PROCESS OPTIMIZATION FOR THE PRODUCTION OF ETHANOL VIA FERMENTATION

A
Dissertation
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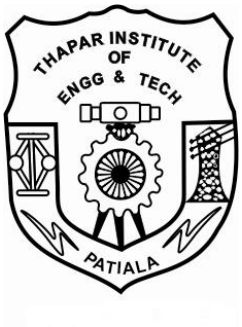
Masters of Science
In
Biotechnology

UNDER THE GUIDANCE OF
Er. Anoop Verma

BY
KADAMBINI GAUR
ROLL. NO.3040010



DEPARTMENT OF BIOTECHNOLOGY AND ENV. SCIENCES
THAPAR INSTITUTE OF ENGG. & TECHNOLOGY
(DEEMED UNIVERSITY)
Patiala-147004

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CERTIFICATE

This is to certify that the thesis entitled “Process optimization for the production of ethanol via fermentation” submitted by Ms. Kadambini Gaur (3040010) in partial fulfillment of the requirements for the award of Degree of Master of Sciences in Biotechnology to Thapar Institute of Engineering and Technology (Deemed university), Patiala, is a record of student’s own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or institute.

(Er. Anoop Verma)
Supervisor
DBTES, TIET
Patiala

(Dr. N.Das)
Head
DBTES, TIET
Patiala

(Dr. T.P.Singh)
Dean (Academic Affairs)
T.I.E.T, Patiala

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Dated: June, 2006, *Kadambini Gaur*

Patiala

ABSTRACT

A fermenting strain of *Saccharomyces cerevisiae* was utilized for alcoholic fermentation using sugarcane molasses. The fermentation of molasses was optimized with respect to temperature, pH and sugar concentration. Results revealed a temperature of 30°C, pH 6.0 and 20% sugar concentration as optimum for fermentation. GC method for estimating percentage of ethanol was employed. After optimizing these parameters, the experiment was scaled to fermenter level. Immobilization of yeast cells was carried out by entrapment in 2% calcium alginate and tested for ethanol production. Under optimized conditions, *S. cerevisiae* produced 11.6% of ethanol. Immobilization resulted in 10.4%ethanol after 48 hours and the same yeast cells were reused to carry out fermentation. The reuse of immobilized cells gave 7.9% ethanol yield.

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CHAPTER 1. INTRODUCTION

1.0 HISTORY

Ethanol has been used by humans since prehistory as the intoxicating ingredient in alcoholic beverages. Dried residues on 9000-year-old pottery found in northern China imply the use of alcoholic beverages even among Neolithic peoples. Its isolation as a relatively pure compound was first achieved by Islamic alchemists. Antoine Lavoisier described ethanol as a compound of carbon, hydrogen, and oxygen, and in 1808, Nicolas-Theodore de Saussure determined ethanol's chemical formula. Ethanol was first prepared synthetically in 1826, through the independent efforts of Henry Hennel in Britain and S.G. Serullas in France. Michael Faraday prepared ethanol by the acid-catalyzed hydration of ethylene in 1828, in a process similar to that used for industrial ethanol synthesis today. With the advent of distillation, which appears to have been discovered first in ancient Arabia, people were able to obtain beverages with higher ethanol content. In its strictest sense, **fermentation** (formerly called **zymosis**) is the anaerobic metabolic breakdown of a nutrient molecule, such as glucose, without net oxidation.Depending on which organism it is taking place in, fermentation may yield lactate, acetic acid, ethanol or other reduced metabolites . Normal fermentation processes typically cease when a beverage has achieved an alcohol content of 10 to 15 percent. Distillation is the process by which ethanol is boiled from the fermented mixture and captured, producing a liquid with a much higher concentration of alcohol.

1.1 PROPERTIES

Ethanol or ethyl alcohol, CH_3CH_2OH, has been described as one of the most exotic synthetic oxygen-containing organic chemicals because of its unique combination of properties as a solvent, a germicide, a beverage, antifreeze, a fuel, a depressant, and especially because of its versatility as a chemical intermediate for other organic chemicals.

Ethanol, also known as **ethyl alcohol** or grain alcohol, is a volatile, flammable, colorless chemical compound. It is a monohydric primary alcohol and it boils at 78.5℃. It is miscible (i.e., mixes without separation) with water in all proportions

and is separated from water only with difficulty; ethanol that is completely free of water is called absolute ethanol. Ethanol forms a constant-boiling mixture, or azeotrope, with water that contains 95% ethanol and 5% water and that boils at 78.15℃. Ethanol is a psychoactive agent and it produces a variety of physiological and behavioral effects.

1.2 PRODUCTION ROUTES

Ethanol is produced both as a petrochemical through the hydration of ethylene, and biologically, by fermenting sugars with yeast. Hydration of ethylene is the primary method for the industrial production of ethyl alcohol while fermentation is the primary method for production of beverage alcohol.

1.2.1 Ethylene hydration

Ethanol for use as industrial feedstock is most often made from petrochemical feedstocks, typically by the acid-catalyzed hydration of ethylene, represented by the chemical equation

$$C_2H_4 + H_2O \rightarrow CH_3CH_2OH$$

The catalyst is most commonly phosphoric acid, adsorbed onto a porous support such as diatomaceous earth or charcoal; this catalyst was first used for large-scale ethanol production by the Shell Oil Company in 1947. Solid catalysts, mostly various metal oxides, have also been mentioned in the chemical literature.

1.2.2 Fermentation

Ethanol for use in alcoholic beverages, and the vast majority of ethanol for use as fuel, is produced by fermentation: when certain species of yeast (most importantly, *Saccharomyces cerevisiae*) metabolize sugar in the absence of oxygen, they produce ethanol and carbon dioxide. The overall chemical reaction conducted by the yeast may be represented by the chemical equation

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$$

The process of culturing yeast under conditions to produce alcohol is referred to as brewing. Brewing can only produce relatively dilute concentrations of ethanol

in water; concentrated ethanol solutions are toxic to yeast. The most ethanol-tolerant strains of yeast can survive in up to about 25% ethanol (by volume).
In order to produce ethanol from starchy materials such as cereal grains, the starch must first be broken down into sugars.

1.3 USES

1.3.1 As a fuel

The largest single use of ethanol is as a motor fuel and fuel additive. The largest national fuel ethanol industries exist in Brazil and the United States. The Brazilian ethanol industry is based on sugarcane; as of 2004, Brazil produces 14 billion liters annually, enough to replace about 40% of its gasoline demand. Also as a result, they have become 80% independent from foreign oil. Most new cars sold in Brazil are flexible-fuel vehicles that can run on ethanol, gasoline, or any blend of the two.

The United States fuel ethanol industry is based largely on corn. Thailand, India, China and Japan have now launched their national gasohol policies. Ethanol with water content of 2% or less can be used as the alcohol in the production of biodiesel, replacing methanol, which is quite dangerous to work with.

1.3.2 Alcoholic beverages

Alcoholic beverages vary considerably in their ethanol content and in the foodstuffs from which they are produced. Most alcoholic beverages can be broadly classified as fermented beverages, beverages made by the action of yeast on sugary foodstuffs, or as distilled beverages, beverages whose preparation involves concentrating the ethanol in fermented beverages by distillation. The ethanol content of a beverage is usually measured in terms of the volume fraction of ethanol in the beverage, expressed either as a percentage or in alcoholic proof units. The proof of an alcohol beverage is equal to twice the percentage of alcohol contained therein.

Fermented beverages can be broadly classified by the foodstuff from which they are fermented. Beers are made from cereal grains or other starchy

materials, wines and ciders from fruit juices, and meads from honey. Fermented beverages may contain up to 15–20% ethanol by volume, the upper limit being set by the yeast's tolerance for ethanol, or by the amount of sugar in the starting material.

