DEVELOPMENT OF OSMOTOLERANT YEAST FOR ETHANOL PRODUCTION FROM MOLASSES

Thesis

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

MASTER OF SCIENCE
in
MICROBIOLOGY
(Minor Subject : Biochemistry)

By

Mandeep Kaur
(L-99-BS-191-M)

Department of Microbiology
College of Basic Sciences & Humanities
PUNJAB AGRICULTURAL UNIVERSITY
LUDHIANA 141 004 INDIA
2001

DEVELOPMENT OF OSMOTOLERANT YEAST FOR ETHANOL PRODUCTION FROM MOLASSES

Thesis

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

MASTER OF SCIENCE
in
MICROBIOLOGY
(Minor Subject : Biochemistry)

By

Mandeep Kaur
(L-99-BS-191-M)

Department of Microbiology
College of Basic Sciences & Humanities
PUNJAB AGRICULTURAL UNIVERSITY
LUDHIANA 141 004 INDIA

2001

CERTIFICATE - I

This is to certify that the thesis entitled, **"Development of osmotolerant yeast for ethanol production from molasses"** submitted for the degree of Master of Science in the subject of Microbiology (Minor subject : Biochemistry) of Punjab Agricultural University, Ludhiana, is a bonafide research carried out by Mandeep Kaur (L-99-BS-191-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

12/10/2001

Major Advisor
(Dr. Gurvinder Singh)
Assistant Microbiologist
Department of Microbiology
Punjab Agricultural University
Ludhiana

CERTIFICATE - II

This is to certify that the thesis entitled, **"Development of osmotolerant yeast for ethanol production from molasses"** submitted by Mandeep Kaur (L-99-BS-191-M) to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of Master of Science in the subject Microbiology (Minor subject : Biochemistry) has been approved by the student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

19/11/2001

Major Advisor
(Dr. Gurvinder Singh)

19/11/2001

External Examiner

Dr. Sunil Pabbi
Senior Scientist
Division of Microbiology
IARI, New Delhi

Head of the Department
(Dr. H.K. Tiwari)

K-S-Sek 27.11.01

Dean, Postgraduate Studies
(Dr. K. S. Sekhon)

ACKNOWLEDGEMENT

It is all by the grace of Almighty God whose blessings furnished the inspiration for undertaking this endeavour.

It gives me pleasure and satisfaction to categorically state that this investigation is not a solo effort. It is very difficult to individualize my gratefulness, here, to all whose contribution has blossomed into this present work still my first gratitude and thanks after God exist for those who has given me the capacity to investigate and complete the present study i.e. my teachers and guides.

I express my sincere gratitude and indebtness to my Major Advisor Dr. Gurvinder Singh, Assistant Microbiologist, Department of Microbiology, Punjab Agricultural University, Ludhiana for his able guidance, direction and constant support in exploration of my present investigation.

I extend my sincere thanks to members of Advisory Committee - Dr. R.P. Gupta, Sr. Microbiologist, Dr. K.L. Kalra, Sr. Microbiologist and Dr. G. Soni, Sr. Biochemist for their valuable and helpful suggestions.

I pay my sublime obeisance to my affectionate parents for sacrificing their today for my better tomorrow and for showering countless blessings upon me. Ever radiant atmosphere, co-operation and affection rendered by my brother and sisters needs a special mention.

A very special thanks is due to my friends - Priya, Pooja, Shivika, Simple and Maninder for their moral support, ever-willing help and for always being there for me.

I am also thankful to Mrs. Mohinder Kaur, Lab attendant for her help during lab work.

I conclude this acknowledgement by thanking all friends, seniors and teachers for their honest participation in the investigation.

Mandeep Kaur.

(MANDEEP KAUR)

Title of the Thesis : Development of osmotolerant yeast for ethanol production from molasses

Name of the student and : Mandeep Kaur

Admission No. (L-99-BS-191-M)

Major Subject : Microbiology

Minor Subject : Biochemistry

Name and Designation of Major Advisor : Dr. Gurvinder Singh
Assistant Microbiologist

Degree to be Awarded : Master of Science

Year of award of Degree : 2001

Total Pages in Thesis : 64

Name of the University : Punjab Agricultural University,
Ludhiana - 141 004, Punjab, India

ABSTRACT

A fermenting strain of *S. cerevisiae* adapted to grow on 22.5% (w/v) glucose grew exponentially between 15-20% (w/v) glucose levels. The strain was tolerant to 10% (v/v) of added ethanol. In comparison, *Z. rouxii* utilized upto 22.5% (w/v) glucose and was tolerant to upto 12% (v/v) added ethanol. The adapted strain of *S. cerevisiae* fermented molasses diluted to 175 and 200 gl^{-1} fermentable sugars producing 62.5 gl^{-1} and 64.6 gl^{-1} ethanol in comparison to 45.5 gl^{-1} by *Z. rouxii* from molasses diluted to 300 gl^{-1} sugars. The fermentation of molasses optimized with respect to temperature, N-sources and inoculum size revealed a temperature of 30°C, 500 ppm of nitrogen in the form of urea as N-source at an inoculum size of 10^8 as optimum for fermentation. Soyabean meal significantly enhanced the fermentation rate as well as ethanol yield (64.7 g/l^{-1} ethanol in 36 h of fermentation). Under optimized conditions, adapted strain of *S. cerevisiae* produced 78.5 gl^{-1} ethanol as compared to 72.3 gl^{-1} of ethanol by unadapted strain, showing overall improvement of 6.2 gl^{-1} in ethanol yield by virtue of adaptation.

12/10/2001

Signature of Major Advisor

Mandeep Kaur

Signature of the Student

CONTENTS

Chapter - I

INTRODUCTION

Ethanol has been used as a component of alcoholic beverages since time immemorial and such beverages contain ethanol from 3-6 per cent in beer to as high as 40% (v/v) in distilled beverages. In addition, ethanol finds use in industry as an important chemical feed stock, a fuel supplement and gasoline extender. Ethanol production for fuel has become industrialized in a number of developing countries like Brazil, Philippines etc. Zimbabwe has an ethanol plant for fuel alcohol production that has been running for over a decade (Rosenchin and Hall 1991). During the past decade a steady progress has been made in developing new technologies for production of industrial alcohol in order to make it available in plenty at a low cost.

Alcohol is mainly produced by fermentation process employing yeast as fermenting organism. Besides alcohol producing industries, other industries like pharmaceuticals and bakeries are also dependent upon yeast and yeast products. Thus yeast is of paramount importance in industries. Among yeasts, *Saccharomyces* (an Ascomycetous yeast) is the best known and exploited as the members of *Saccharomyces* have been associated with man ever since he knowingly or unknowingly used these for the production of fermented beverages.

At present, most of the bioethanol is produced by batch fermentation although various innovative techniques such as continuous fermentation

(Hospodke 1968), rapid fermentation (Nagodawithana and Steinkraus 1976), fermentation of concentrated wort (Rose 1976), vacuum fermentation (Ramaligham and Finn 1977), vacuum fermentation with recycling (Cysewski and Wilke 1978) and cell recycling (Verma *et al* 1983) have been proposed from time to time in order to improve ethanol productivity. Bioethanol is produced mainly by fermentation of sugary solutions or hydrolyzed starch. In our country, sugarcane molasses which is a byproduct of sugarcane industry has been a substrate of choice for industrial production of ethanol. This is because India is the biggest sugarcane producing country and molasses are easily available in plenty at a low cost.

Because of increasing demand for ethanol, there is a need to develop yeast strains with capacity to produce high ethanol concentration. Efforts are also being made to improve fermentation technology, exploit alternate substrates for ethanol production, use of highly concentrated substrate and to develop improved industrial yeast strains for making ethanol in plenty at a low cost.

As far as fermenting yeast is concerned the ethanol production capacity, rate and tolerance are important features of an efficient distillers yeast. Use of fast and efficient yeast (Bertolini *et al* 1991) can substantially reduce the cost of production since it reduces both time required for fermentation as well as yield higher amounts of ethanol per unit time. Recently there has been interest in developing yeast strains that can ferment high gravity solutions. This is because the effect of osmotic pressure becomes pronounced at high sugar concentration which affects the growth and metabolism of yeast and consequently affects the rate of ethanol production (Thatipamala *et al* 1992). Therefore, fermentation of such solutions needs a sugar tolerant yeast that can withstand the high osmotic pressure of such solutions.

Very high gravity fermentation not only requires an efficient sugar tolerant or osmotolerant yeast but also an ethanol tolerant yeast for high alcohol yield. Alcohol production and tolerance in yeast will be a problem that the distilleries have to face for many years. Even if the efficient yeast strains are available, their performance is restricted by the rise in temperature during fermentation. Temperature of fermentation process is brought down by spraying cold water on fermentors which adds to the cost of fermentation process. Fermentation at high temperature using thermotolerant yeast has advantages such as facility of ethanol recovery and significant savings on operational costs of refrigeration in distilleries, thus decreasing the overall production cost.

Further, distilleries produce huge amount of wastewater which poses a threat to environment, thus improved techniques must be adopted to reduce the effluent volume. Use of osmotolerant and thermotolerant yeast contributes to reduction in effluent volume as they reduce the requirement of process water needed for dilution and cooling purposes during fermentation. Thus the osmotolerance, ethanol tolerance, temperature tolerance, addition of nutrients etc. are the parameters which need to be standardized to improve ethanol production.

Keeping in view the above problem of distillery, the present study has been carried out for developing an osmotolerant yeast by adaptation on concentrated molasses with objective of optimizing the fermentation process which may reduce the overall production cost as well as effluent volume.

Chapter - II

REVIEW OF LITERATURE

Ethanol production by yeast is an important industry as ethanol offers a wide range of use namely as a solvent, a basic raw material for many chemicals in pharmaceuticals and above all as an alcoholic beverage. Now a days, it's use in automobiles as an alternate fuel has attracted worldwide attention for it's production on a large scale while maintaining the economic status of a country. In present state of energy crises, efforts are being made to reduce the dependence upon non-renewable energy sources through development of domestic energy sources, one of which is fuel alcohol produced by fermentation of agricultural/agroindustrial wastes and byproducts. An efficient ethanol production requires four components : a fermentable carbohydrates, an efficient yeast strain, a few nutrients and simple cultural conditions. As India is one of the largest sugarcane producing countries, molasses, a byproduct of sugarcane industry available in plenty at cheap rate is mostly used as a raw material for fermentation (Sharma and Tauro 1986). In order to produce ethanol in large quantities at reasonable costs, the optimization of various physio-chemical parameters depending upon particular geographical situation is very important. Besides, a number of factors like high temperature, low ethanol and sugar tolerance of the yeast limit the industrial production of ethanol at low production costs. The high volume of distillery waste water produced as process water also imposes ecological implications as the effluent puts high BOD and COD values. Therefore, a combination of high temperature, ethanol and sugar tolerance are clearly desirable yeast characteristics for an economical fermentation process. This particular chapter deals in the latest about the above mentioned four basic factors for increasing the fermentation yield and productivity.

2.1 RAW MATERIALS

Alcoholic fermentation has been carried out using a number of sugary materials depending upon their availability and suitability in a particular geographic situations. Various raw materials like sugarcane juice and molasses (Morimura *et al* 1997 and Agrawal *et al* 1998), sugar beet, beet molasses (El-Diwany *et al* 1992 and Agrawal *et al* 1998), Sweet sorghum (Bulawayo *et al* 1996) and starchy materials like sweet potato (Sree *et al* 1999). Corn cobs and hulls (Beall *et al* 1992 and Arni *et al* 1999). Cellulosic materials like cocoa, pineapples and sugarcane waste (Othman *et al* 1992) and milk/cheese/whey using lactose hydrolyzing fermenting strains (Silva *et al* 1995, Ghaly and Ben-Hassan 1995) have been reported. Of these, simple sugar bearing materials are the easiest to process, since the yeast ferment these directly while other carbohydrates like starch/cellulose have to be first hydrolyzed to fermentable sugars using current commercial technologies (Physio-chemical/enzymatic preparation) before they can be fermented to yield ethanol. Ethanol in India and other developing countries is mainly produced by fermentation of diluted molasses at ambient temperature of 25-35°C employing *Saccharomyces cerevisiae* (Sharma and Tauro 1986, Bulawayo *et al* 1996). Cane molasses is a complex mixture that varies in composition according to geographical sources, agricultural practices and sugarcane mill operations (Harrison and Graham 1970, Dhamija *et al* 1986). The average composition of Indian cane molasses (Dahiya and Rose 1986) is :

Moisture = 28.2
Total reducing sugars = 51%
a) Fermentable = 45.0%
b) Non-fermentable = 6%
Total nitrogen = 0.36
Volatile acidity = 0.18
Total sulphur dioxide = 0.05
Total ash = 11.0

The fermentable carbohydrates in molasses are sucrose and other sugar mainly glucose and fructose. The non-sugars may consist of nitrogenous substances like gums, polysaccharides, wax, sterols, pigments and salts of calcium, potassium and magnesium (Rao 1983).

2.2 ORGANISM

In fermentation of the various ethanol producing micro-organisms (Bhatt *et al* 1987, Laplace *et al* 1992 and 1993) yeast belonging to *Saccharomyces cerevisiae* have been used most commonly. Ok and Hashinaga (1997) isolated yeast from spoiled high sugar foods. These yeast are not merely undesirable micro-organism but they are osmophilic yeasts thus creating a potential for industrial applications (Groleau *et al* 1995).

Uma and Polasa (1990) isolated *S. cerevisiae* from palm wine which produced increased amounts of ethanol in yeast extract peptone dextrose medium. Bertolini *et al* (1991) isolated new strains of *S. cerevisiae* on basal medium containing 48% sucrose from fermenting sample collected from Brazilian alcohol factories. Isolated strains fermented concentrated sugarcane syrups as well as high sucrose solution in synthetic medium with conversion efficiency of 89-92%.

Laluce *et al* (1993) studied the thermotolerant behaviour of wild type yeast strains selected under pressure of temperature, high sugar and added alcohol using fermentation of sugar syrup. Sree *et al* (2000) reported isolation of four osmotolerant, thermotolerant, flocculating fermenting yeast strains from soil samples collected from thermal power plant in India.

