedly improved the rate of ethanol production, efficiency; and have drastically reduced the alcoholic fermentation period.

3. Unconventional methods were attempted to improve the yield of ethanol by employing different categories of yeasts into fermentation process. A combination of binary cultures like top and bottom yeast was used to accelerate the rate of ethanol formation. This binary culture has accelerated the rate of ethanol without any additives in cane molasses batch fermentation.

4. A search was carried out for cheaper production of yeast cells and minimum inocula size required in alcoholic fermentation. A fairly sufficient yeast cells were collected from a cheaper medium `MUMY', which contained molasses sugars, urea, magnesium sulfate and yeast extract. Likewise requirement of minimum but sufficient inoculum size has been worked out that will complete the fermentation activity within an expected period in molasses batch fermentation.

APPLICATIONS

1. The waste materials like chitin or fungal mycelium can be used as powerful catalysts to accelerate the rate and productivity of ethanol in alcoholic fermentation.

2. The novel additives can be used directly in batch or yeast recycle alcoholic fermentation to speed up the ethanol productivity.

3. The supplements can be applied directly to the traditional batch-fermentation technology and there will be no need for any modification to existing fermentors.

4. The fermentation period has been drastically reduced and hence more batches of fermentation can be operated.

5. The use of chitin or fungal mycelium will result in saving of labour, power and inocula size in alcoholic fermentation. Similarly use of a cheaper medium for yeast cells production, will reduce considerably the cost of ethanol production.

6. A new small scale industry may emerge for the utilization of waste materials like chitin or waste mycelium from antibiotic plants and thereby a problem of waste disposal can be solved indirectly.

7. The novel procedure of employing costless binary cultures in alcoholic fermentation, has accelerated the rate of ethanol production and fermentation period has been reduced to two-third.

8. Two promising yeast strains (Sacch. cerevisiae) have been made available through authentic culture collections (ATCC, NCYC OR NCIM) for industrial application.

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