1.3.3 Other uses

It is easily soluble in water in all proportions. Absolute ethanol and 95% ethanol are themselves good solvents, somewhat less polar than water and used in perfumes, paints and tinctures. Alcoholic drinks have a large variety of tastes because various flavor compounds are dissolved during brewing.

Ethanol is used in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62%. Ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi, and many viruses, but is ineffective against bacterial spores. Wine with less than 16% ethanol cannot protect itself against bacteria. It is also used in preservation of biological specimens.

Objectives

As yeast ferments different sugars at different rates depends on the process conditions, thus there is a need to optimize the production of alcohol so as to economize the project. This is the aim of the project which covers the following objectives.

1.Process optimization for production of ethanol at lab scale and in the fermenter.

2.Comparison of ethanol production through immobilized yeast cells at lab scale.

CHAPTER 2. REVIEW OF LITERATURE

On account of limited global supply of oil, ethanol has emerged as an alternative for petroleum based liquid fuels. Now a days, it's use in automobiles as an alternative fuel has attracted worldwide attention for its 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, one of which is fuel alcohol produced by fermentation of agricultural/agroindustrial wastes and byproducts. An efficient ethanol production requires four components: fermentable carbohydrates, an efficient yeast strain, a few nutrients and simple culture conditions. Approximately 80% of world supply of alcohol is produced by fermentation of sugar and starch containing crops or byproducts from industries based on such crops. Among the widely used substrates for ethanol production are the molasses of sugarcane and sugar beet (Bose and Ghose, 1973). This is because they are ready for conversion with limited pre-treatments as compared with starchy or cellulosic materials. In India at present there are 285 distilleries producing ethanol by traditional batch fermentation process. However use of this system followed by distillation to recover the ethanol is rather uneconomical. In order to produce ethanol in large quantities and reasonable costs, the optimization of various physico-chemical parameters is important. Immobilization offers advantages of modern technique of continuous fermentation along with low cost design & optimum utilization of available expertise. Of the important parameters that could affect ethyl alcohol fermentation may be mentioned: availability and fermentability of the substrate, possible isolation of new potent strain and improvement of the available strain towards higher productivity, improvement in fermentation technology and reduction in by-product formed during the fermentation process. 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). This chapter deals in 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 particular geographic situations. Various raw materials like sugarcane juice and molasses (Morimura *et al* 1997 and Agrawal *et al* 1998), sugar beet, beet molasses (EI-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.

Dabas *et al* studied ethanol production from wheat starch. Hydrolyzed wheat starch was used as a substrate for ethanol production using 2 strains of *S.cerevisiae*. Wheat flour slurry (25%w/v) was gelatinized and conditions were standardized for saccharification and fermentation of wheat starch for ethanol production.

Ethanol in India and other developing countries is mainly produced by fermentation of dilute molasses at ambient temperature of 25-35^{o}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 sugar mill operations.

Oderinde *et al* (1986) showed that the removal of metal ions from molasses enhanced ethanol production.

Yadav *et al* (1997) studied the effect of pretreatment of sugarcane molasses for ethanol production by yeast. The effect of pretreatment of molasses with H_2SO_4 and $K_4Fe(CN)_6$ on ethanol production by different yeast strain was studied in order to find an effective method to reduce the load of various inhibitory substances and to select a suitable yeast strain for fermentation of pretreated

molasses. Pretreatment resulted in decreased level of inhibitory substances like Ca, Cu, and Fe in the molasses solution with improved ethanol production. The inhibitory effect of these constituents was confirmed by supplementation of synthetic medium with residues from different pretreatments and the inhibitory level for various constituents was found to be Ca>0.5%, iron> 46ppm and Cu >5.4ppm.

The fermentable carbohydrates in molasses are sucrose and other sugars 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).

The average composition of Indian cane molasses (Dahiya and Rose1986) In **Table 1** is:

Table 1: showing average composition of Indian cane molasses

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

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 Hashinanga (1997) isolated yeast from spoiled high sugar foods.

Skotnicki *et al* (1981) compared the rates of growth and ethanol production by 11 different strains of *Zymomonas* , with some strains being more tolerant of high sugar or ethanol concentration and high incubation temperature than others. One of the most promising ethanol producing organism is the bacterium *Zymomonas mobilis* which is used to make palm wines. This bacterium can produce upto 1.9 mol of ethanol from each mole of glucose fermented.

Renu Bansal and R.S. Singh (2003) did a comparative study on ethanol production from molasses using *Saccharomyces cerevisiae* & *Zymomonas mobilis.* Yeast was found to be more ethanol tolerant and produced more ethanol at sugar concentration above 15% (v/v).

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%.

Most of the distilleries in India operate at a low efficiency because the yeast strains used are not of good quality. Fermentation efficiencies less than 90% are quite common while it should be 95% on an average. Secondly, exact conditions of temperature, pH and nutrients, which are essential for yeast fermentation, are not vigorously maintained. The **Table 2** below lists some of the yeast strains used in distilleries and the amount of alcohol they produce.

Table 2: Different types of ethanol producing strains

Strain	% Ethanol produced
S.cerevisiae	5.8-11.16
Zygosaccharomyces sp.	4.2
S.ellipsoids	9.7
Schizo.pombe	8.7
Schizo.mallaeri	7.8

(ref. Recycling, residues of agriculture and industry, pp202, M.S.Kalra)

2.3 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 of calculating fermentation efficiency.

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

Glucose — Ethanol

(1 Kg) — (0.511 kg) (0.489 kg)

2.3.1 ALCOHOLIC FERMENTATION

2.3.1a) SUGAR

One of the main constraints in obtaining higher rates of ethanol production is the inhibition of yeast metabolism by both high concentration of sugar substrate as well as the end product. Generally in industrial alcohol production an initial of 16-18% sugar is used and when substrate concentration increases, osmotic pressure becomes pronounced which seriously effects fermentation efficiency.

Janssens *et al* (1983) studied lipid enhanced ethanol production by *Kluyveromyces fragilis* from lactose while maintaining initial sugar concentration between 5-20%, increasing wort concentration was reported to have a detrimental effect on fermentation performance, adversely affecting yeast physiology and altering the physical and flavor properties of beer product Brothwick *et al* (1997), D'Amore (1992), Younis and Stewart (1997). The fermentation ability of a strain of *Kluyveromyces fragilis*, already selected for rapid lactose fermenting capability, was improved by the addition of unsaturated fatty acids and ergosterol to the medium.

Xin *et al* (2003) observed at 45 percent (w/v) glucose, bacterial growth was inhibited while maximum 16.5 percent (w/v) ethanol was produced at 35 percent glucose concentration. Fermentation conditions for production of ethanol from sago starch were optimized by Ratnam *et al* (2003).

2.3.1b) TEMPERATURE

Temperature exerts a profound effect on all aspects of growth, metabolism, survival of fermenting organism and fermentation. Fermentation in industry is usually carried out at ambient temperature (25-30^{o}C).

Mauricio *et al* (1989) observed that high volatile acidity was produced at low temperature. Fermentation ceased at 30^{o}C with 342 g/l sugar in the medium before whole of sugar fermented while 25-30^{o}C caused a negative effect on survival of *Saccharomyces cerevisiae*. Better fermentation rate and efficiency were reported by Saeki *et al* (1997) in a continuous process at higher temperature by thermotolerant bacterial strains which turned out to be 2-3 times higher than that of mesophillic strains at 30°C. Torija *et al* (2001) observed a mixed response to fermentation temperature (15-35^{o}C) on mixed strain population of *S.cerevisiae*. Some strains performed better at higher temperature, while others did so at lower temperature. Alcohol yield was higher at lower temperature while at higher temperature secondary metabolites increased.

Phisalaphong *et al* (2005) developed a mathematical model to describe the effects of temperature on the kinetic parameters of ethanol fermentation by the flocculating yeast, *Saccharomyces cerevisiae* M30, using cane molasses as the substrate. A high temperature led to a decrease in the ethanol and cell yields. The inhibition effect of the initial sugar concentration on cell growth was clearly observed. The adopted mathematical model could describe very well the dynamics of ethanol fermentation from the beginning upto the stationary phase.