2.3 GROWTH OF YEAST

2.3.1 Sugar tolerance and growth

Although *Saccharomyces* spp. have been used to ferment molasses for ethanol production, tolerance to high sugar concentration limits the ethanol production during fermentation. High sugar concentration in fermentation medium may promote a decrease in the specific growth rate and metabolism of industrial yeast strains (Morais *et al* 1996). Therefore yeast must be able to grow well in concentrated fermentation media in order to get maximum possible ethanol yield. Ekunsanmi and Odunfa (1990) isolated 13 yeast strains from fermenting cassava tubers and screened them for ethanol tolerance, also found to be sugar tolerant. Sugar tolerance was tested with reference to growth in a medium containing glucose at the rate of 100, 150, 200 and 250 g per litre. Three among the thirteen isolated strains were able to grow in all sugar concentrations of upto 25% (w/v). Groleau *et al* (1995) reported that yeast *Zygosaccharomyces rouxii* ATCC 12572 grew well in concentrated glucose media containing sugar upto 20% (w/v) or above and was selected for ethanol and polyols production from concentrated media.

Morais *et al* (1996) observed that the strains of *Saccharomyces* spp. isolated from tropical substrates show high osmotolerance toward sugars. The yeast were inoculated in Sabouraud broth added with 2, 10, 18, 20, 25, 30 or 50% glucose and incubated in a water bath at 28°C. Measure of cell growth and viability were obtained, indicating that all strains could tolerate sucrose upto 50% (w/v) and showed 98% viability. Ok and Hashinaga (1997) isolated sugar tolerant yeasts from high sugar fermented vegetable extracts. Nearly 90% of the identified strains belonged to *Zygosaccharomyces rouxii.* All strains grew well in 50% (w/w) glucose medium and among them 2 strains grew on 60% (w/w) glucose medium. One strain of *Z. rouxii* V19 grew even upto 80% (w/w) glucose in liquid medium. Bulawayo *et al* (1996) studied growth of various yeast strains at the sugar concentration of 100 to 500 g/l. While 6 of the 10 strains had maximum growth rate at 15% sucrose, 4 yeast isolates could tolerate high concentration of sugar and showed maximum growth at 30% (w/v) sucrose. All strains used in this study showed marked fall in growth rate when sugar concentration increased beyond 15% (w/v) in the fermentation medium.

Kocher and Sedha (1999) studied growth characteristic of an osmophilic yeast isolated from a mixed fruit jam. Isolated yeast was identified as a strain of *Zygosaccharomyces rouxii.* Yeast was grown in the GYE broth containing 10-50% (w/v) glucose. Maximum growth was observed upto 30% glucose and the growth at 40 and 50% glucose showed a lag phase of 24 h.

2.3.1.1 Improvement in osmotolerance of *S. cerevisiae*

Osmotolerance of yeast may be improved by adaptation on concentrated media, by mutations or by immobilizing cells.

Zelinkarja - Tkachenko *et al* (1982) isolated osmophilic yeast strains from concentrated fermentation media based on molasses or raw cane sugars, which were progressively adapted to 30° Brix media by sub-culturing about 100 times in 5 months. Eventually, a strain with significantly better tolerance towards high Brix and ethanol was obtained which produced 13.11% ethanol by volume instead of 12.5% from 30° brix media with unfermented sugar concentration of only 6.7 g/litre.

Chanderkala (1993) used UV mutagenesis to improve thermotolerance of *S. cerevisiae* and obtained two mutants that produce 5.4% (v/v) ethanol from 20% (w/v) molasses. Dale *et al* (1994) studied the effects of high concentrations of whey permeate on *Kluyveromyces marxianus* using both free and immobilized cell reactors. The osmolality of sweet whey permeate was determined and found to inhibit the cell growth of free and immobilized yeast strongly. The immobilized cells showed more tolerance to high osmolalities if osmolality was increased slowly over time, indicating adaptation by the immobilized cells.

Morimura *et al* (1997) improved the salt tolerance of *Saccharomyces cerevisiae* strains by maintaining a high concentration of KCl in the medium. Of the adaptive mutants obtained, strain K211 was found to have highest cell viability and ethanol productivity in a molasses medium containing 25% (w/v) total sugars.

Sree *et al* (2000) used immobilized osmotolerant *S. cerevisiae* for ethanol production. Immobilized cells were kept in medium containing 50, 200 and 250 g l^{-1}. From the exhausted media cells were subsequently replaced into fresh media, having same glucose concentration. Cells grew well and successfully fermented the media upto 25% (w/v) sugars.

2.3.2 Ethanol tolerance and growth

Effect of ethanol on the growth of fermenting organism is primary limiting factor in ethanol production. It inhibits yeast cell multiplication and metabolism. The accumulation of ethanol in yeast cultures leads to decrease in viability, growth and ethanol fermentation (Ingram and Buttake 1984). Luong (1985) reported that yeast cells do not grow above 14% (v/v) ethanol and there were no striking differences between response of growth and ethanol fermentation. Jimenez and Benitez (1987) reported that growing the cells of a highly ethanol tolerant *Saccharomyces* wine strain in the presence of ethanol, efficiently improves it's ethanol tolerance. However, membrane of less ethanol tolerant *Saccharomyces* laboratory strain after growing in the presence of ethanol becomes increasingly sensitive. Reversion to the higher tolerance occurs only after the addition of easily utilizable energy sources. Ethanol produced by micro-organisms during fermentation has been reported to be more toxic than ethanol added to the culture (Nagodawithana and Steinkraus 1976). The reason could be intracellular accumulation of produced ethanol, low sugar concentration used in ethanol tolerance studies or some nutrient limitations. In another study, cells of *Saccharomyces cerevisiae* grown in the presence of ethanol were shown to increase the amount of unsaturated fatty acyl residues in the membrane lipid which increases the membrane fluidity (Mishra and Prasad 1989) and this was supposed to be an adaptation to ethanol stress. Dasari *et al* (1990) studied the reason using plate count viability technique after exposing the cells to gradually increasing levels of ethanol upto 124 gl^{-1}. It was suggested that high rate of fall of viability when ethanol is rapidly produced is partly due to inability of cells to adapt quickly to ethanol and partly due to increased demand of magnesium at high ethanol concentrations.

With regard to the amount of ethanol tolerated, a number of studies have been carried out. In one such study, Ekunsanmi and Odunfa (1990) isolated 13 yeast strains from steep water of fermenting cassava tubers which were screened for ethanol tolerance. Three strains showed measurable growth in medium containing 10% (v/v) ethanol. Minimum concentration of ethanol was

11% and 12% which just inhibit the growth. Bulawayo *et al* (1996) observed ethanol tolerance in ten strains of *Saccharomyces* spp. Ethanol tolerance was studied in terms of growth rate in YEPS media containing ethanol at the rate of 5, 10, 15 and 20% (v/v). Results indicated that with no exogenous ethanol added the generation time was less than 10 h for all strains. In 10% (v/v) ethanol the lowest generation time was 13.9 h for N96 strain followed by 23.1 h for Z7 and Z-15 then 34.7 h for Z 11 and ATCC 20002 strains. All other strain used in this study had generation time above 40h and 15% (v/v) ethanol was inhibitory to all strains used. Morais *et al* (1996) reported that strains of *Saccharomyces* spp. isolated from tropical substrates exhibit tolerance towards ethanol. Ethanol tolerance was determined in terms of growth in Sabouraud broth at 28°C upto 96 h. The strain TD 200 was the most ethanol tolerant wild strain and grew in the presence of 20 g/l of ethanol maintaining a high viability after 96 h. Kajiwara *et al* (1996) developed transformants of *Saccharomyces cerevisiae* cells which could tolerate ethanol upto 15% (v/v). Ethanol tolerance was determined in terms of viability. Cells were grown in presence of zero, 5, 10, 15 and 20% added ethanol. Cells remained viable upto 15% added ethanol. However, when cell suspension was exposed to 20% ethanol, cells died within 1 h of incubation.

Alcohol tolerance of thermotolerant yeast strains was also studied using rich medium containing peptone and yeast extract plus added alcohol by Peres and Laluce (1998). The growth studies were carried out in order to take into account the adaptation of the strains to the external ethanol and it's effect on growth. Concentration of added ethanol in the medium varied from 0 to 9%. The final biomass formation declined for all ethanol additions. The biomass diminished markedly above 4% initial ethanol added. At 9% ethanol growth rate become constant i.e. yeast strain showed no growth on 9% ethanol containing medium. Chi and Arneberg (2000) have shown that yeast strains with different degrees of ethanol tolerance exhibit different adaptive response to produced ethanol during fermentation.

2.4 FERMENTATION

Indian cane molasses contain about 50% fermentable sugars principally sucrose, glucose or fructose that can be easily fermented by yeast. Yeast cells have an enzyme invertase which acts on sucrose and converts it into fermentable sugars that are fermented to produce ethyl alcohol through Embden - Meyerhof-Paranas (EMP) pathway. The pyruvic acid formed from glucose is in turn decarboxylated by pyruvate decarboxylase to acetaldehyde which is then reduced to ethanol.

The overall reaction of this fermentation of hexose sugar (glucose) by yeast has been expressed by Gay-Lussac which forms the basis for calculating fermentation efficiency.

$$C_6H_{12}0_6 \xrightarrow{\qquad\qquad} 2C_2H_5OH + 2CO_2$$

Glucose	Ethanol	
(1 kg)	(0.511 kg)	(0.489 kg)

Along with the ethanol, minor products such as glycerol, lactic acid and succinic acid are also formed. According to the equation, theoretical yield of

51% w/v (64% v/v) ethanol is possible, which however, is biologically unobtainable because of various physio-chemical and biological variations.

One of the main constraints in obtaining higher rates of ethanol production is the inhibition of yeast metabolism by both a high concentration of the sugar substrate as well as the end product. Exposure of osmosensitive yeast to high sugar concentrations results in an increase in osmotic pressure which significantly affects ethanol production as well as yeast viability. Generally in industrial alcohol production an initial of 16-18% sugar is used and when substrate concentration increases, osmotic pressure becomes pronounced which seriously affects fermentation efficiency (Panchal and Steward 1980).

Indian distilleries operate round the clock to meet the demand of ethanol, thus producing large volume of effluents. This effluent made to flow either to land surface or into drains causing health hazards and environmental pollution. This necessitates the use of concentrated substrate for ethanol production which will reduce the water requirements for dilution of molasses thus leading to reduction in effluent volume.

Also during fermentation, there is steady increase in the concentration of ethanol and this adversely affects yeast activity and inhibits bioethanol production (Panchal and Stewart 1980). Alcohol sensitivity expresses itself in pre-mature cessation of fermentation rather than a reduction in the fermentation rate.

Depending upon the yeast strain and the medium, the quantity of yeast initially added affects the fermentation rate in many ways. It has been observed that fermentation time gets lowered as inoculum concentration is increased (Ghosh and Tyagi 1979). If yeast resistance to ethanol is increased then media with a higher initial sugar concentration can be fermented with higher inoculum size resulting in improved economy. Further, simple raw materials like molasses are deficient in nitrogen and phosphorous that has to be supplemented in the fermentation media in the form of urea and Na_2HPO_4 (Dahiya and Rose 1986). Lots of work is being carried out to lower the nutrient cost. Therefore a plethora of factors primarily yeast strain, nutrients, inoculum size and environmental factors like pH, temperature, substrate and ethanol concentration affect the fermentation efficiency. The following section discusses in detail these factors.

2.5 FACTORS AFFECTING FERMENTATION

2.5.1 Effect of sugar concentration

Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation as well as maintain the economic status. It reduces the dilution water requirements, suppresses the osmosensitive contaminants and helps in decreasing distillation cost. However, high substrate concentrations are inhibitory to fermentation (Jones *et al* 1981) due to osmotic stress. D'Amore *et al* (1988) and Cachot *et al* (1991) reported similar results in a study on kinetics of volatile metabolites during alcoholic fermentation of cane molasses by *Saccharomyces cerevisiae*. Maximum accumulation of ethanol, acetaldehyde, ethyl acetate and fuel alcohols was found to be related to their initial concentration of the medium and inoculum dose. Bertolini *et al* (1991) isolated yeast strains from sample collected from Brazilian alcohol factories. These strains were capable of fermenting upto 30% of sucrose efficiently. Among these isolated strains, 8 strains (osmo-1 to osmo-8) were selected on the basis of sugar consumed and cell viability after 24 h of fermentation. The efficiency of selected strains varied from 89 to 92% depending upon the utilization of total sugar available in the medium. A maximum amount of 19.7% (v/v) ethanol accumulated from fermentation of 30% sugar as compared to 2 reference strains which produced 18.0 (v/v) and 15.6% (v/v). No residual sugars were left at the end of fermentation in the selected strains while 14 and 15% sugars remained unused in case of reference strains. Effect of some fermentation parameter on ethanol production from beet molasses by *Saccharomyces cervisiae* was studied by El-Diwany *et al* (1992). Among various parameters, effect of initial sugar concentration in molasses medium was also studied. They observed that with 20% (w/v) initial sugars, sugar assimilation 89.2% in 2 days giving 5.4% ethanol with a fermentation efficiency of 93% is observed. Thatipalama *et al* (1992) observed that product inhibition does not have any effect on product yield whereas substrate inhibition significantly affects the product yield from 0.45 to 0.30 as the initial substrate concentration increases from 150 to 280 g l^{-1}. These results are expected to have a significant influence in fermentating optimum fermentor design variables and in developing an efficient control strategy for optimizing ethanol productivity.