2.4 FACTORS AFFECTING FERMENTATION

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.

2.4.1 Effect of sugar concentration

Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. However high substrate concentrations are inhibitory to fermentation (Jones *et al* 1981) due to osmotic stress.

Borzani *et al* (1993) studied fermentation with various initial concentrations of sugar. They also demonstrated the logarithmic relationship between time of fermentation and initial concentrations of sugar. 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. 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).

A repeated batch fermentation system was used to produce ethanol using an osmotolerant *S.cerevisiae* (US3) immobilized on calcium alginate. (Sree *et al* 2000). Fermentation was carried out with initial concentration of 150, 200, 250 g glucose per litre at 30ºC .The maximum amount of ethanol produced by immobilization VS3 cells using 150, 200 and 250 g/l glucose was 72.5, 93 and 83 g ethanol per litre at 30ºC after 48h. Maximum yield was obtained at initial sugar of 20% with fermentation efficiency of 90%.

Converti *et al* (1998) studied the inhibition of the fermentation of oak hemicellulose acid hydrolysates by minor sugars. Synthetic xylose media and detoxified oak hemicellulose acid hydrolysates were fermented batchwise. Maximum productivity was calculated from the experimental data of ethanol concentration. The kinetic parameters calculated for the fermentation of both carbon sources indicate that a competitive inhibition is exerted by the minor sugars (arabinose, rhamnose and galactose) that are metabolized slowly or not at all.

2.4.2 Effect of temperature

The fermentation process is always accompanied with evolution of heat that raises the temperature of the fermenter. As a result it becomes necessary to cool the large fermenters 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 o the fermenting organism. Fermentation in industries is usually carried out at ambient temperature of 25-35ºC but temperature exceeds 40ºC during fermentation especially in northern regions 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 costs of refrigeration control in distilleries for alcohol production. Therefore many studies have been carried out for development of yeast to ferment at high temperature of upto 40-45°C.

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

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.

Morimura *et al* (1997) made an attempt to improve the salt tolerance of the thermotolerant flocculating yeast *Saccharomyces cerevisiae* strain KF-7 by maintaining a high concentration of KCl in the molasses medium. Among selected strains, K211 had the highest cell viability and ethanol productivity in a molasses medium containing 25% (w/v) total sugar at 35°C. As a result of repeated batch fermentation tests with K211, stable ethanol production was achieved with an ethanol concentration of 92g/l and a productivity of 3.5 g/l-h at 33°C in 22% molasses medium. Even at higher temperature of 35°C, strain K211 gave stable ethanol concentration of 91 g/l and productivity of 2.7g/l-h.

2.5 IMMOBILIZATION

Immobilization is the technique for the physical or chemical fixation of cells, enzymes or other proteins onto a solid support, into a solid matrix or retained by a matrix in order to increase their stability and make possible their repeated or continued use.

Immobilized cells exhibit many advantages over free cells such as relative ease of product separation, reuse of biocatalyst, high volumetric productivity, improved process control and reduce susceptibility of cells to contamination. Among the different cell immobilization techniques, entrapment in calcium alginate gel is the most used matrix for whole cell entrapment due to its simplicity and non-toxic character.

Yadav *et al* studied high ethanol productivity in an immobilized cell reactor. Calcium alginate immobilized cells of *S.cerevisiae* HAU 1 were used to carry out fermentation studies using synthetic medium in glass column reactors. Immobilization of yeast cells (10-40% wet w/v) in 1.5% calcium alginate was accomplished and these were tested for ethanol production at varying temperature and pH of the feed. Maximum ethanol productivity (40 g l^{-1} h^{-1}) was achieved at 30ºC, pH 4.5 at a dilution rate 0.20h^{-1}. Incorporation of brick powder in the beads resulted in increased ethanol productivity. The maximum amount of cells that could be immobilized in 1.5% Ca alginate gel was found to be 50%(wet wt basis) but the gel beads containing 30%(w/v) cells resulted in maximum productivity.

Roukas investigated ethanol production from non-sterilized beet molasses by free and immobilized *S.cerevisiae* cells using fed batch culture. Fed batch culture proved to be a better fermentation system for the production of ethanol than batch culture. In fed batch culture, both free and immobilized *S.cerevisiae* gave the same maximum ethanol concentration (53 g/l) at an initial sugar concentration of 250g/l. In the free cell system, the maximum ethanol productivity of 3.5g/l h was obtained at substrate concentration of 250 g/l with 30.6% ethanol yield and 80% sugars utilization. In the immobilized cells system, a maximum ethanol productivity of 3.5g/l h was achieved at an initial sugar concentration of 250g/l with 31.5% ethanol yield and 73.3% sugars utilization.

CHAPTER 3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 A fermenting yeast procured from MTCC, Institute of microbial technology, Chandigarh was used in the present study. Alcohol producing strain was utilized.

Saccharomyces cerevisiae (MTCC NO. 171)

Growth medium	YEPD
Growth condition	Aerobic
Temperature	30C
Incubation time	24 hrs
Subculture	60 days

Special feature: distillery strain, no vitamin requirement, cylindrical plate assay for nystatin, and production of ethanol.

3.2 METHODS

3.2.1 Maintenance of culture

The yeast cultures were maintained by subculturing them every 15 days on YEPD agar plates, incubating for 24 hours at 30ºC and thereafter storing in a refrigrator at 4ºC till further use.

Composition YEPD

Yeast extract	3.0g
Peptone	10.0g
Dextrose	20.0g
Distilled water	1.0L
Agar	15.0g

The medium was sterilized in an autoclave at 15psi for 15minutes.

3.3 Inoculum and inoculation

The yeast inoculum was prepared in YEPD broth. A loopful of twenty four hour old culture was inoculated at 28ºC on a rotary shaker (200rpm) for twenty-four hours. This inoculum was used at 10 percent or as specified to inoculate sterilized molasses broth.

3.4 Fermentation of molasses

Molasses was procured from Patiala distilleries and manufactures limited, Mann village Patiala and it was estimated for total fermentable sugars by the method of Miller (1959) and found to contain 42% reducing sugars. Molasses were diluted to prepare different concentrations of sugars. The production medium was supplemented with nitrogen and phosphorus. The pH of the medium was adjusted to 5.0. For fermentation studies 24 h old inoculum was used to inoculate the production media and the effect of variable parameters like pH, temperature and total reducing sugars was studied. The primary inoculum prepared in YEPD broth was transferred to sterilized production media taken in 250ml flask and incubated at 30ºC under shaking conditions.

Media composition

KH_2PO_4	0.1%
$(NH_4)_2SO_4$	0.5%
$MgSO_4.7H_2O$	0.05%
Yeast extract	0.1%
pH	5

3.5 Optimization of fermentation process

Fermentation process carried out by yeast is 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 so that the production efficiency increases. Various factors were investigated affecting ethanol production from molasses.

3.5.1. Effect of sugar concentration

To study the effect of sugar concentration on ethanol production by *S.cerevisiae*, the production media was prepared by diluting molasses to sugar concentration of 5,10,15,20,25,30 percent with distilled water and filtered through ordinary filter paper to remove suspended particles. Fermentation was carried out in 250 ml conical flasks. A twenty four hour old inoculum of yeast was added at the rate of 6 percent to the medium. Samples were withdrawn after every 12-hour interval and estimated for residual sugars (Miller, 1959) as well as ethanol content in the media (Caputi et al 1968). GC method for estimating the percentage of ethanol was employed. The initial sugar concentration that was efficiently utilized by the yeast for ethanol production was selected and maintained in fermentation media for further use.

3.5.2 Effect of pH

pH of 5.0, 6.0, 7.0 and 8.0 were tested for fermentation using molasses with 20% sugar concentration (best of previous experiment) and temperature of 29 ± 1ºC. Low pH inhibits the yeast multiplication.