Borzani *et al* (1993) studied fermentation with various initial concentrations of sugar. They also demonstrated the logarithmic relationship between time of fermentation and the initial concentrations of sugar. Yusuf *et al* (1993) monitored the ethanol concentration and yield during fermentation of cane molasses by *S. cerevisiae* BTCC 281 at 26°C in order to optimize the initial pH and initial sucrose concentration in the substrate. The highest ethanol yield obtained was 68.4 g l^{-1} from the medium with sucrose concentration 16.75% using initial pH 4.5. Szopa *et al* (1993) studied the physiological and technological characteristics of 24 yeast strains for alcoholic fermentation of molasses at higher sugar concentration. Among 24 strains, only 4 strains showed enough osmotolerance for adequate fermentation of 25-30° Brix molasses worts. Esuoso *et al* (1993) worked on optimization of alcoholic fermentation. Red Star DADY active dried yeast was used for fermentation at 16% (w/v) sugars. Groleau *et al* (1995) observed that *Zygosaccharomyces rouxii* ATCC 12572 has ability to produce appreciate levels of ethanol and of various polyols from concentrated glucose media containing 20% sugar (w/v). Depending upon fermentation conditions *Z. rouxii* ATCC 12572 produced ethanol or various polyols as main fermentation product and either of the product could be favoured by simple manipulation of fermentation conditions. Techniques for fermenting higher glucose concentrations upto 30% (w/v) with *S. cerevisiae* were studied by Xu *et al* (1996) in order to increase the final ethanol concentration from 5.9 to 9.6% (v/v) by addition of 8% yeast extract and 1% peptone and to 12.8% (v/v) using

30% initial glucose concentration by immobilizing yeast in alginate. They further increased in the ethanol yield to 15% by starting with 20 g glucose plus 160 g nutrient medium and adding 30 lots of 20g glucose at intervals of 12 h. Morimura *et al* (1997) reported that yeast strain K211 is capable of fermenting molasses medium containing 25% (w/v) total sugar at 35°C. The strain K211 had high viability and ethanol productivity as it produced 92 g/l of ethanol at 33°C in 22% molasses medium with a productivity of 3.5 $gl^{-1}h^{-1}$, at 35°C ethanol production was 9g l^{-1} with a productivity of 2.7 $gl^{-1}h^{-1}$. Ramadan *et al* (1998) reported that an increase in sugar concentration leads to marked decrease in yeast biomass due to suppression of O_2 uptake and consequent inhibition of respiratory enzymes. But this high osmotic stress due to increased sugar results in increased production of glycerol and ethanol.

Sree *et al* (2000) isolated strains of *S. cerevisiae* from soil samples which were able to tolerate glucose concentration upto 350 gl^{-1}. Maximum ethanol yield obtained from 150 g l^{-1} glucose were 75 and 60 g l^{-1} using different strains.

A repeated batch fermentation system was used to produce ethanol using an osmotolerant *Saccharomyces cerevisiae* (US3) immobolized in calcium alginate (Sree *et al* 2000). Fermentation was carried out with initial concentration 150, 200, 250 g glucose per litre at 30°C. The maximum amount of ethanol produced by immobilized VS3 cells using 150, 200 and 250 gl^{-1} glucose was 72.5, 93 and 83 g ethanol per litre at 30°C after 48 h. Maximum yield was obtained at initial sugar of 20% with fermentation efficiency of 90%.

2.5.2 Effect of temperature

Fermentation process is always accompanied with evolution of heat which raises the temperature of the fermentors. As a result it becomes necessary to cool the large fermentors in the distilleries. This necessity often becomes a major operation and a cost factor in the production of ethanol. Temperature exerts a profound effect on growth, metabolism and survival of the fermenting organism. Fermentation in industries is usually carried out at ambient temperature of 25-35°C but temperature exceeds 40°C during fermention especially in northern region which decreases the cell viability and productivity. Maintenance of high cell viability is a major characteristic of fermentation to get high ethanol yield. Fermentation at 35-40°C or above has advantages such as ethanol recovery and significant savings on operational cost of refrigeration control in distilleries for alcohol production. Therefore many studies have been carried out for development of yeast to ferment at high temperatures of upto 40°C/45°C.

Raghav *et al* (1989) studied ethanol production from cane molasses using standard *S. cerevisiae* and a flocculent strain of *S. uvarum* at 30, 35, 40 and 45°C. The data suggested that the temperature toxicity was higher in the terminal stages of fermentation when the ethanol concentration was high.

Screening of yeast for glucose fermentation was done by D' Amore *et al* (1989) who reported that a strain of *S. diastaticus* was the most thermotolerant yeast out of a total of 65 yeast strains of various genera. Temperature adaptation in yeast involved changes in fatty acid composition

(Suutari 1990). The growth rates were determined over a range of temperature with the minimum temperature of 10°C. Experiments showed that the growth rate increases with increase in temperature upto 30°C and then decreases sharply with further increase in temperature. Among all, strains of *Saccharomyces cerevisiae* grew well at 35°C and *Candida utilis* showed fair growth rate at 40°C.

D'Amore *et al* (1990) reported the effect of temperature on glucose fermentation by fusion product, *Saccharomyces* sp. strain 1400 obtained by the fusion of *S. diastaticus* and *S. uvarum.* Even with 10% glucose the fusion product could not completely ferment the substrate at 41°C, leaving about 2% glucose in the medium. However addition of more nutrients to the medium resulted in complete utilization of the glucose and maximum ethanol production.

Laluce *et al* (1991) studied the effect of temperature on the fermentation capacity of 3 strains 19G, 78I and baker yeast in complete medium and sugarcane juice broth containing 15% total sugar. Complete conversion of total sugar to ethanol was observed after 12 h fermentation at 39-40°C. Above 40°C a strong inhibitory effect of temperature on ethanol formation in all classes was observed.

Further, optimum temperature for growth and rate of ethanol formation were found to depend on medium composition and strain. At high sugarcane syrup concentrations (20% w/v and above), a temperature of 35°C was found to be the best temperature for ethanol formation strain 78I.

The thermotolerant alcohol producing yeast strain *Kluyveromyces* *marxianus* IMB 3 was shown to grow on media containing 10% sucrose (w/v) at 45°C. At 45°C temperature, ethanol yield was 33 g/litre (Fleming *et al* 1993). Laluce *et al* (1993) examined yeast strains for their ability to grow, retain cell viability and ferment diluted sugarcane juice to ethanol at 40°C. Ethanol production, biomass production and viability was taken as criterion of thermotolerance determination. Osmo-6 strain used in this study maintained ethanol yield even at 38°C only exhibiting reduction when temperature reached 40°C. Temperature not only effects the biomass but ethanol production as well. At 40°C almost all strains showed marked fall in biomass and ethanol yield. Thermotolerance was studied under shaking as well as stationary conditions. Under shaking conditions improved biomass yield was observed possibly because of the increased level of aeration. In repeated batch fermentation using a thermotolerant flocculating yeast constructed by protoplast fusion, Kida *et al* (1997) found stable fermentations in molasses medium containing 20% (w/v) total sugar with an ethanol productivity of 5 g $l^{-1}h^{-1}$ at 30°C. Even at 35°C an ethanol productivity of 3.6gl^{-1} h^{-1} was achieved. Morimura *et al* (1997) observed ethanol production in concentrated molasses media at high temperature of 35°C. Stable repeated batch fermentations were possible at 35°C in molasses medium containing 20% (w/v) total sugar by *S. cerevisiae* strains developed by protoplast fusion. However stable fermentation could not be performed above 33°C in 22% molasses. This indicated that temperature tolerance depends on sugar concentration in the medium. Repeated batch fermentation was started at 30°C when fermentation temperature was increased to 33°C, ethanol was still produced at fairly good level though the fermentation time increased from 13 to 20 h.

The role of heat shock proteins in temperature tolerance mechanism in yeast cells have been reported by Banat *et al* (1998). They found that fatty acid composition of yeasts also changes in response to temperature and ethanol concentration during fermentation at elevated temperatures. Singh *et al* (1998) further studied the ethanol production at elevated temperatures. They isolated a number of strains of *Kluyveromyces marxiaus* var. *marxianses* capable of growth at high temperatures coupled with production of high alcohol concentrations by fermentation of glucose and molasses.

Sree *et al* (1999) used thermotolerant *Saccharomyces cerevisiae* strain for high alcohol production from starchy substrates. Fermentation was carried out at 37°C and 42°C. After 96 h, maximum ethanol produced from starchy substrates using VS3 was 10g and 3.5g/100g rice starch and 8.2 g and 7.5 g/100g sweet sorghum at 37°C and 42°C respectively.

Pellegrini *et al* (1999) screened 457 cultures of *Saccharomyces cerevisiae* conserved in the Industrial Yeasts Collection DB VPG for their ability to ferment glucose at supra optimal temperatures. Out of total 457, 43 strains were able to grow at 41°C, 13 at 42°C and one at 43°C. Among them, further 9 isolates showed higher ethanol yield and cell viability than other at these temperatures.

Sree *et al* (2000) isolated strains of *Saccharomyces cerevisiae* from soil samples were able to grow well at 44°C. Strains VS1 and VS3 showed better growth than other strains. Maximum ethanol yields obtained from 150g/litre glucose were 75 and 60g/litre using VS3 at 30°C and 40°C, respectively. Both growth as well as ethanol production decreased at 44°C.

2.5.3 Effect of Nutrients

Fermentation of concentrated substrates containing sugars above 18% leads to problems of decreased yeast viability and slow or incomplete fermentation. The level of new cell synthesis must be increased to have rapid fermentation. Therefore the concentration of the nutrients must be increased in such medium. It has been found that addition of nitrogen and phosphorus to molasses improves the fermentation rate and ethanol production (Sedha *et al* 1984 and Dhamija *et al* 1986). Fermentation of sucrose based on concentrated media by thermotolerant yeast was studied by Laluce *et al* (1991) and it was reported that supplementation of the medium with soyabean flour extract increased the rate of fermentation. In their studies, a maximum ethanol concentration of 13.5 - 14.8% (v/v) from diluted sugarcane syrup (22.5-33.5% sugars) was obtained.

Gokhale *et al* (1992) reported that the fermentation activity of *S.cerevisiae* NCIM 3509 was nearly complete in 60h at 30°C in presence of supplements like Glucosamine, Sodium stearate and Oleic acid along with an ethanol concentration of 6.9 - 7.4 (w/v) in comparison to 5% ethanol when no supplements were added.

Strel *et al* (1993) studied the fed batch fermentation of *Saccharomyces uvarum* on beet molasses in order to optimize growth conditions and raise fermentation activity. It was observed that medium supplemented with optimized amounts of $(NH_4)_2 SO_4$, $(NH_4)_2 PO_4$ and D-biotin results in increased biomass from 6-7 to >30 g/litre with specific growth rate 0.1 h^{-1}. fermentation activity also increased with supplementation of N-sources. Ezeogu and Okolo (1994) reported a significant increase in ethanol production from molasses when supplemented with soyabean oil meal, groundnut oil meal or castor oil meal.

N-sources applied at various concentrations affect the synthesis of enzymes in yeast during fermentation of highly concentrated substrates (Thomas *et al* 1996). Yeast activities were measured in the medium containing upto 35% (w/v) glucose and 1, 10 or 20 mM $(NH_4)_2 SO_4$. It was found that flux of carbon through the glycolytic pathway in *Saccharomyces cerevisiae* is greater under nitrogen limiting than under nitrogen excess growth conditions, conditions which stimulate the glycolytic pathway depress the flow of carbon through hexose monophosphate pathway and vice versa. Hence a higher concentration of assimilable N is needed to maintain a given glycolytic flux when fermentation at higher sugar concentration, yet excess nitrogen reported to impairs the fermenting capacity of the yeast.

Ethanol yield could be improved by providing the yeast with amino acids as N-source (Albers *et al* 1996). It was observed that addition of amino acids reduces the glycerol formation and leads to increased ethanol production. The glycerol yield obtained with glutamic acid and with mixture of other amino acids as nitrogen source was clearly lower than those for ammonium growth cultures. Ethanol yield increased for growth in both glutamic acid by 9% and mixture of amino acids by 14%.

Patil *et al* (1998) used tamarind waste generated from tartaric acid extraction as supplement in ethanol production from molasses using *S. cerevisiae* cultures. They obtained an ethanol of 9.7% (w/v) compared to 6.45% (w/v) in supplemented and unsupplemented molasses respectively from 22.5% (w/v) molasses after 72h of fermentation Ari and Donmoz (2000) studied the effect of soyabean flour as N-source on alcohol and cell concentration in alcoholic fermentation of molasses. Soyabean flour affected fermentation time, alcohol concentration and total cell count with all strains used in this study but the degree of this effect differed among strains. The maximum ethanol concentration was 63.9 g/litre in *S. cerevisiae* M 3 strain furthermore, soyabean flour increased cell counts in all strains and the increment level varied with the concentration of soyabean flour and strain used. Addition of soyabean flour also resulted in approximately 15% decrease in the fermentation time in all of the strains.

2.5.3.1 Urea as nitrogen source

Urea is most commonly employed N-source in ethanolic fermentation. It is one of the most important N-source as it supports cell viability during fermentation and hence increased the ethanol productivity.

Gattas and Doin (1989) studied the effect of urea along with KH_2PO_4 and yeast extract on alcoholic fermentation. pH of medium varied least with urea and most without nutrients. With addition of nutrients hourly rate of ethanol production increased by 4%.

Depending upon the urea concentration employed to the fermented media, fermentation efficiency varies (Sengupta and Sadhukan 1992). They studied the fermentation efficiency in relation to urea concentration. Cane molasses containing 48.6% fermentable sugars was diluted and supplemented with urea @ 0.025-0.15%. Optimum fermentation efficiency of 89.35%, was obtained with 0.09% urea. But the fementation of medium having high fermentable sugars upto 300g/l, addition of urea failed to increase the fermentation rate or final ethanol concentration. Herrera *et al* (1995) observed that addition of urea to the fermented sugarcane juice results in increased biomass production with high protein content.

In very high gravity fermentation technology using media containing 300g dissolved solids, addition of yeast extract or urea to worts based on sugarcane juice or molasses failed to increase the fermentation rate or final ethanol concentration (Jones *et al* 1994).

Pereira *et al* (1996) studied the effect of different amounts of urea on microbial growth in sugarcane fermented in solid state urea was added at the rate of 0.5, 1.0 1.5 or 2.0% urea to the substrate. protein content and so the growth were found to increase with increasing urea concentration.

Fedel (1998) also reported that urea in the medium affects biomass yield and it's made protein contents while it only had a slight effect on ethanol production. Urea at > 0.2% has detrimental effect on biomass and ethanol yield.