3.5.3 Effect of temperature on fermentation of molasses

To optimize the fermentation temperature, fermenation was carried out at 15, 20, 25, 30 and 35ºC. Molasses diluted to 20% sugars and supplemented with nitrogen and phosphorus were used as production media and fermentation was carried out at different temperatures. The periodic samples were analyzed for reducing sugars and ethanol content.

3.5.4 Effect of immobilization

To study the effect of immobilization, previously grown culture of *S.cerevisiae* was immobilized by entrapment in calcium alginate. After accomplishment of immobilization of yeast cells in 2% calcium alginate, these were tested for ethanol production at 30°C, pH 6 and sugar concentration maintained at 20%.

3.6 Analytical methods

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

One millilitre of the fermented wash was taken in 500ml 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 62.5ºC for 20 minutes. The flasks were cooled to room temperature and the volume raised to 50 ml. Five ml of this was diluted with 5ml of distilled water for measuring the optical density at 600nm using a spectrophotometer.

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. Ethanol content of each sample was estimated and graph was made.

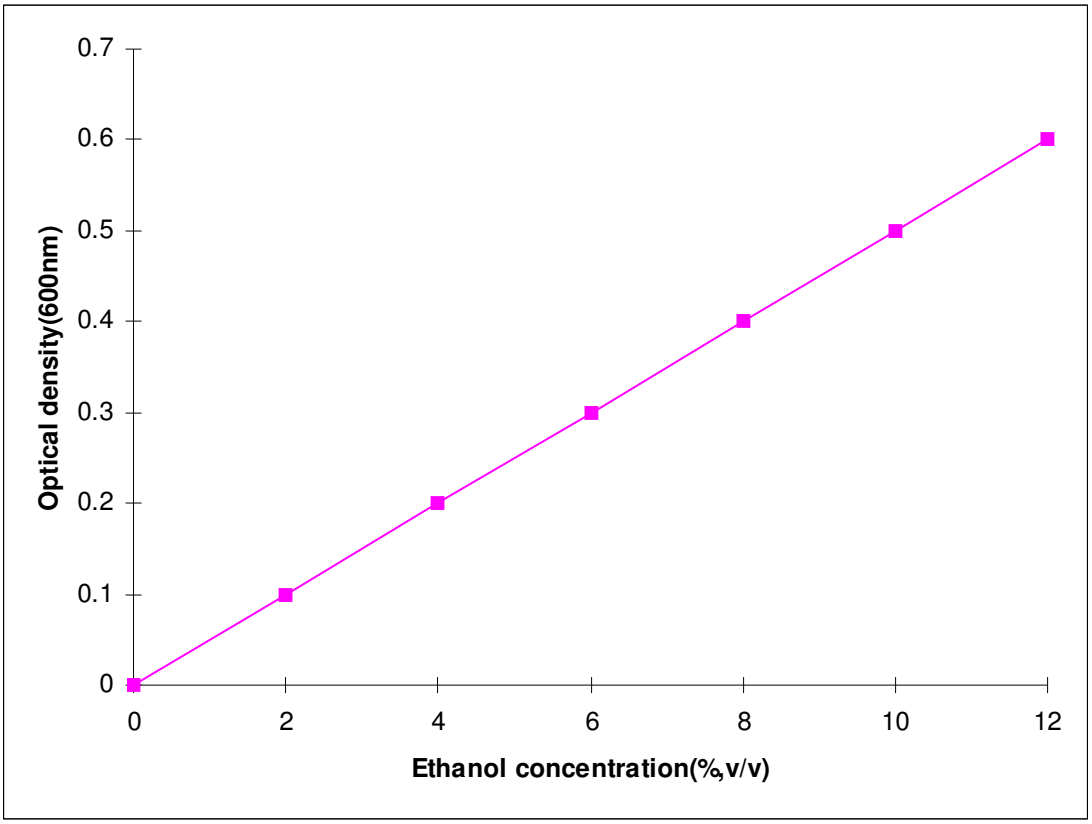


Figure 3.6.1(a) Standard curve for ethanol estimation

Fermentation efficiency

It was calculated as

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

Theoretical recovery = Total sugars χ 0.64

Actual ethanol recovery = Actual ethanol obtained

3.6.2 Estimation of reducing sugars

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

3.6.2.1 Reagents

1. **Substrate solution:** Standard solution of 1000 µ g/ml concentration was prepared by dissolving 100 mg of glucose in 100ml of distilled water.
2. **3,5 dinitrosalicylic acid (DNS) solution:** Reagent was prepared by dissolving 10.0g of 3,5-DNS, 2.0g 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 colored bottle.
3. **Potassium sodium tartarate (Rochelle salt) :** 40 g of potassium sodium tartarate was dissolved in distilled water and the volume was made to 100ml.

3.6.2.2 Procedure

One ml of appropriately diluted solution (500-1000 µg ml^{-1}) sample was taken in a test tube to which 3ml 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 test 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 100-1000 g concentration prepared in distilled water.