2.5.5 Effect of Inoculum Size

To prevent contamination during industrial molasses fermentation, large volume of active yeast is added. Depending upon the yeast strain and the medium, the quantity of yeast initially added affects the fermentation rate in many ways. In case of low inoculum, the sugars may be wasted in producing yeast biomass rather than producing ethanol. Therefore, inoculum size should be adequate to achieve maximum possible ethanol yield.

Nain and Rana (1988) found 10% (v/v) inoculum to be optimum for ethanol production from sugarbeet juice.

Cachot *et al* (1991) reported maximum concentrations of ethanol, aldehyde, ethylacetate and fuel alcohols during batch and fed batch and fed batch fermentation of Brazilian cane molasses media by commercial *S. cerevisiae* were related to the initial concentrations of medium, inoculum dose and the feed rate in the fed batch mode.

El-Diwany *et al* (1992) studied effect of some of fermentation parameters on ethanol production from beet molasses by *S. cerevisiae* Y-7 and reported an increase in cell concentration (inoculum size) of the yeast above 3.6×10^5 cells/ml decreased the ethanol yield.

Kaur (1995) studied the alcoholic fermentation at higher substrate concentrations and high inoculum size. It was observed that for substrate concentration of 20% total sugars with fermentation temperature 37°C, an initial cell population of 1.0×10^7 cells/ml was optimum for maximum ethanol yield with fermentation efficiency of 88% in batch fermentation. Kida *et al* (1997) in this fermentation studies in a 2-tower fermentor maintained a cell count of > 10^9 cells/ml for obtaining an ethanol yield of 9 gl^{-1} from molasses containing 22.5% sugars at a temperature of 35°C.

Ari and Donmez (2000) in their studies on fermentation of molasses showed that soyabean flour increases fermentation efficiency by decreasing fermentation time as it increases cell growth.

Chapter - III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 A fermenting yeast procured from the department of Microbiology, PAU, Ludhiana was used in the present study.

3.1.2 Molasses

Molasses were procured from Jagatjit Distillery, Hamira. Appropriate dilution of molasses used in the fermentation were filtered through ordinary filter paper prior to their use in fermentation.

3.2 METHODS

3.2.1 Maintenance of culture

The yeast cultures were maintained by sub-culturing them every 15 days on glucose yeast extract (GYE) agar slants, incubating for 24h at 30°C and thereafter storing in a refrigerator at 4°C till further use.

Composition of GYE agar medium (gl^{-1}) :

Glucose	10.0
Peptone	5.0
Yeast extract	5.0
Agar	20.0
pH	5.5

Distilled water to make volume 1 litre. The medium was sterilized in an autoclave at 15 psi for 20 min.

3.2.2 Adaptation of yeast

The *Saccharomyces cerevisiae* isolate was improved to grow on glucose yeast extract agar/broth containing increasing glucose levels. Erlenmeyer flasks containing GYE broth having 10-25% glucose with an increment of 2.5% were prepared and the *S. cerevisiae* strain was inoculated @ 10^5 cells/ml in the flasks having 10% glucose and incubated at 28°C under shake flask conditions. The culture was passaged in each glucose concentration twice after an incubation period of 24 h in each passage. The *S. cerevisiae* cells were counted with hemocytometer and periodically transferred to increasing glucose levels till 25% glucose. Total and viable cell counts were taken after incubation in every passage and compared with initial cell counts. The direct cell counts were taken by the method of Lee *et al* (1981) using methylene blue reagent to stain the cells. Viable and dead cells were distinguished with the latter taking up colour. The number of total cells were calculated as $n = Y \times 25 \times 10^4 \times$ dilution factor.

Where, Y is an average no. of cells per big square

3.2.3 Growth studies

The growth kinetics of adapted strain of *S. cerevisiae* were studied at different glucose levels in GYE containing 10, 15, 17.5, 20 and 22.5% glucose.

For growth studies, tubes containing 10 ml each of GYE broth were inoculated with a loopful of 24h old slant-culture of yeast and incubated for 16 h at 28 ± 2°C. Thereafter 1 ml of this primary inoculum was transferred into 250 ml of Erlenmeyer flasks each containing 50 ml of GYE broth and incubated at 30°C for 16 h under shake culture conditions. This raised culture was used to inoculate Erlenmeyer flasks (250 ml) containing 100 ml of GYE of appropriate glucose concentration in duplicate @ 10^6 cells ml^{-1}. The flasks were incubated at 28±2°C under shake culture (140 rpm) conditions. Periodic samples were drawn aseptically and used for analysis of growth in terms of increase in optical density at 540 nm on Bausch and Lomb Spectronic - 20 and direct microscopic counts. The latter were determined by the method of Lee *et al* (1981) as described earlier.

The periodic samples were centrifuged at 8000 rpm for 5 min and supernatant was used for estimation of reducing sugars (Miller 1959).

3.2.4 Ethanol Tolerance of *S. cerevisiae*

Ethanol tolerance of *S. cerevisiae* was studied in GYE broth supplemented with 6 to 12% of ethanol. The inoculum of adapted strain of *S. cerevisiae* was prepared in 50 ml GYE broth as described in section 3.2.3 and used to inoculate Erlenmeyer flasks (250 ml) containing 100 ml medium with various concentrations (6, 8, 10 and 12%) of added alcohol. Flask containing only GYE broth, without supplementation of ethanol was also run alongwith for comparing growth in the presence and absence of alcohol. All the flasks were incubated at 30°C under shake flask culture conditions. Periodic samples were drawn every 3 h and used to determine growth in terms of optical density at 540 nm using Bausch and Lomb Spectronic - 20.

3.2.5 Fermentation of Molasses

Molasses procured from Jagatjit distilleries, Hamira were estimated for total fermentable sugars by the method of Miller (1959) and found to contain 48% reducing sugars. Molasses were diluted to prepare seed medium (8% sugar) and production medium supplemented with nitrogen @ 500 ppm in the form of urea and phosphorus @ 250 ppm in the form of Na_2HPO_4. The pH of media was adjusted to 5.0. For fermentation studies inoculum was raised in seed medium. One ml of primary inoculum prepared in GYE broth was transferred to sterilized seed medium taken in 250 ml Erlenmeyer flasks and incubated at 30°C under shaking culture conditions. This raised inoculum was used to inoculate production medium (120 ml taken in a 100 ml measuring cylinder of total capacity 150 ml).

3.2.6 Optimization of Fermentation Process

Fermentation processes carried out by yeast are known to vary with respect to substrate concentration, temperature, N-source and inoculum size. It is therefore imperative to optimize the fermentation conditions for yeast cells.

3.2.6.1 Effect of substrate concentration

To study the effect of substrate concentration on ethanol production by *S. cerevisiae*, the production medium was prepared by diluting molasses to sugar concentration of 15, 17.5, 20 and 22.5% (w/v) with distilled water and filtered through ordinary filter paper to remove suspended particles. Fermentation was carried out in cylindrical vessels of total 150 ml capacity and 120 ml working volume. Cylinders containing production medium supplemented with 500 ppm of nitrogen (in urea) and 250 ppm of phosphorus (in Na_2HPO_4) inoculated with seed culture @ 1.0×10^7 cells/ml and fermentation was carried out at 30°C upto 60 h.

Samples were drawn after every 12 h intervals and estimated for residual sugars (Miller 1959) as well as ethanol content in the media (Caputi *et al* 1968). Specific gravity was measured every 12 h during fermentation with hydrometer. Initial and final counts were also taken by the method of Lee *et al* (1981) as described earlier.

3.2.6.2 Effect of different of N-sources

Production medium containing 17.5% fermentable sugars supplemented with different N-sources like urea, $(NH_4)_2$ SO_4 and soyabean meal at the rate of 500 ppm of nitrogen each. Fermentation was carried out at 30°C for 48h in 100 ml cylinder as described earlier. Samples were drawn for estimation of reducing sugars as well as ethanol content at regular interval of 12h. Specific gravity was also measured at 12 h intervals by hydrometer (1100-1200 and 1000-1100). Initial and final cell counts were also determined as described earlier.

3.2.6.3 Effect of different concentration of urea

Urea found to be the best N-source in this study was supplemented @ 250 ppm, 500 pm and 1000 ppm and 1500 ppm of nitrogen in the production medium containing 17.5% fermentable sugars. All analyses, specific gravity every 12 h, cell counts, reducing sugars and ethanol content were performed on the periodic samples as described earlier.

3.2.6.4 Effect of temperature on fermentation of molasses

To optimize the fermentation temperature, fermentation was carried out at 15°, 25°, 30° and 37°C. Molasses diluted to 17.5% sugars and supplemented with 500 ppm of nitrogen (in urea) and 250 ppm of phosphorus (Na_2HPO_4) were used as production media and fermentation was carried out at different temperatures. The periodic samples were analysed for reducing sugars and ethanol content. Cell counts and hydrometer readings were also taken appropriately.

3.2.6.5 Effect of inoculum size

To study the effect of inoculum size on final ethanol produced, an initial cell concentration of 1×10^6, 1×10^7 and 1×10^8 cells/ml of seed medium was used. Production medium diluted to 17.5% initial sugars was taken in cylindrical vessels and inoculated with the inoculum prepared as described earlier. Fermentation was carried out at 30°C upto 48 h. The fall in specific gravity was monitored after every 12 h and 5.0 ml samples were also drawn simultaneously which were analysed for residual sugars and ethanol content.

3.2.7 Analytical methods

3.2.7.1 Spectrophotometric determination of ethanol (Caputi *et al* 1968)

One millilitre of the fermented wash was taken in 500 ml pyrex distillation flask containing 30 ml of distilled water. The distillate was collected in 50 ml flask containing 25 ml of potassium dichromate solution (33.768 g of $K_2Cr_2O_7$ dissolved in 400 ml of distilled water with 325 ml of sulphuric acid and volume raised to 1 litre). About 20 ml of distillate was collected in each sample and the flasks were kept in a water bath maintained at 62.5°C for 20 min. The flasks were cooled to room temperature and the volume raised to 50 ml. Five ml of this was diluted with 5 ml of distilled water for measuring the optical density at 600 nm using a Bausch and Lomb Spectronic - 20.

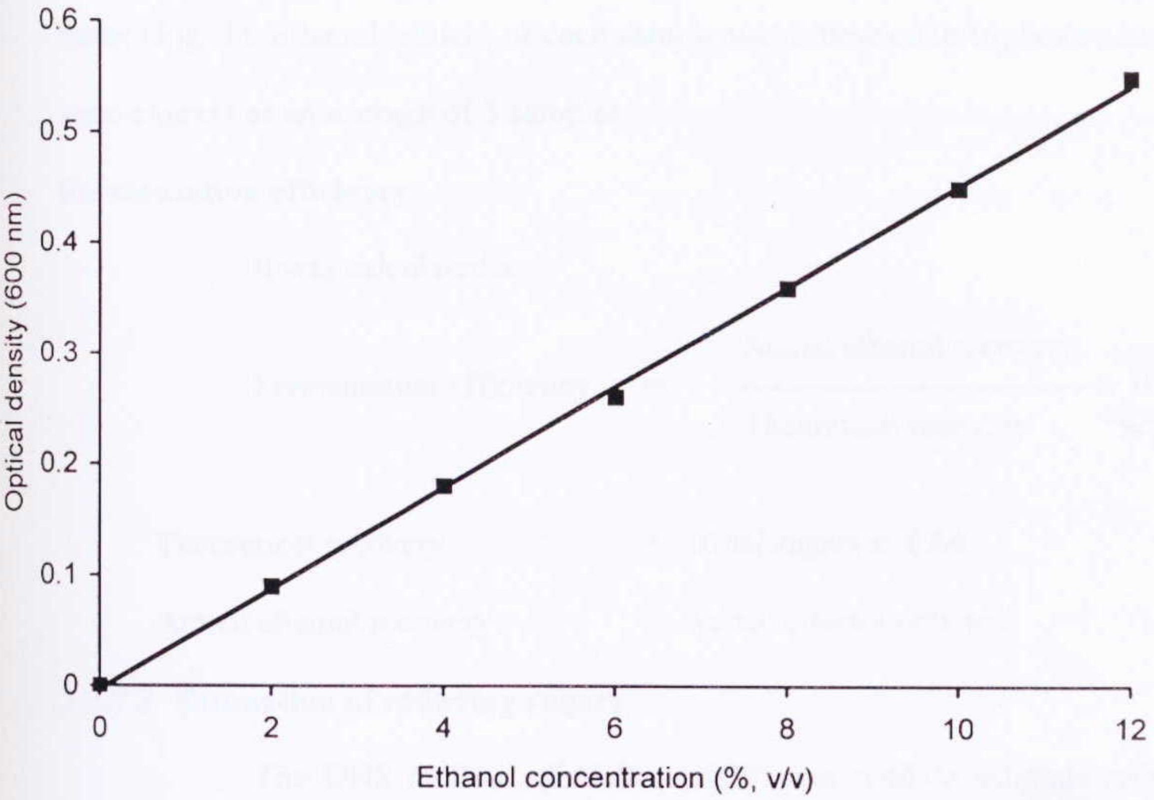

Fig. 1 Standard curve for ethanol estimation

A standard curve was prepared under similar set of conditions by using standard solution of ethanol containing 2 to 12% (v/v) ethanol in distilled water (Fig. 1). Ethanol content of each sample was estimated in triplicates and has been express as an average of 3 samples.

Fermentation efficiency

It was calculated as

$$\text{Fermentation efficiency} = \frac{\text{Actual ethanol recovery}}{\text{Theoretical recovery}} \times 100$$

Theoretical recovery = Total sugars x 0.64

Actual ethanol recovery = Actual ethanol obtained

3.2.7.2 Estimation of reducing sugars

The DNS method of Miller (1959) was used to estimate reducing sugars.

3.2.7.2.1 Reagents

1. **Substrate solution :** Standard solution of 1000 µg/ml concentration was prepared by dissolving 100 mg of glucose in 100 ml of distilled water.
2. **3, 5 dinitrosalicylic acid (DNS) solution :** Reagent was prepared by dissolving 10.0 g of 3, 5-DNS, 2.0 g of phenol and 0.5 g of sodium sulphite in 500 ml of 2% NaOH solution and then diluting it to 1 litre with distilled water. The reagent was filled and stored in dark coloured bottle.
3. **Potassium sodium tartarate (Rochelle salt) :** 40g of potassium sodium tartarate was dissolved in distilled water and the volume was made to 100 ml.

3.2.7.2.2 Procedure

One ml of appropriately diluted (500-1000 µg ml^{-1}) sample was taken in a test tube to which 3 ml of DNS reagent was added. The tubes were boiled in a boiling water bath for 15 minutes. One ml of Rochelle salt was added to these tubes and tubes were cooled to room temperature and used for measuring optical density at 575 nm.

A standard curve of glucose was prepared by using 100-1000µg ml^{-1} concentration prepared in distilled water (Fig. 2).

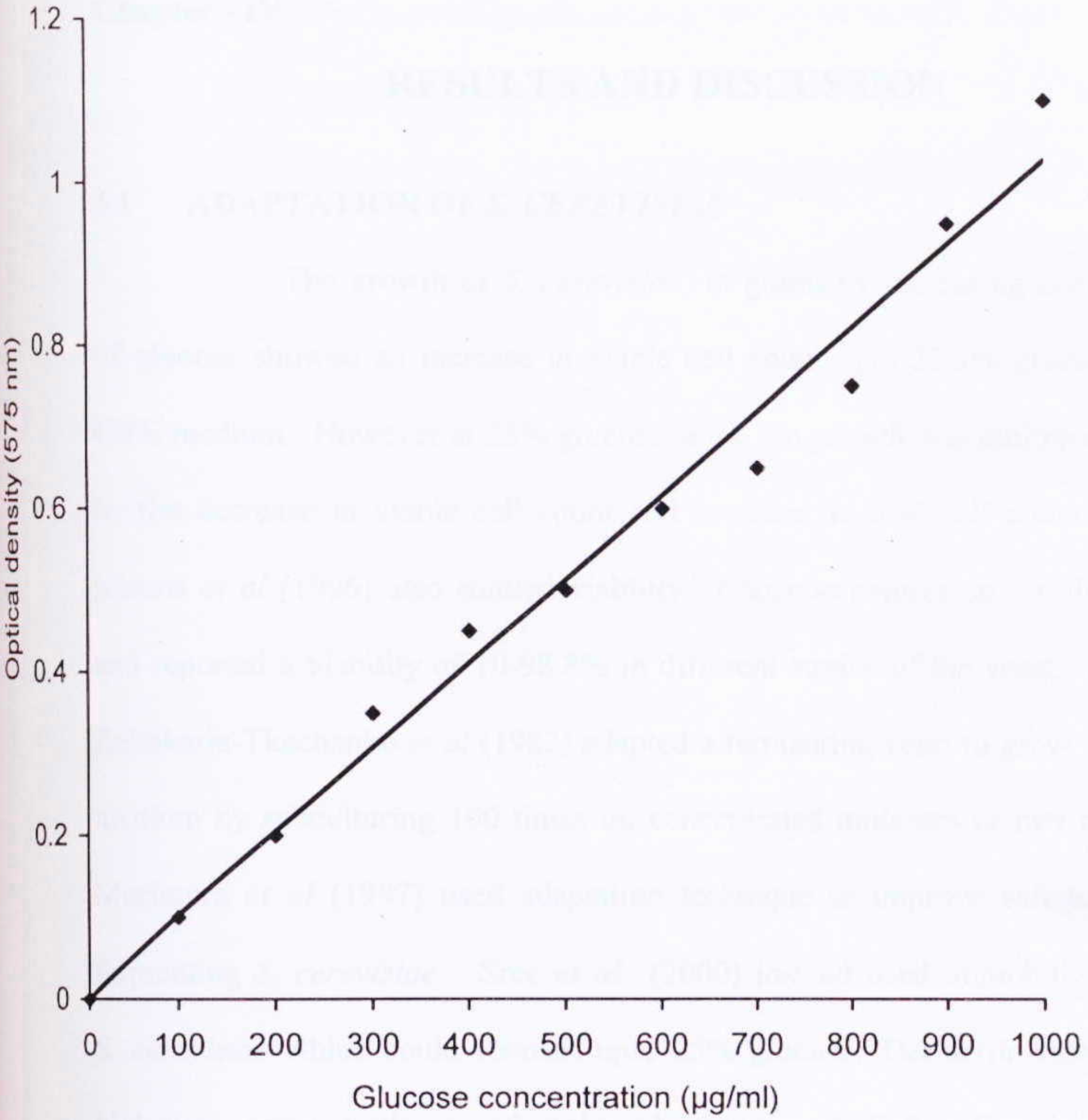

Fig. 2 Standard curve for estimation of reducing sugars (Glucose)

Chapter - IV

RESULTS AND DISCUSSION

4.1 ADAPTATION OF *S. CEREVISIAE*

The growth of *S. cerevisiae* in gradually increasing concentrations of glucose showed an increase in viable cell count upto 22.5% glucose level in GYE medium. However at 25% glucose level, the growth was inhibited as shown by the decrease in viable cell count and increase in dead cell count (Table 1). Morais *et al* (1996) also studied viability of *Saccharomyces* sp. in 50% glucose and reported a viability of 10-98.8% in different strains of the yeast. Elsewhere, Zelinkarja-Tkachanko *et al* (1982) adapted a fermenting yeast to grow on 30° brix medium by subculturing 100 times on concentrated molasses or raw cane sugar. Morimura *et al* (1997) used adaptation technique to improve salt tolerance of fermenting *S. cerevisiae* Sree *et al* (2000) instead used immobilized cells of *S. cerevisiae* which could ferment upto 25% glucose. The detrimental effect of high sugar concentration on ethanol production was studied by Gough *et al* (1996) in *Kluyveromyces marxianus* and a sucrose concentration more than 23% in molasses was found to affect ethanol production. Therefore, in the present study growth and fermentation studies were carried out with sugar concentrations of upto 22.5% (w/v).

4.2 GROWTH STUDIES

The adapted strain of *S. cerevisiae* was studied for its ability to grow and build a requisite biomass in 100 ml of GYE broth containing 15, 17.5, 20 and

Table 1 Effect of increasing glucose concentration on cells of *Saccharomyces cerevisiae*

Glucose (%)	Hemocytometer count	
	Viable cells	Dead cells
10	5.00×10^7	-
12.5	3.20×10^7	-
15	1.50×10^8	-
17.5	1.35×10^8	-
20	1.60×10^8	3.1×10^3
22.5	3.90×10^7	5.1×10^4
25	1.50×10^6	1.3×10^6

Initial count at each glucose concentration was 10^5 cells ml^{-1}

-, No dead cells observed

22.5% (w/v) concentrations of glucose. The growth kinetics indicated that cell number increases exponentially without a lag phase upto 15h of incubation in 15, 17.5 and 20% (w/v) glucose (Fig.3). However in 22.5% glucose containing GYE broth, exponential increase in number of cells continued even upto 24h. Ekunsanmi and Odunfa (1990) also reported exponential growth of an ethanol tolerant yeast strain YL3 on 10 to 25% glucose without a noticeable lag phase. The growth rates of *S. cerevisiae* w.r.t. increase in number of cells were found to be 0.78, 0.75, 0.75 and 0.636 h^{-1} at 15, 17.5, 20 and 22.5% (w/v) glucose with generation times of 55, 55.3, 53.2 and 65.4 min respectively (Fig.3). On the other hand, growth studies of *Zygosaccharomyces rouxii* (an osmotolerant yeast already identified in our laboratory) showed exponential increase in number of cells upto 24h at all sugar levels with a lag phase of 12h in 22.5% glucose supplemented GYE medium (Fig.3). The growth rate of *Z. rouxii* calculated at each sugar level was 0.812, 0.787, 0.781 and 0.775 h^{-1} with generation times of 51.2, 52.3, 53.2 and 53.6 min at 15, 17.5, 20 and 22.5% (w/v) glucose respectively (Fig.3).

Growth kinetics of both adapted *S. cerevisiae* and *Z. rouxii* was compared. The generation time of adapted *S. cerevisiae* at 22.5% glucose level was significantly higher than at other glucose concentrations whereas generation time of *Z. rouxii* was more or less same between 10 to 22.5% sugar levels. Earlier also Kocher and Sedha (1999) reported a constant cell productivity upto 30% glucose level in *Z. rouxii* with a decrease thereafter. The specific growth rates have been found to be inversely proportional to increasing sugar concentrations in

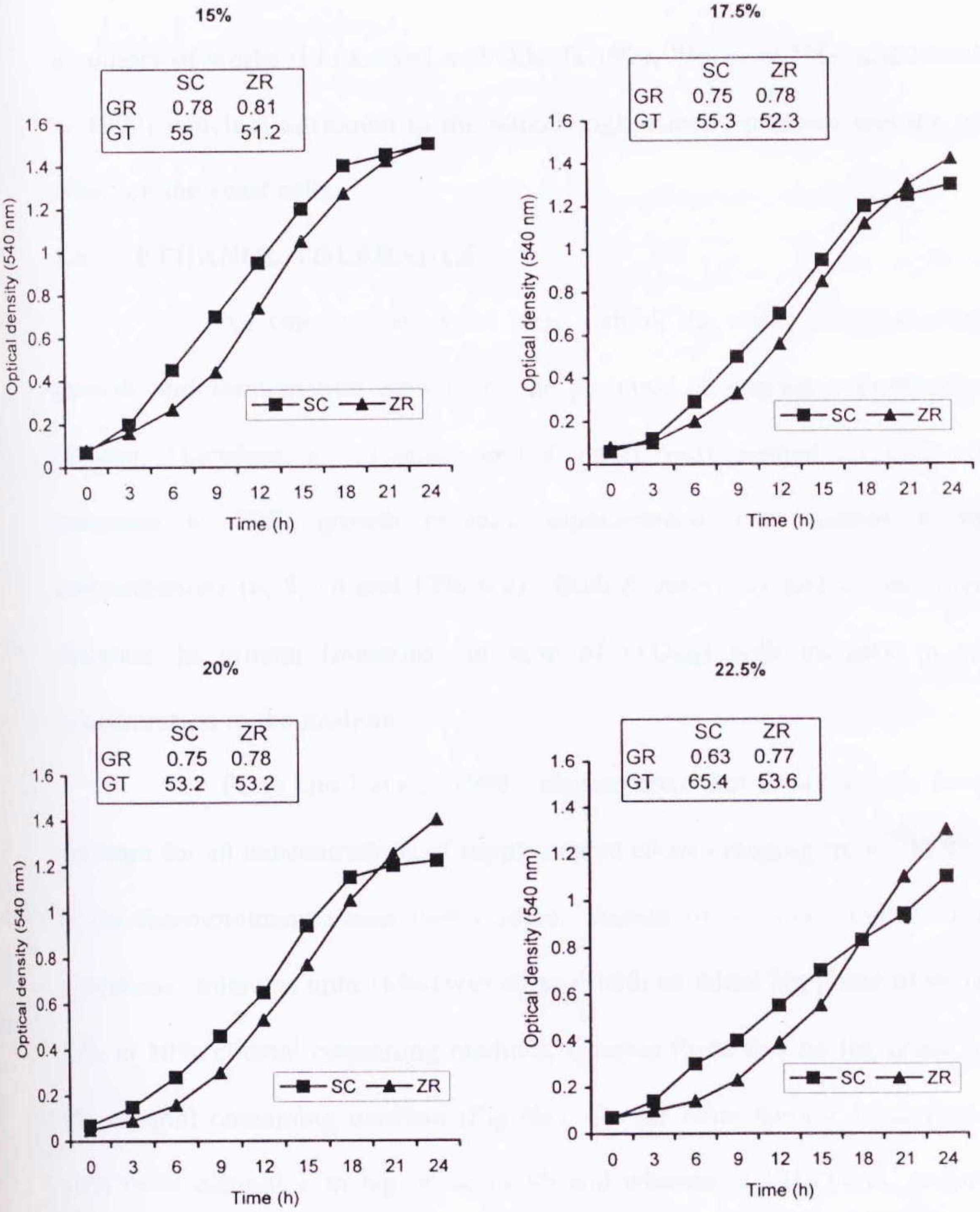

Fig. 3 **Growth kinetics of *S. cerevisiae* and *Z. rouxii* at different glucose concentration (15, 17.5, 20 and 22.5%). GR represents growth rate (h^{-1}) and GT represents generation time (min). Both growth rate and generation time have been calculated from viable cell counts.**

a number of works (Ekunsanmi and Odunfa 1990, Win *et al* 1996 and Membre *et al* 1999) which is attributed to the latter's high osmotic pressure and it's relative affect on the yeast cells.

4.3 ETHANOL TOLERANCE

An osmotolerant yeast must exhibit the ability to retain viability, growth and fermentation activity in the presence of varying concentrations of ethanol. Therefore, *S. cerevisiae* and *Z. rouxii* were studied for their ethanol tolerance in GYE growth medium supplemented with ethanol at various concentrations (6, 8, 10 and 12% v/v). Both *S. cerevisiae* and *Z. rouxii* showed decrease in growth (measured in term of $O.D_{540}$) with increase in ethanol concentration in the medium.