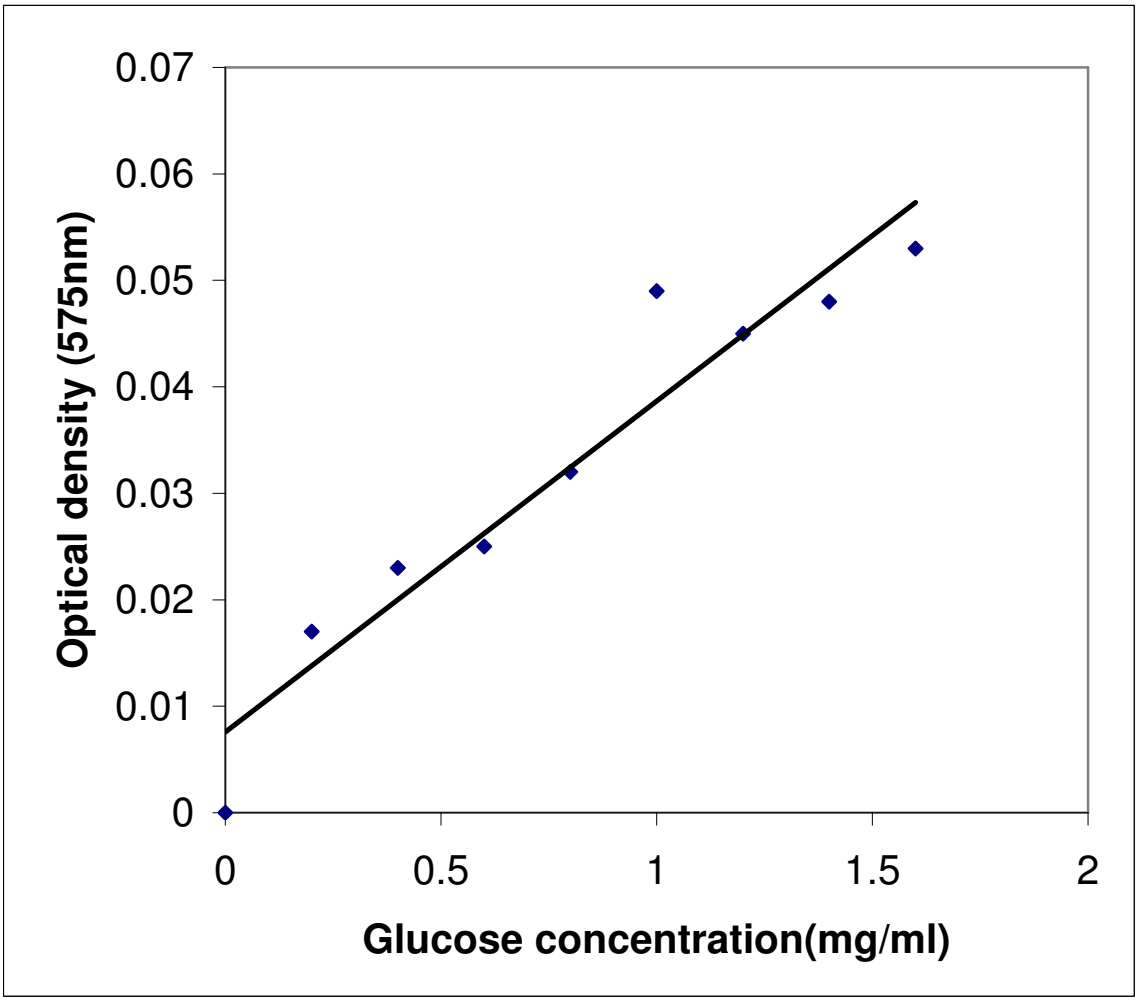


Figure 3.6.2.2(a) Standard curve for estimation of reducing sugars

3.6.3 Gas chromatography

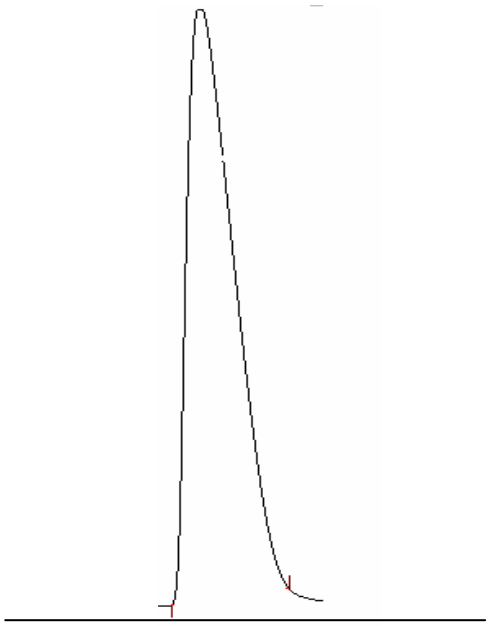
Ethanol in the fermentation broth was estimated by gas chromatography method. A computer related Nucon series gas chromatograph equipped with flame ionization detector (FID) was employed for the separation and quantification of ethanol. A stainless steel column (5m × 2mm) was fitted into the instrument to provide on column injection. The column packing was Porapak Q. The detector and injector temperature was maintained at 200°C. The gas

chromatograph was connected to an integrator and computer system to determine area of ethanol and internal standard peak. For analysis of ethanol the following program has been standardized:

Nature of the column	Porapak Q
Dimensions	5m × 2mm
Material	Stainless steel
Carrier Gas	Nitrogen (flow rate 50 kg/cm^2)
Hydrogen/oxygen ratio	2: 1

PROGRAM:		
	Oven 1 temperature	75°C
	Rate 1	10°/minute
	Oven 2 temperature	200°C
	Injection temperature	200°C
	Detection temperature	200°C

Standard solutions of ethanol were prepared. The standards contain 2,3,4,5,6,7,8% ethanol. The standard curve was prepared with retention time 7.6 minutes. The area under peak was determined for samples and by comparing with standard curve; the percentage of ethanol was measured.



Time (minutes)

Fig 3.6.3(a): GC chromatogram of standard alcohol.

3.7 IMMOBILIZATION

3.7.1 Procedure

To carry out immobilization, 2% of $CaCl_2$ solution was prepared and kept at 4°C for chilling. 30-40ml of previously grown culture of *S.cerevisiae* was centrifuged at 500rpm for 15 minutes. The supernatant was discarded and the pellet washed with saline water. Again centrifugation was carried out at 500rpm for five minutes to obtain the final pellet that was washed and then air-dried and weighed. The next step was to dissolve 2g of sodium alginate in hot water with constant stirring on magnetic stirrer. After cooling sodium alginate solution, 2g of yeast biomass was added to the slurry under stirring conditions for even dispersal. The slurry solution, with yeast biomass was dispersed drop wise into 2% chilled $CaCl_2$ solution. Spherical beads were formed which were washed with 0.2% chilled $CaCl_2$ solution and stored at 4°C for further use to carry out fermentation.

CHAPTER 4.RESULTS AND DISCUSSION

Optimization studies on fermentation of molasses by *S.cerevisiae.*

4.1. Growth studies and effect of sugar concentration

The growth of *S.cerevisiae* in gradually increasing concentrations of sugar showed an increase in optical density upto 20% sugar concentration in YEPD medium as shown in **Table 1**. However on increasing the sugar concentration beyond 20%, the growth was inhibited as shown by the optical density measured. Samples were taken every 12 hours for the study of growth kinetics. The growth was measured at 600nm.

Moaris *et al* (1996) also studied viability of *Saccharomyces* sp. in 50% glucose and reported a viability of 10-98.8% in different strains of yeast. 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 were carried out with sugar concentrations upto 20%.

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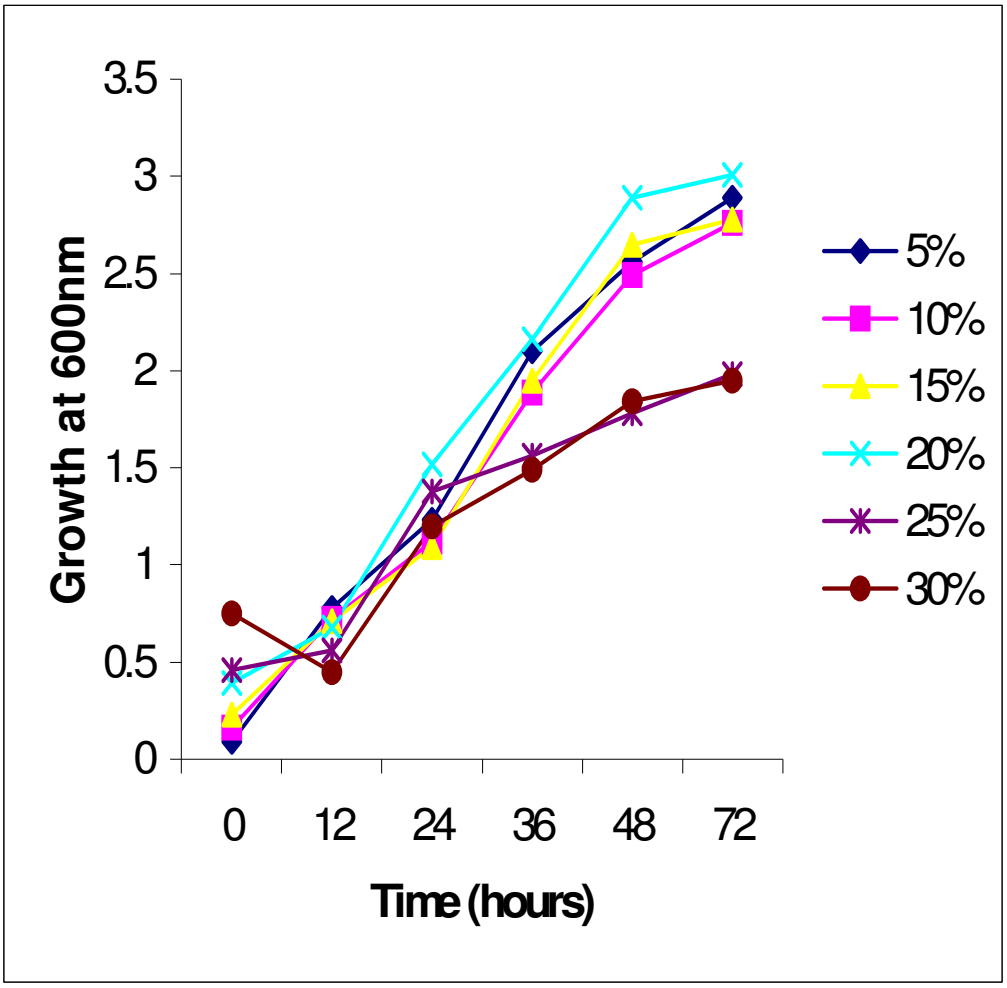
Table1 Effect of increasing sugar concentration on *S.cerevisiae*

Time (hrs)	Sugar concentration (%)					
	5%	**10%**	**15%**	**20%**	**25%**	**30%**
0	0.09	0.16	0.23	0.39	0.46	0.75
12	0.78	0.72	0.71	0.68	0.56	0.45
24	1.23	1.12	1.09	1.52	1.38	1.2
36	2.1	1.89	1.95	2.16	1.56	1.49
48	2.56	2.49	2.65	2.89	1.78	1.84
72	2.89	2.76	2.78	3.01	1.98	1.95

Graph 1. Effect of increasing sugar concentration on growth of *S.cerevisiae* at 6 pH and 30°C temperature



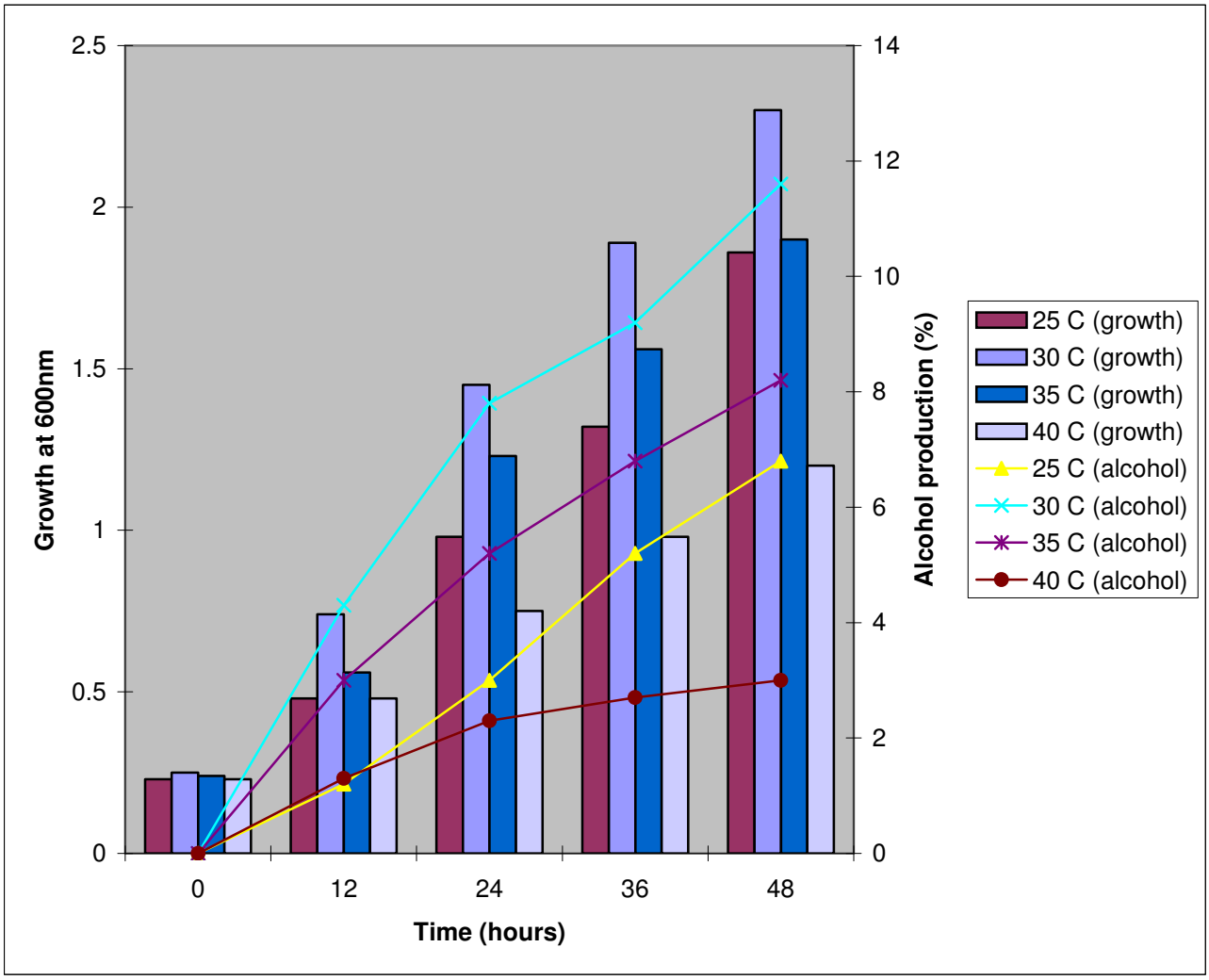
4.2 Effect of temperature on ethanol yield

Temperature is one of the major constraints that determines the ethanol production. To know the optimum temperature for ethanol fermentation, the solutions were kept at 25, 30, 35 and 40°C with 20% initial sugar concentration. The molasses were diluted upto 20% sugars and fermentation was carried out in 250ml flasks. Two parameters were simultaneously studied, the growth of *S.cerevisiae* and the ethanol yield. Samples were withdrawn every 12 hours and the fermentation was carried out for 48 hours. A low ethanol yield of 6.8% was observed at 25°C in 48 hours. As shown in **Table 2** at 30°C ethanol yield was maximum and turned out to be 11%. However increasing the temperature beyond 30°C the growth as well as concentration of alcohol decreased.this decrease was pronounced at 40°C so 30°Cwas selected as optimum temperature for ethanol production.

Temperature tolerance was also been found to depend upon sugar concentrations 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 2 Effect of temperature on ethanol production

Time (hrs)	Growth (ln O.D.)				Alcohol (in %)			
	25°C	30°C	35°C	40°C	25°C	30°C	35°C	40°C
0	0.23	0.25	0.24	0.23	0	0	0	0
12	0.48	0.74	0.56	0.48	1.2	4.3	3	1.3
24	0.98	1.45	1.23	0.75	3	7.8	5.2	2.3
36	1.32	1.89	1.56	0.98	5.2	9.2	6.8	2.7
48	1.86	2.3	1.9	1.2	6.8	11.6	8.2	3



Graph 2 Effect of temperature on alcohol production at pH 6and 20%sugar concentration.

4.3 Effect of pH on ethanol yield

Initial sugar concentration of 20% and optimum temperature of 30°C was selected for further studies and subjected to pH treatments 5, 6, 7 and 8. The results are shown in table 3.