Peres and Laluce (1998) also reported that final biomass formation declines for all concentrations of supplemented ethanol ranging from 0 to 9% (v/v) in the thermotolerant yeasts they studied. Results of our study indicated that *S. cerevisiae* tolerates upto 10% (v/v) ethanol with an initial lag phase of more than 12h in 10% ethanol containing medium, whereas there was no lag phase in 6 & 8% ethanol containing medium (Fig. 4a). On the other hand *Z. rouxii* tolerated 10% (v/v) ethanol with lag phase of 9h and whereas at 12% (v/v), growth was poor throughout 48 h of incubation. However, there was no lag phase in the presence of 6% and 8% added ethanol (Fig. 4b). Infact ethanol tolerance is strain dependent and ethanol adaptive responses vary from strain to strain (Chi and Arneberg 2000). Various studies reported measurable growth in medium

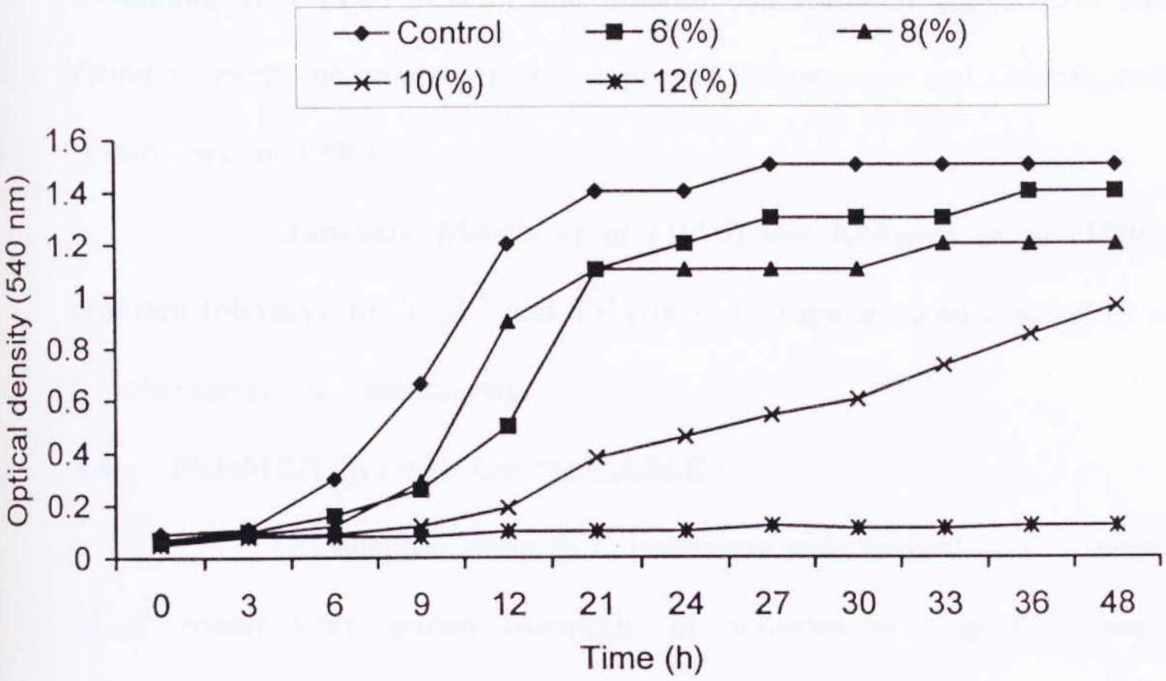

Fig. 4a Ethanol tolerance of *S. cerevisiae*

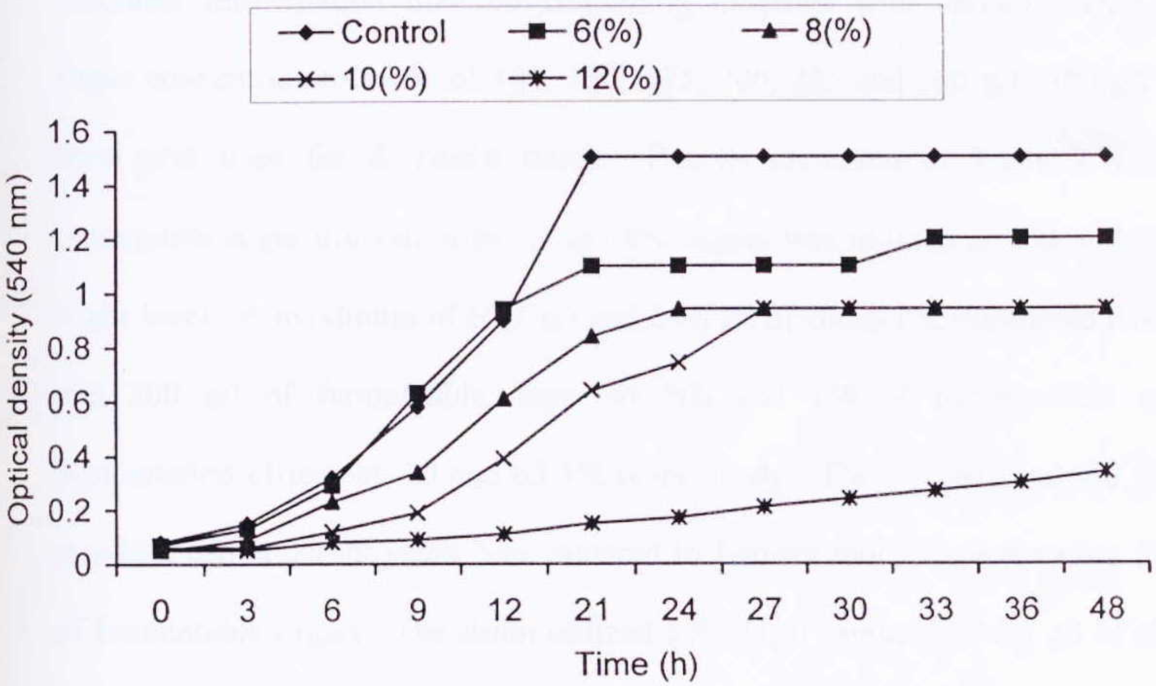

Fig. 4b Ethanol tolerance of *Z. rouxii*

containing 10% (v/v) ethanol and ethanol concentration above 10% has been found to exert inhibitory effects on growth (Ekunsanmi and Odunfa 1990 and Bulawayo *et al* 1996).

Elsewhere Morais *et al* (1996) and Kajiwara *et al* (1996) have reported tolerance to 20 gl^{-1} and 15% (v/v) of supplemented ethanol in case of *Saccharomyces* sp. respectively.

4.4 FERMENTATION OF MOLASSES

The adapted strain of *S. cerevisiae* and standard osmotolerant strain of *Z. rouxii* were grown overnight in molasses seed medium containing fermentable sugar concentration of 10%. Cells @ 1×10^7 cells/ml were used to inoculate fermentation medium containing molasses with variable fermentable sugar concentration levels of 100, 150, 175, 200, 225 and 300 g/l (300 g/l sugar level was used for *Z. rouxii* only). Results presented in Table 2 show an incomplete sugar utilization as 72 to 78% sugars was utilized at 200 and 225 g/l sugar level. A maximum of 62.5 g/l and 64.6 g/l of ethanol accumulated from 175 and 200 g/l of fermentable sugar in 60h and 48h of fermentation with a fermentation efficiency 70 and 63.3% respectively. The *Z. rouxii* culture used as standard osmotolerant yeast, was allowed to ferment molasses containing 300 g/l of fermentable sugars. The strain utilized 80% sugar producing 45.5 g/l of ethanol in 72h of fermentation with a fermentation efficiency of 30%. This showed that *Z. rouxii* although fermented a high 300 g/l of sugar but ethanol produced was much less than the adapted strain of *S. cerevisiae* which showed maximum alcohol

Table 2 Fermentation of molasses diluted to different sugar levels by *S. cerevisiae*

Time (h)	Sugar concentration in molasses (g/l)											
	100		150		175		200		225		300*	
	RS	Et	RS	Et	RS	Et	RS	Et	RS	Et	RS	Et
0	100	0.0	150	0.0	175	0.0	200	0.0	225	0.00	300	0.00
12	79.4	7.3	102.5	6.2	146.3	-	173	7.3	200.5	7.3	275	3.19
24	31.4	20.8	69.5	25.0	126.4	13.5	101.8	29.2	115.0	31.2	243	8.78
36	30.0	27.1	23.7	45.8	47.7	25.0	92.9	43.7	60.0	60.4	210	17.5
48	18.3	30.2	15.3	45.8	27.7	59.4	64.0	64.6	50.0	62.4	173.7	31.9
60	16.7	33.3	12.8	45.8	27.7	62.5	57.3	64.6	50.0	62.4	112.5	42.3
72	--	--	--	--	--	--	--	--	--	--	57.5	45.5
FE (%)	**65.2**		**60.0**		**70.0**		**63.3**		**54.2**		**30**	

- Inoculum size at start was 1 x 10^7 cells/ml and at the end was 1.63 x 10^8, 1.31 x 10^8, 1.64 x 10^8, 2.02 x 10^8, 1.16 x 10^8 cells/ml and 1.7 x 10^8 cells/ml at 100, 150, 175, 200, 225 and 300 g l^{-1} sugar levels.
- FE (Fermentation efficiency) is defined as the actual ethanol produced divided by theoretical possible ethanol.
- RS (Reducing sugar) and Et (Ethanol)values in g/l.
- *Fermentation of molasses employing *Z. rouxii*.

production of 64.6 g/l with 200 g/l of sugar requiring lesser time for fermentation.

An incomplete sugar consumption during fermentation has been reported in different studies (Bertolini *et al* 1991, Morais *et al* 1996, Bulawayo *et al* 1996). Morais *et al* (1996) reported sugar conversion of 55 to 95% in different strains of *S. cerevisiae*. As observed in our studies, Bulawayo *et al* (1996) also reported an initial rapid sugar conversion followed by it's levelling off at 30h of fermentation. We observed this trend at all sugar levels at 36h of fermentation. This was the phase when rapid accumulation of ethanol was observed which might be responsible for inhibition of invertase activity of the yeast thus preventing the hydrolysis of sucrose (Bulawayo *et al* 1996).

Maximum ethanol accumulation during fermentation has been found to be related to initial sugar concentration as ethanol yield decreases significantly with increase in substrate concentration (Thatipalama *et al* 1992). Different fermentation studies have reported efficient utilization of 150 to 300 g/l sugars with ethanol yield of 6.7 to 19.7% (v/v) from 200 and 300 g/l sugars respectively (Bertolini *et al* 1991, El-Diwany *et al* 1992). Sree *et al* (2000) also reported such decrease from 93 g/l to 87 g/l when initial sugar was increased from 200 to 250 g/l.

The low amount of 45.5 g/l ethanol from 300 g/l of initial sugars in *Z. rouxii* could be attributed to production of various polyols in addition to ethanol (Groleau *et al* 1995). Keeping in view, the higher fermentation efficiency of our adapted strain *S. cerevisiae* in comparison to *Z. rouxii*, fermentation optimization studies were concentrated on *S. cerevisiae* only using molasses diluted to containing 175 g/l of fermentable sugars.

4.5 OPTIMIZATION STUDIES ON FERMENTATION OF MOLASSES BY ADAPTED STRAIN OF *S. CEREVISIAE*

4.5.1 Effect of Temperature

Temperature is one of the major constraints that determines the bioethanol production. In the present study, the effect of temperature on fermentation of molasses diluted to 175 g/l sugars was carried out in cylindrical vessels at 15, 20, 30 and 35°C and the rate of fermentation was monitored by studying periodic fall in specific gravity, fermentable sugars and ethanol accumulation. A parallel fall in specific gravity and fermentable sugars of fermentation molasses medium was observed between 25°C and 35°C whereas decrease in specific gravity and fermentable sugars at 15°C was significantly low (Table 3). A comparatively similar accumulation of 56.2 g l^{-1} and 59.8 g l^{-1} of ethanol at 25°C and 30°C respectively was observed. On the other hand, ethanol accumulated at 15°C and 35°C was very low (Table 3). The thermotolerance is a strain dependent character and ethanol yield decreases with increase in temperature (Laluce *et al* 1993).

Temperature tolerance was also been found to depend upon sugar concentration of the medium as Morimura *et al* (1997) observed that fermentation of molasses at 35°C was possible when sugar concentration was 20% (w/v) with no fermentation when sugar concentration was 22% (w/v).

Table 3 Effect of temperature on fermentation of molasses (175 gl^{-1} sugars) by *S. cerevisiae*

Time (h)	Fermentation Temperature (°C)							
	15		25		30		35	
	SG	Et	SG	Et	SG	Et	SG	Et
0	1115	0.0	1110	0.0	1121	0.0	1115	0.0
12	1110	4.2	1107	5.0	1117	4.7	1090	10.0
24	1100	16.7	1097	30.2	1090	39.1	1063	35.4
36	1095	30.2	1068	50.0	1065	57.0	1058	39.6
48	1088	31.2	1061	56.2	1065	59.8	1058	41.6
FE (%)	**34.9**		**62.8**		**66.9**		**46.5**	

- Inoculum was added @ 10^7 cells ml^{-1} and final cell counts were 3.9×10^7, 1.2×10^8, 1.6×10^8 and 3.01×10^8 cells/ml at 15°, 25°C, 30° and 35° respectively.
- FE (Fermentation efficiency)
- SG (Specific gravity measured by hydrometer) and Et (Ethanol in $g\ l^{-1}$)

4.5.2 Effect of Nitrogen on Fermentation

4.5.2.1 Effect of Urea

Urea is popularly used as a N-source in fermentation of molasses and its different concentrations have been shown to effect fermentation rates. In our present work, effect of nitrogen supplemented @ 250, 500, 1000 and 1500 mg/l in the form of urea on fermentation of molasses was studied. Results presented in Table 4 show 500 mg/l as the optimum urea concentration that produced 61.2 g/l ethanol in 36h of fermentation whereas ethanol yield decreased when urea concentration was increased. Earlier, Sengupta and Sadhukan (1992) and Fedel (1998) reported increased fermentation efficiency with urea in the right concentration as N-source. Fedel (1998) reported decreased ethanol yield when urea was added @ of more than 0.2%. Therefore further studies on the effect of different N-sources on ethanol yield were carried out by supplementing nitrogen @ 500 mg/l in the fermentation media.