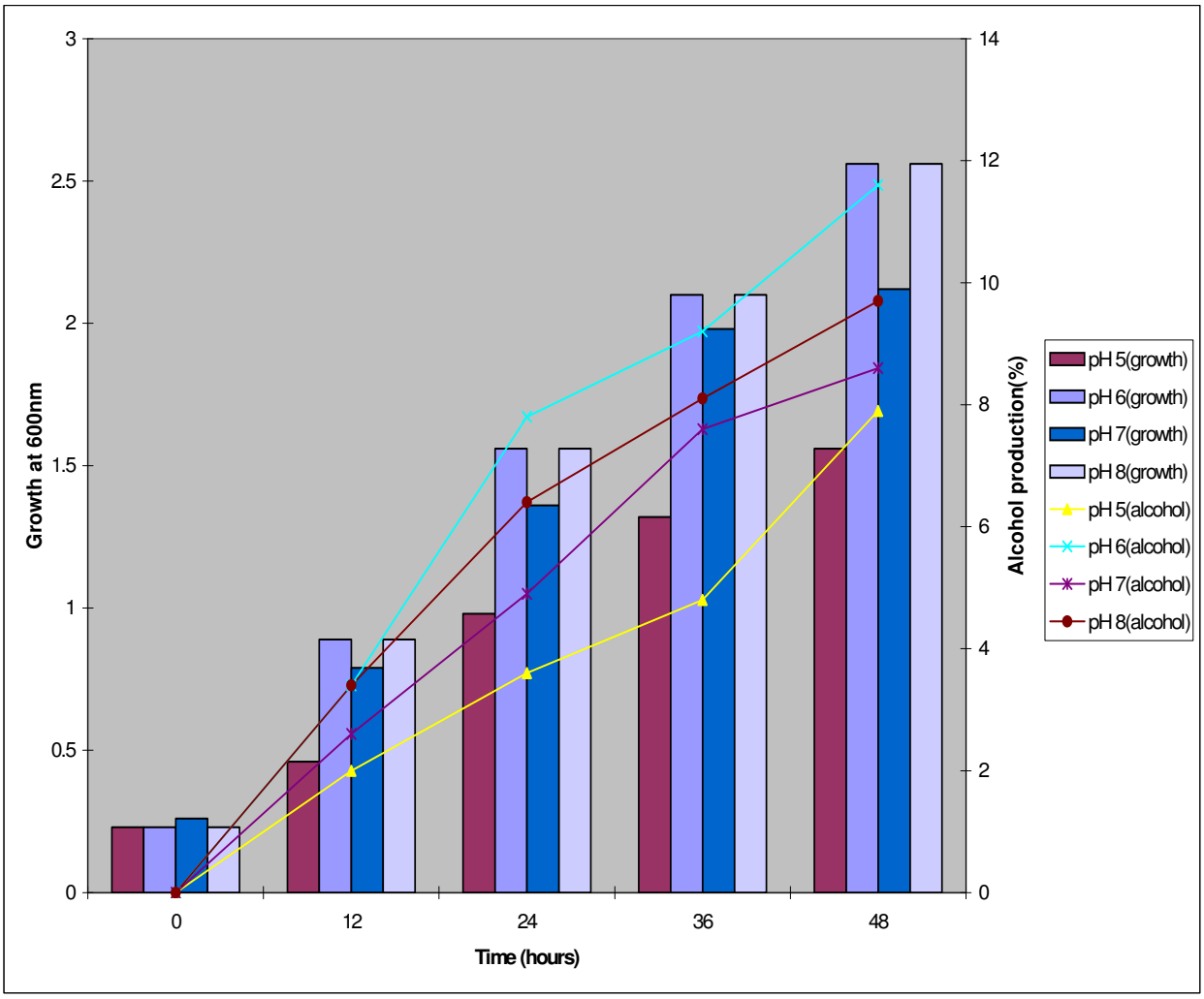
At pH 5, fermentation took place but it gave low ethanol content. Best results were obtained at pH 6 where maximum ethanol production was noticed.

Yadav *et al* (1997) found an increase in alcohol concentration, productivity as well as efficiency with an increase in pH from 4.0-5.0 and found that the optimum pH range for *S.cerevisiae* strain HAU-1 to be between pH 4.5-5.0.

Based on fermentation efficiency the pH 6 was selected for further experimentation.

Table 3 Effect of pH on ethanol production

Time (hrs)	Growth				Alcohol			
	pH 5	pH 6	pH 7	pH 8	pH 5	pH 6	pH 7	pH 8
0	0.23	0.23	0.26	0.23	0	0	0	0
12	0.46	0.89	0.79	0.89	2	3.4	2.6	3.4
24	0.98	1.56	1.36	1.56	3.6	7.8	4.9	6.4
36	1.32	2.1	1.98	2.1	4.8	9.2	7.6	8.1
48	1.56	2.56	2.12	2.56	7.9	11.6	8.6	9.7



Graph 3 Effect of pH on ethanol production at 20% sugar concentration and 30°C temperature

4.2 Effect of immobilization on ethanol yield

Immobilization is the restriction of cell mobility within a defined space.

Sree *et al* (2000) used immobilized cells of *S.cerevisiae* that could ferment upto 25% glucose. Yadav *et al* studied high ethanol productivity in an immobilized cell reactor. Maximum ethanol productivity (40 g l^{-1} h^{-1}) was achieved at 30ºC, pH 4.5 at a dilution rate 0.20h^{-1}. The maximum amount of cells that could be immobilized in 1.5% Ca alginate gel was found to be 50%(wet wt basis) but the gel beads containing 30%(w/v) cells resulted in maximum productivity.

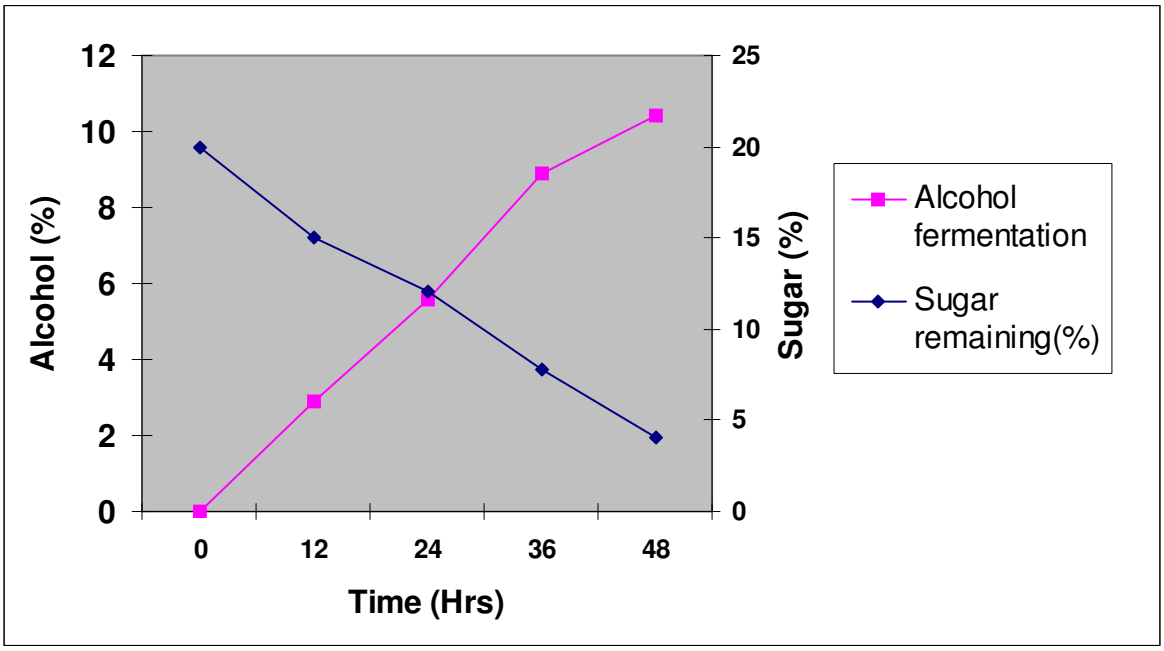
After carrying out immobilization of *S.cerevisiae*, the immobilized beads were used to carry out the fermentation. After 48hrs of fermentation the samples were filtered and the beads removed. This was followed by washing the beads with saline water and again carrying out fermentation by reusing the beads. Samples were withdrawn and checked for the production of ethanol by using GC. Continuous production of ethanol was noticed though the production was less as compared to the fermentation carried out using fresh immobilized culture of *S.cerevisiae.*

Table 4 Effect of immobilization on ethanol yield

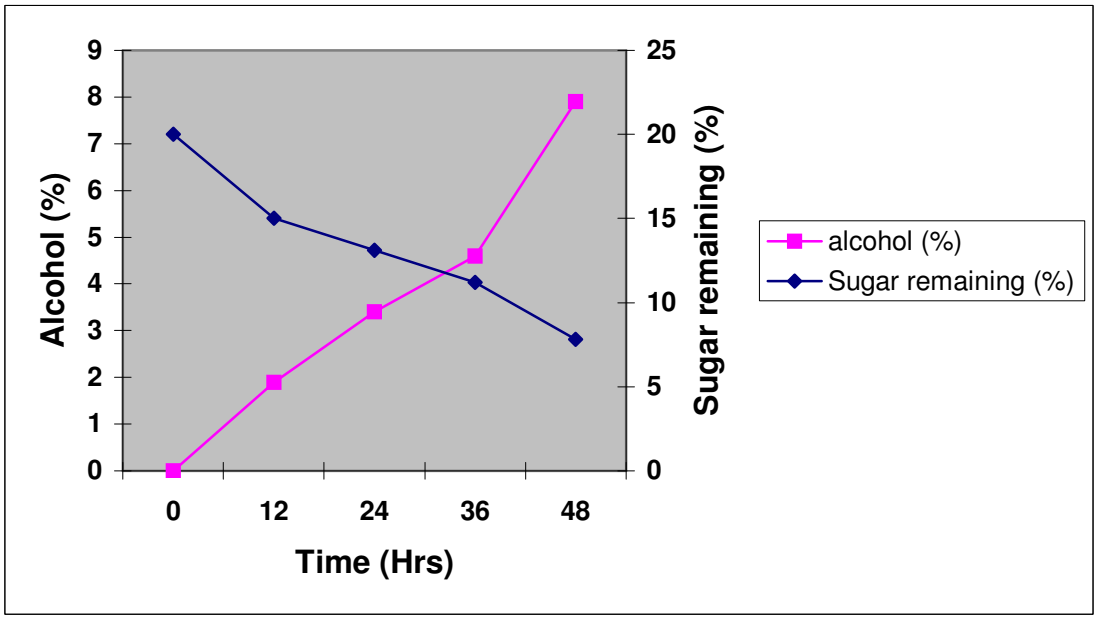
Time(hrs)	Alcohol(%)	Sugars remaining
0	0	20
2	2.9	15
24	5.6	12.1
36	8.9	7.8
48	10.4	4.1

Table 5 Effect of reuse of immobilized cells

Time (hrs)	Alcohol (%)	Sugars remaining
0	0	20
12	1.9	15
24	3.4	13.1
36	4.6	11.2
48	7.9	7.8



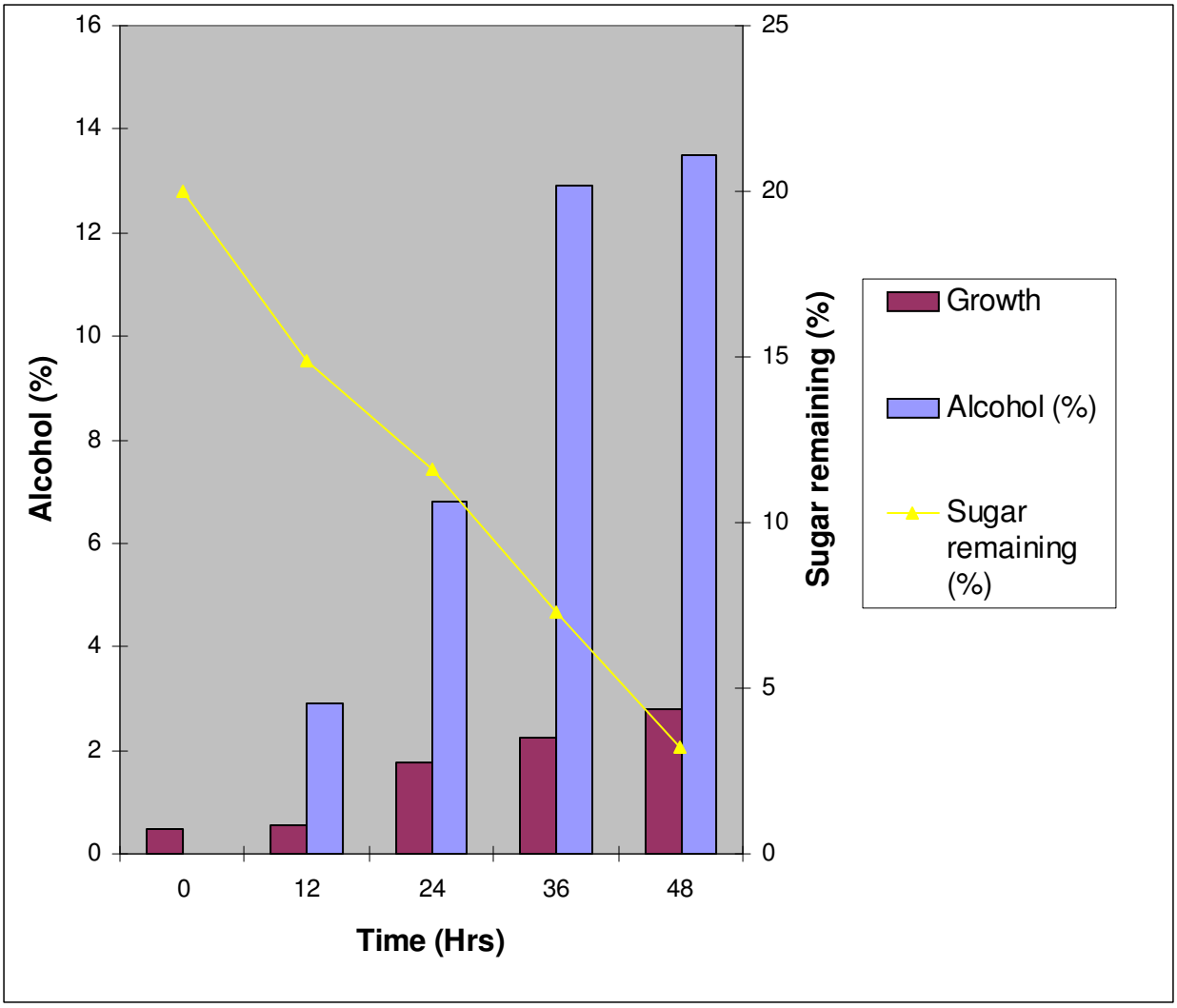
Graph 4 Effect of immobilization on ethanol yield at 6pH, 30°C and 20% sugar concentration



Graph 5 Reuse of immobilized cells under same set of conditions

4.3 Increase in ethanol yield using fermenter

After optimizing the various parameters like pH, temperature, sugar concentration etc. the experiment was scaled up from shake flask to fermenter. The optimum of previous experiments was taken i.e. the sugar concentration of 20%, pH 6 and temperature 30°C to further carry the experiment on fermenter. Fermenters are designed to provide best possible growth and biosynthesis conditions for industrially important microbial cultures. In fermenter, it is easier to control various parameters like temperature, pH that increases the ease of obtaining the desired product, ethanol in present study. After carrying out the fermentation, the samples were analysed using GC and compared with the standard run of absolute ethanol. From the broadness of peak it was inferred that there was continuous increase in ethanol production till 48 hrs. Graph 6 clearly shows the hike in ethanol production as the sugar is being consumed.



Graph 6 Ethanol yield using fermenter with pH6, sugar concentrarion 20% and temperature 30°C

SUMMARY

The fermentation of molasses using *S.cerevisiae* (distillery strain) under optimized conditions i.e. pH 6, sugar concentration 20% and temperature 30°C revealed an increase in ethanol production with good fermentation efficiency. However fermentation efficiency deceases after 48 hours of fermentation time. This might be due the either substrate limitations or due to product inhibition. *S.cerevisiae* reportedly showed the decrease in growth with increase in ethanol concentration in the medium.

Peres and Laluce 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.

Further studies involving fermentation of molasses using optimized conditions in the fermenter (2 L working volume) showed an increase in the ethanol production. However the removal and discard of used cells remained a problem in the alcoholic fermentations. This obstacle was removed by carefully immobilizing the cells using calcium alginate process. Again with the same optimized conditions used in previous experiments, fermentation was then carried out using immobilized cells and good results were obtained. Same immobilized cells after first fermentation were used for second fermentation and results were acceptable.

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