4.5.2.2 Effect of Different Nitrogen Sources

Supplementation of nitrogen and phosphorous to molasses has been found to improve fermentation of molasses (Dhamija *et al* 1986). We studied the effect of different N-sources (Ammonium sulphate, Soyabean meal, Urea each @ 500 mg/l) on fermentation of molasses diluted to 175 g/l sugars. Results presented in Table 5 show a comparatively similar ethanol accumulation with $(NH_4)_2\,SO_4$ and urea as N-source. One the other hand, soyabean meal increased fermentation rate and accumulated 64.7 g/l ethanol in 36h of fermentation. Earlier

Table 4 Effect of different concentrations of urea on fermentation of molasses (175 g l^{-1}) by *S. cerevisiae*

Time (h)	Urea (mg of nitrogen per litre)							
	250		500		1000		1500	
	SG	Et	SG	Et	SG	Et	SG	Et
0	1100	0.0	1100	0.0	1105	0.0	1108	0.0
12	1098	5.3	1095	4.8	1102	5.3	1102	5.3
24	1085	26.6	1083	29.3	1090	23.9	1085	26.6
36	1067	29.3	1065	61.2	1078	53.2	1067	53.2
48	1065	53.2	1062	61.2	1070	53.2	1067	53.2
FE (%)	59.5		68.4		59.5		59.5	

- Inoculum size (cell ml^{-1}) at start was 1 x 10^7 and at the end of fermentation was 9.8 x 10^7, 1.1 x 10^8, 1.15 x 10^8 and 1.3 x 10^8 with 250, 500, 1000 and 1500 μg ml^{-1} of urea concentration respectively.
- SG (Specific gravity measured by hydrometer) and Et (Ethanol in g l^{-1})
- FE (Fermentation Efficiency)

Table 5 Effect of different nitrogen sources on fermentation of molasses (175 gl^{-1} sugars) by *S. cerevisiae*

Time (h)	Nitrogen sources supplementended @ 500 mg of nitrogen per litre					
	Ammonium sulphate		Urea		Soyabean meal	
	SG	Et	SG	Et	SG	Et
Zero	1120	0.0	1121	0.0	1126	0.00
12	1111	7.2	1117	4.8	1120	15.9
24	1077	39.1	1090	39.1	1089	63.0
36	1063	55.1	1065	57.1	1068	64.7
48	1063	56.0	1065	59.8	1068	64.7
FE (%)	**62.5**		**67.1**		**72.3**	

- Inoculum was added @ 10^7 cells/ml and final cell counts were 1.4×10^8, 1.3×10^8 and 1.3×10^8 with $(NH_4)_2SO_4$, urea and soyabean meal respectively.
- FE (Fermentation efficiency)
- SG (Specific gravity measured by hydrometer) and Et (Ethanol in g/l)

Laluce *et al* (1991) and Ari and Donmoz (2000) reported increase in rate of fermentation with soyabean four as the N-source.

The use of soyabean meal as N-source in our studies showed a maximum ethanol accumulation of 63 g/l in 24h of fermentation whereas 39.1 g/l ethanol had accumulated in the same time when $(NH_4)_2 SO_4$ and urea were used as N-source (Table 5). Ari and Donmoz (2000) also reported a 15% decrease in fermentation time when soyabean flour was used as N-source. Similarly Patil *et al* (1998) reported tamarind waste as a supplement in ethanol production from molasses.

4.5.3 Effect of Inoculum size on Fermentation

A study was carried out to monitor the effect of different inoculum sizes on bioethanol production. Molasses diluted to 175 g/l fermentable sugars were taken in cylinders and inoculum was added @ 10^6, 10^7 and 10^8. Results presented in Table 6 showed that ethanol yield increases with increase in inoculum size. A maximum bioethanol of 67.9 g/l was produced using 10^8 cells with a fermentation efficiency of 76% while 59.8 g/l and 50.0 g/l ethanol was reported with inoculum size of 10^7 and 10^6 respectively in 48h of fermentation.

An adequate inoculum size is a must for maximum possible ethanol yield because in case of low inoculum, sugars are wasted in producing yeast biomass rather than alcohol (Cachot *et al* 1991). Nain and Rana (1988) and El-Diwany *et al* (1992) reported inoculum sizes of 10% (v/v) and $3.6x10^5$ cells/ml as optimum for ethanol production from sugarbeet juice and beet molasses

Table 6 Effect of inoculum size on fermentation of molasses (175 gl^{-1} sugars) by *S. cerevisiae*

Time (h)	Inoculum size (viable cells)					
	10^6		10^7		10^8	
	SG	Et	SG	Et	SG	Et
0	1119	0.0	1121	0.0	1110	0.0
12	1105	3.2	1117	4.7	1102	5.2
24	1075	37.5	1090	39.1	1080	66.7
36	1068	45.2	1065	57.0	1045	66.7
48	1066	50.0	1065	59.8	1044	67.9
FE (%)	**56**		**66.9**		**76**	

- SG (Specific gravity measured by hydrometer) and Et (Ethanol in g/l)
- FE (Fermentation efficiency)

respectively. In an earlier study in this Lab, Kaur (1995) reported an inoculum size of 10^7 cells/ml of a strain of *S. cerevisiae* as optimum for ethanol production using 200 g/l molasses.

4.6 FERMENTATION OF MOLASSES UNDER OPTIMIZED CONDITIONS

The fermentation of molasses by adapted strain of *S. cerevisiae* as optimized in the earlier sections showed a substrate concentration between 175 to 200 g/l, nitrogen @ 500 mg/l in the form of urea, fermentation temperature of 30°C and inoculum size of 10^8 as the best for fermentation of molasses. The over all ethanol yield improved significantly from 64.6 g/l to 78.5 g/l with 200 g/l sugars (Table 2 & 7). With a high inoculum size, fermentation time also decreased from 48 to 24h (Tables 6 & 7). Therefore the adapted strain of *S. cerevisiae* could produce a maximum of 78.5 g/l ethanol with a fermentation efficiency of 77% in terms of total sugars added. However, unadapted strain of *S. cerevisiae* run along side showed an overall ethanol production of 72.3 gl^{-1} with a fermentation efficiency of 70.9%. The low fermentation efficiency is attributed to the sensitivity of yeast to ethanol. Although the present yeast is tolerant to 10% (v/v) of supplemented alcohol (Fig. 2a), ethanol produced during fermentation is more toxic to the ethanol added to culture due to it's intracellular accumulation or requirements of magnesium/nutrients (Nagodathiwana and Steinkraus 1976, Dasari *et al* 1990). However, the present study shows an increase in ethanol yield by 6.2 gl^{-1} with 200 gl^{-1} of fermentation sugars.

Table 7 **Comparative fermentation of molasses by adapted and unadapted strain of *S. cerevisiae* under optimum conditions of temperature (30°C) inoculum size (10^8), substrate concentration of (200 gl^{-1}) and nitrogen source (urea @ 500 $mg\ l^{-1}$)**

Time (h)	**Adapted strain**		**Unadapted strain**	
	SG	**Et**	**SG**	**Et**
0	1120	0	1120	0
12	1102	8.0	1112	6.7
24	1058	77.9	1065	71.7
36	1055	78.5	1061	72.0
48	1052	78.5	1059	72.3
FE (%)	77.0		70.9	

Inoculum size (cell ml^{-1}) added @ 1.0×10^8
SG (specific gravity measured by hydrometer), Et (Ethanol $g\ l^{-1}$)
FE (Fermentation Efficiency)

Chapter - V

SUMMARY

A fermenting yeast, *S. cerevisiae* procured from Department of Microbiology, PAU was adapted to grow on high concentrations of glucose (10% to 22.5% w/v). The adapted strain of *S. cerevisiae* was compared with an already identified osmotolerant strain of *Z. rouxii* for it's growth on 15, 17.5, 20 and 22.5% (w/v) glucose supplemented growth medium. The strain was found to grow actively at 15, 17.5 and 20% (w/v) glucose concentration levels with lesser growth at 22.5% (w/v). On the other hand, *Z. rouxii* showed more or less similar growth at all sugar levels as reflected by growth rates and generation times of the two strains. Ethanol tolerance of both adapted *S. cerevisiae* and *Z. rouxii* showed that while *Z. rouxii* tolerated 12% (v/v) ethanol in the GYE medium, *S. cerevisiae* tolerated upto 10% added ethanol. The adapted strain of *S. cerevisiae* was used for ethanol production from concentrated molasses as *Z. rouxii* produced significantly low ethanol (45.5 g/l ethanol from 300 g/l fermenting sugars) compared to *S. cerevisiae*. *S. cerevisiae* was found to produce maximum ethanol of 62.5 g/l and 64.6 g/l from molasses diluted to 175 and 200 g/l sugars. The fermentation of concentrated molasses was optimized with respect to temperature, nitrogen sources and inoculum size. A temperature of 30°C was found to be optimum for fermentation as fermentation temperatures below and above 30°C led to fall in ethanol yield. In order to enhance the ethanol productivity besides urea which is most commonly used. N-source, other N-sources like soyabean meal and ammonium sulphate were used.

The final ethanol accumulation with 500 mg/l of nitrogen in the form of urea (59.8 g/l ethanol) and ammonium sulphate (56 g/l ethanol) in 48h of fermentation was comparatively same. However soyabean meal significantly enhanced the fermentation rate as well as ethanol yield (64.7 g/l ethanol in 36 h of fermentation). Ethanol yield was found to vary significantly with different inoculum sizes. Among the inoculum sizes of 10^6, 10^7 and 10^8 studies, maximum ethanol produced with 10^8 cells/ml was found to be 67.9 g/l in 48 h of fermentation. The fermentation of molasses under optimum conditions of sugar concentration, temperature, N-source and inoculum size, was carried out with both adapted and unadapted strain of *S. cerevisiae*. Adapted strain produced 78.5 gl^{-1} ethanol as compared to 72.3 gl^{-1} of ethanol by unadapted strain, showing overall improvement of 6.2 gl^{-1} in ethanol yield.

REFERENCES

Agrawal P K, Kumar S and Kumar S (1998) Studies on alcohol production from sugarcane juice, sugarcane molasses, sugarbeet juice and sugarbeet molasses, *Saccharomyces cerevisiae* NSI-113. *Proceedings of the 60th Annual Convention of the Sugar Technologists Association of India*, Shimla, India (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 19990307654).

Albers E, Larsson C, Liden G, Niklassen C and Gustafsson L (1996) Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. *Appl Environ Microbiol* **62** : 3187-95.

Arni S, Molinari M, Borghi M del, Converti A (1999) Improvement of alcohol fermentation of a corn starch hydrolysate by viscosity raising additives. *Starch-Starke* **51** : 218-24 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 20000310547).

Ari T and Donmoz S (2000). The effect of soyabean flour on alcohol and cell concentration in alcohol fermentation of molasses. *Turkish-Journal of Biology* **24** : 573-84 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 20000313166).

Banat I M, Nigam P, Singh D, Marchant R and Mchale A P (1998) Ethanol production at elevated temperatures and alcohol concentrations; Part 1-yeasts in general. *World J Microbiol Biotechnol* **14** : 809-21.

Beall D S, Ingram L O, Bassat A B, Doran J B, Fowler D E, Hall R G and Wood B E (1992) Conversion of hydrolysate of corn cobs and hulls into ethanol by recombinant *E. coli* B containing integrated genes for ethanol production. *Biotechnol Lett* **14** : 857.

Bertolini M C, Ernandes J R and Laluce C (1991) New yeast strains for alcoholic fermentation at higher sugar concentration. *Biotechnol Lett* **13** : 197-202.

Bhatt B, Rana R S and Nain L R (1987) Ethanol production from mixed fruit juice of damaged guava and banana. *J Fd Sci Technol* **24** : 192.

Borzani W, Garab A, Pires M H, Piplovic R, De-Ia Higuera G A (1993) Batch ethanol fermentation of molasses : a correlation between the time necessary to complete the fermentation and the initial concentrations of sugar and yeast cells. *World J Microbiol Biotechnol* **9** : 265-68.

Bulawayo B, Brochora J M, munzondo M I and Zvauya R (1996) Ethanol production by fermentation of sweet sorghum juice using various yeast strains. *World J Microbiol Biotechnol* **12** : 357-60.

Cachot T, Muller M and Pons M N (1991) Kinetics of volatile metabolites during alcohol fermentation of cane molasses by *Saccharomyces cerevisiae. Appl Microbiol Biotechnol* **35** : 450.

Caputi A, Ueda M and Brown T (1968) Spectrophotometric determination of ethanol in wine. *Am J Enol Vitic* **19** : 160-65.

Chanderkala (1993) Studies on bioethanol production from cane molasses using thermoresistant yeast. M.Sc. Thesis, Punjab Agricultural University, Ludhiana, India.

Chi Z and Arneberg N (2000) *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance exhibit different adaptive responses to produced ethanol. *J Indust Microbiol Biotechnol* **24** : 75-78.

Cysewski G R and Wilke C R (1978) Process design and economic studies of alternative fermentation methods for production of ethanol. *Biotechnol Bioeng* **20** : 1421.

Dahiya D S and Rose A H (1986) Factors affecting ethanol sensitivity and production by yeasts. In : Vashishat R K and Tauro P (ed) *Yeast Biotecnology.* pp 37-48. Haryana Agricultural University Press, Hisar, India.

Dale M C, Eagger A and Okos M R (1994) Osmotic inhibition of free and immoblized *K. maxianus* anaerobic growth and ethanol productivity in whey permeate concentrate. *Proc Biochem* **24** : 535-44.

D'Amore T, Celotto G, Russell I and Stewart G G (1989) Selection and optimization of yeast suitable for ethanol production at 40°C. *Enzyme Microb Technol* **11** : 411-16.

D'Amore T, Panchal C J and Stewart G G (1988) Intracellular ethanol accumulation in *Saccharomyces cerevisiae* during fermentation. *Appl Environ Microbiol* **54** : 110-14.

D'Amore T, Panchal C J, Russell I and Stewart G G (1990) A study of ethanol tolerance in yeast. *Crit Rev Biotechnol* **9** : 305.

Dasari G, Worth M A, Connor M A and Pamment N B (1990) Reasons for the apparent difference in the effects of produced and added ethanol on culture viability during rapid fermentations by *Saccharomyces cerevisiae. Biotechnol Bioeng* **35** : 109-122.

Dhamija S S, Dahiya D S and Tauro P (1986) Effect of molasses composition on ethanol fermentation. *J Fd Sci Technol* **23** : 162-64.

El-Diwany A I, El-Abyad M S and EL Rafai A H, Sallam L A and Allam R P (1992) Effect of some fermentation parameters on ethanol production from beet molasses by *Saccharomyces cerevisiae* Y-7. *Biores Technol* **42** : 191.

Ekunsanmi T J and Odunfa S A (1990) Ethanol tolerance, sugar tolerance and invertase activities of some yeast strains isolated from steep water of fermenting cassava tubers. *J Appl Bacteriol* **69** : 672-75.

Esuoso K O, Oderinde R A, Vega-Catalan F J and Bamiro F O (1993) Optimization of batch alcoholic fermentation of *Cyperus esculentus. Nahrung* **37** : 274-76 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 952310098).

Ezeogu L I and Okolo B N (1994) Sedimentation characteristics and effect of molasses concentration and medium supplementation on the ethanol productivity of an ethanol tolerant palm wine *Saccharomyces. Biotechnol Lett* **16** : 101-06.

Fedel M (1998) Production of ethanol and yeast biomass from whey permeate and starch waste. *Egyptian J Dairy Sci* **26** : 339-50 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 990507926).

Fleming M, Borrow N, Mchale L, Marchant R and Mchale A P (1993) Studies on the growth of a thermotolerant yeast strain, *Kluyveromyces maxianus* IMB, on sucrose containing media. *Biotechnol Lett* **15** : 1195-98.

Gattas E A D L and Doin P A (1989) Effect of added nutrients on alcoholic fermentation of a culture medium prepared from cassava starch. *Alimentos-e-Nutricao* **1** : 61-69 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 910210813).

Ghaly A E and Ben-Hassan R M (1995) Kinetics of batch production of single cell protein from cheese whey. *Appl Biochem Biotechnol - Part A, Enz Engin Biotechnol* **50** : 79-92.

Ghosh T K and Tyagi R D (1979) Rapid ethanol fermentation of cellulose hydrolysate. I. Batch Versus continuous systems. *Biotech Bioeng* **21** : 1387-2000.

Gokhale D V, Bastowde K B, Patil B G and Patil S G (1992) Glucosamine supplement activates ethanol production activity in cane molasses fermentation. *Abstract in National Symposium on Yeast Biology held at Hisar*.

Gough S, Flynn O, Hack C J and Marchant R (1996) Fermentation of molasses using a thermotolerant yeast, Kluyveromyces marxianus IMB3 : Simplex optimization of media supplements. *Appl Microbiol Biotechnol* **46** : 187-90.

Groleau D, Chevalier P and Yuen T L S Tse Hing (1995) Production of polyols and ethanol by the osmophilic yeast *Zygosaccharomyces rouxii*. *Biotechnol Lett* **17** : 315-20.

Harrison J S and Graham J C J (1970) Yeast in distillery practice. In : Rose A H and Harrison J S (ed) *The Yeast*. pp 283-348. Academic Press, London

Herrera - Coello N, Garcia-Rodriguez A, Vento-oliva Y and Obregon -Luna J (1995) Culture of micro-organisms to produce biomass from sugarcane juice. *Centro Azucar* **22** : 3-9 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 970306233).

Hospodke J (1968) Theoretical and methodological basis of continuous culture of micro-organism. *Czechoslovakia, Acad Sci Progue* (Original not seen. Cited by Chanderkala (1993) Studies on Bioethanol production from cane molasses using thermo-resistant yeast. M.Sc. Thesis, Punjab Agricultural University, Ludhiana).

Ingram L O and Buttake T M (1984) Effects of alcohol on micro-organisms. *Adv Microb Physiol* **25** : 253.

Jimenez J and Benitez T (1987) Adaptation of yeast cell membranes to ethanol. *Appl Environ Microbiol* **53** : 196-98.

Jones A M, Thomas K C and Inglew W M (1994) Ethanolic fermentation of blackstrap molasses and sugarcane juice using very high gravity technology. *J Agric Chem* **42 :** 1242-46.

Jones R P, Pamment N and Greenfield P F (1981) Alcohol fermentation by yeasts-the effect of environmental and other variables. *Proc Biochem* **16** : 42-49.

Kajiwara S, Shirai A, Fujii T, Toguri T, Nakamura K and Ontaguchi K (1996) Polyunsaturated fatty acid biosynthesis in *Saccharomyces cerevisiae* : Expression of ethanol tolerance and the FAD 2 gene from *Arabidopsis thaliana*. *Appl Environ Microbiol* **62 :** 4309-13.

Kaur U (1995) Studies on alcoholic fermentation at higher substrate concentration. M.Sc. thesis, Punjab Agricultural University, Ludhiana, India.

Kida K, Morimura S, Thong Y and Thong Y L (1997) Production of ethanol from molasses by flocculating yeast for use as a alternative energy source. *Seibutsu-Kogaku-Kaishi* **75** : 15-34 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 970310673).

Kocher G S and Sedha R K (1999) Growth studies on an osmophilic yeast isolated from a mixed fruit jam. *Ind J Microbiol* **39** : 119-20.

Laluce C, Abud C J, Greenhalf W and Sanches Peres M F (1993) Thermotolerance behaviour in sugarcane syrup fermentations of wild type yeast strains selected under pressures of temperature, high sugar and added ethanol. *Biotechnol Lett* **15** : 609-14.

Laluce C, Palmieri M C and Cruz R C L D (1991) Growth and fermentation characteristics of new selected strains of saccharomyces at high temperature and high cell densities. *Biotechnol Lett* **37** : 528-36.

Laplace J M, Delgenes J P, Moletta R and Navarro J M (1992) Alcoholic glucose and xylose fermentations by co-culture process : Compatibility and typing of associated strains. *Can J Microbiol* **38** : 654.

Laplace J M, Delgenes J P, Moletta R and Navarro J M (1993) Effects of culture conditions on the co-fermentation of *Saccharomyces diastaticus* associated with *Pichia stipitis*. *Microbiol Biotechnol* **39** : 760.

Lee S S, Robinson F M and Wang H Y (1981) Rapid determination of yeast viability. *Biotechnol Bioeng Symp* **11** : 641.

Luong J H T (1985) Kinetics of ethanol inhibition in alcohol fermentation. *Biotechnol Bioeng* **27** : 280-85.

Membre J M, Kubaczka M and Chene C (1999) Combined effects of pH and sugar on growth rate of *Zygosaccharomyces rouxii,* a Bakery product spoilage yeast. *Appl environ Microbiol* **65** : 4921-25.

Miller G L (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31** : 426-28.

Mishra P and Prasad R (1989) Relationship between ethanol tolerance and fatty acyl composition of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **30** : 294-98.

Morais P B, Rosa C A, Linardi V R, Carazza F and Monato E A (1996) Production of fuel alcohol by *Saccharomyces* strains from tropical habitats. *Biotechnol Lett* **18** : 1351-56.

Morimura S, Ling Z Y and Kida K (1997) Ethanol production by repeated batch fermentation at high temperature in a molasses medium containing a high concentration of total sugar by a thermotolerant flocculating yeast with improved salt tolerance. *J Ferment Bioeng* **83** : 271-74.

Nagodawithana T W and Steinkraus K H (1976) Influence of rate of ethanol production and accumulation on the viability of *S. cerevisiae* in rapid fermentation. *App Environ Microbiol* **31** : 158.

Nain L R and Rana R S (1988) Ethanol production from sugarbeet juice by *S. cerevisiae*. Nutrient optimization studies. *J Maharastra Agric Univ* **13** : 141.

Ok T and Hashinaga F (1997) Identification of sugar tolerant yeasts isolated from high sugar fermented vegetable extracts. *J Gen Appl Microbiol* **43** : 39-47.

Othman A S, Othaman M N, Abdulrahim A R and Bapar S A (1992) Cocoa, Pineapples, Sugarcane Waste for ethanol production. *Planter* **68** : 125.

Panchal C J and Stewart G G (1980) Effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast-strain. *J Inst of Brewing* **86** : 207-10.

Patil B G, Gakhole D V, Bastawde K B, puntambekar U S and patil S G (1998) The use of tamarind waste to improve ethanol production from cane molasses. *J Indust Microbiol Biotechnol* **21** : 307-10.

Pellegrini L, Candinali G and Mantini A (1999) Selection of *Saccharomyces cerevisiae* strains able to ferment at supraoptimal temperatures. *Annali-di-Microbiologia-ed-Enzimologia* **49** : 55-65 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 20000310461).

Peres M F S and Laluce C (1998) Ethanol tolerance of themotolerant yeasts cultivated on mixtures of sucrose and ethanol. *J Ferment Bioeng* **85** : 388-97.

Pereira O G, Garcia R, Valadares-Filho -S-de-C, Loures E G, Cecon P R and Ribeiro S (1996). Effect of different amounts of urea on microbial growth (true protein) in sugarcane fermented in the solid state. *Revista-da-Sociedade-Brasileira-de-Zootecnia.* **25** : 741-49 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 20000310461).

Raghav R, Sivaraman M, Gokhale D V and Rao B S (1989) Ethanol fermentation of cane molasses by a flocculent yeasts. *Biotechnol Lett* **11** : 739-44.

Ramadan E M, Gamal R F, Abdelhady H M and Galal G F (1998) Effect of osmotic potential of sugar on the biological activity of yeast. *Anal Agric Sci Cairo* **45** : 73-98 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 980313313).

Ramalingham A and Finn R K (1977) The vacuferm. Process : A new approach to fermentation alcohol. *Biotechnol Bioeng* **19** : 583.

Rao P J M (1983) Raw materials for production of ethyl alcohol and their comparative economics. *Paper presented in International Specialized Symp on Yeast held at Bombay.*

Rose D (1976) Yeasts for molasses alcohol. *Proc Biochem* **11** : 10.

Rosenschein D A and Hall D O (1991) Energy analysis of ethanol production from sugarcane in Zimbabwe. *Biomass and Bioenergy* **1** : 41-46.

Sedha R K, Verma G, Gupta R P and Tewari H K (1984) Ethanol production from molasses using cell recycling of *S. cerevisiae. J Ferment Technol* **62** : 471-76.

Sengupta D K and Sadhukan R K (1992) By-product utilization and optimization of different external factors in ethanol fermentation of sugarcane molasses in batch process. *Ind Chem Engin* **34** : 85-90 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 930325491).

Sharma S and Tauro P (1986) Control of ethanol production by *Saccharomyces cerevisiae. Proceedings of National Symposium on yeast Biotechnology held at Haryana Agricultural University*, Hisar, India.

Silva-CA-da, Castro-Gomez R J H, Abercio -da-Silva C and Gomez R J H C (1995) Study of the fermentation process using milk whey and the yeast *Kluyveromyces fragitis Semina Londrina* **16 :** 17-21 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 9604250).

Singh D, Nigam P, Merchant R and Mchole A P (1998) Ethanol production at elevated temperatures and alcohol concentration : Part II - use of *Kluyveromyces maxianus* IMB3. *World J Microbiol Biotechnol* **14**:823-34.

Sree N K, Sridhar M, Suresh K, Bharat I M and Rao L V (2000) High alcohol production by repeated batch fermentation using an immobilized osmotolerant *Saccharomyces cerevisiae. J Indust Microbiol Biotechnol* **24** : 222-26.

Sree N K, Sridhar M, Suresh K and Rao L V (1999) High alcohol production by solid substrate fermentation from starchy substrates using thermotolerant *Saccharomyces cerevisiae. Bioproc-Engin* **20** : 561-63.

Sree N K, Sridhar M, Suresh K, Banat I M and Rao L V (2000) Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. *Biores Technol* **72** : 43-46.

Strel B, Grba S and Maric V (1993) Enhancement of biomass and fermentation activity of surplus brewers yeast in a fed batch process. *Appl Microbiol Biotechnol* **39** : 53-57.

Suutari M, Liukkonen K and Laakso S (1990) Temperature adaptation in yeasts : the role of fatty acids. *J Gen Microbiol* **136** : 1404-74.

Szopa J S, Nielepkowicz C A and Gancarz M (1993) New yeast strains for alcoholic fermentation of molasses at higher sugars concentration. *Zeszyty - Naukowe-Politechniki-Lodzkiej-Technologia-i-Chemia-Spozyw* **50**:113-25 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 940301063).

Thatipamala R, Rohani S and Hill G A (1992) Effects of high product and substrate inhibition on the kinetics, biomass and product yields during ethanol batch fermentation. *Biotechnol Bioeng* **40** : 289.

Thomas K C, Hynes S H and Ingledew W M (1996) Effect of nitrogen limitation on synthesis of enzymes in *Saccharomyces cerevisiae* during fermentation of high concentration of carbohydrates. *Biotechnol Lett* **18** : 1165-68.

Uma V and Polasa H (1990) *Saccharomyces cerevisiae* of palm wine enhanced ethanol production by using mutagens. *J Microb Biotechnol* **5** : 1-4.

Verma G, Sedha R K and Gupta R P (1983) Use of yeast cell recycling for rapid ethanol production from molasses. *J Ferment Technol* **61** : 523-31.

Win S S, Impoolsup A and Noomhorm A (1996) Growth kinetics of *Saccharomyces cerevisiae* in batch and fed batch cultivation using sugarcane molasses and glucose syrup from cassava starch. *J Indust Microbiol* **16** : 117-23.

Xu P, Thomas A and Gilson C D (1996) Combined use of three methods for high concentration ethanol production by *Saccharomyces cerevisiae. Biotechnol Lett* **18** : 1439-40.

Yusuf Z, Islam K A, Ahmed A, Hossain N, Zaki Y, Afsaruddin A and Nillufer H (1993) Bench scale study of ethyl alcohol production from molasses using *Saccharomyces cerevisiae. Chemical-Engineering-Research-Bulletin* **10** : 45-51 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 94030428).

Zelinkarja-Tkachenko *et al* (1982) Yeasts fermenting concentrated molasses solution. *Mikrobiologicheskii Zn* **44 :** 19-23.

VITA

Name of the student : Mandeep Kaur

Father's name : S. Mukhtiar Singh Gill

Mother's name : Mrs. Jagtar Kaur Gill

Nationality : Indian

Date of birth : 06.01.1980

Permanent home address : Ward No. 6, House No. 108,
Basti Gobindgarh,
Moga - 142 001 (Punjab).

EDUCATIONAL QUALIFICATION

Bachelor degree : B.Sc. (Medical)
University and year of award : Panjab University, Chandigarh. 1999

% marks : 73.3%

Master's degree : M.Sc. (Microbiology)
University and year of award : Punjab Agricultural University, Ludhiana, 2001

OCPA : 7.23/10.00

Title of Master's Thesis : "Development of osmotolerant yeast for ethanol production from molasses"