

**Production of ethanol from cheese waste water using**  
*Kluyveromyces marxianus var marxianus*

by

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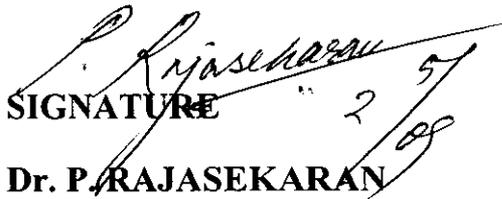
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**BONAFIDE CERTIFICATE**

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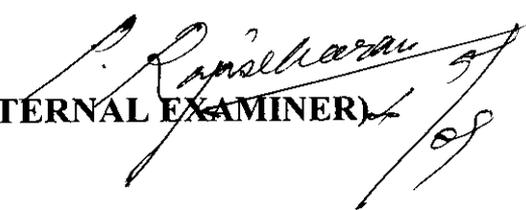
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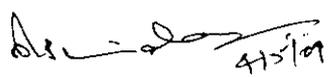
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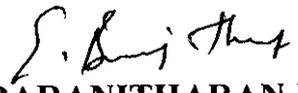
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## LIST OF ABBREVIATIONS

YEPD	Yeast extract peptone dextrose medium
°C	Degree Celsius
DNS	Dinitrosalicylic acid
kDa	Kilo Dalton
COD	Chemical oxygen demand
OD	Optical density
%	Percentage
w/v	Weight/volume
v/v	Volume/volume
ppm	Parts per million
nm	Nanometre

***ABSTRACT***

## ABSTRACT

Cheese whey contains 5–6% lactose (w/v), 0.8–1% protein, and 0.06% fat constituting an inexpensive and nutritionally rich raw material for ethanol fermentation. Cheese whey has been used by many investigators as the raw material for ethanol fermentations because of its high carbohydrate content and availability. Fermenting strains of *Kluyveromyces marxianus var marxianus* was utilized for alcoholic fermentation using cheese whey. The fermentation of cheese whey was optimized with respect to temperature, pH, inoculum ratio and sugar concentration. Results revealed a temperature of 30°C, pH 6.0, inoculum ratio of 2% v/v and 20% sugar concentration as optimum for fermentation. The low percentage of lactose in cheese whey (5–6% lactose (w/v)) causes low ethanol yield. Therefore, cheese whey water is concentrated to produce high ethanol yield by converting it to cheese whey powder.

# ***INTRODUCTION***

## 1. INTRODUCTION

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 of ethanol

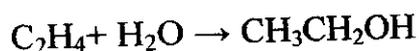
Ethanol or ethyl alcohol,  $\text{CH}_3\text{CH}_2\text{OH}$ , has been described as one of the most exotic synthetic oxygen-containing organic chemicals because of its unique combination of properties as a solvent, a germicide, a beverage, antifreeze, a fuel, a depressant, and especially because of 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  $78.5^\circ\text{C}$ . 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  $78.15^\circ\text{C}$ . 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 microorganisms. 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



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 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



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.

**Table 1.1: Annual ethanol production in various countries**

**Total Annual Ethanol Production (All Annual fuel ethanol production by grades)by country (2004-2006) Top 15 country (2007) Top 15 countries countries (Millions of (U.S. liquid gallons) (Millions of U.S. liquid gallons)**

World rank	Country	2006	2005	2004	World rank	Country/Region	2007
1	United States	4,855	4,264	3,535	1	United States	6,498.6
2	Brazil	4,491	4,227	3,989	2	Brazil	5,019.2
3	China	1,017	1,004	964	3	European Union	570.3
4	India	502	449	462	4	China	486.0
5	France	251	240	219	5	Canada	211.3
6	Germany	202	114	71	6	Thailand	79.2
7	Russia	171	198	198	7	Colombia	74.9

8	Canada	153	61	61	8	India	52.8
9	Spain	122	93	79	9	Centra America	39.6
10	South Africa	102	103	110	10	Australia	26.4
11	Thailand	93	79	74	11	Turkey	15.8
12	United Kingdom	74	92	106	12	Pakistan	9.2
13	Ukraine	71	65	66	13	Peru	7.9
14	Poland	66	58	53	14	Argentina	5.2
15	Saudi Arabia	52	32	79	15	Paraguay	4.7
	World Total	13,489	12,150	10,770		World Total	13,101.7

**Table 1.2:** Ethanol Production from different sources

<b>Crop</b>	<b>Annual (Liters/hectare)</b>	<b>Annual yield (US gal/acre)</b>	<b>Annual yield (US gal/acre)</b>	<b>Greenhouse- gas savings (% vs. petrol)</b>	<b>Comments</b>
<i>Miscanthus</i>	7300	780	780	37–73	Low-input perennial grass. Ethanol production depends on development of cellulosic technology.
Switchgrass	3100–7600	330–810	330–810	37–73	Low-input perennial grass. Ethanol production depends on development of cellulosic technology. Breeding efforts underway to increase yields. Higher biomass production possible with mixed species of perennial grasses.
Poplar	3700–6000	400–640	400–640	51–100	Fast-growing tree. Ethanol production depends on

Sugarcane	6800–8000	727–870 87–96	<p>development of cellulosic technology. Completion of genomic sequencing project will aid breeding efforts to increase yields.</p> <p>Long-season annual grass. Used as feedstock for most bioethanol produced in Brazil. Newer processing plants burn residues not used for ethanol to generate electricity. Only grows in tropical and subtropical climates.</p>
Sweet sorghum	2500–7000	270–750 No data	<p>Low-input annual grass. Ethanol production possible using existing technology. Grows in tropical and temperate climates, but highest ethanol yield estimates assume multiple crops per year (only possible in</p>

tropical climates). Does not store well.

High-input annual grass. Used as feedstock for most bioethanol produced in USA. Only kernels can be processed using available technology; development of commercial cellulosic technology would allow stover to be used and increase ethanol yield by 1,100 - 2,000 litres/ha.

Corn	3100–4000	330–424	10–20
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Source: Savings of GHG emissions assuming no land use change (using existing crop lands), *Nature* 444 (December 7, 2006): 673-676.

### **1.3 Fuel System Problems**

Several of the outstanding ethanol fuel issues are linked specifically to fuel systems. Fuels with more than 10% ethanol are not compatible with non E85-ready fuel system components and may cause corrosion of ferrous components. Ethanol fuel can negatively affect electric fuel pumps by increasing internal wear, cause undesirable spark generation, and is not compatible with capacitance fuel level gauging indicators and may cause erroneous fuel quantity indications in vehicles that employ that system. It is also not always compatible with marine craft, especially those that use fiberglass fuel tanks. Ethanol is also not used in aircraft for these same reasons. Using 100% ethanol fuel decreases fuel-economy by 15-30% over using 100% gasoline; this can be avoided using certain modifications that would, however, render the engine inoperable on regular petrol without the addition of an adjustable ECU. Tough materials are needed to accommodate a higher compression ratio to make an ethanol engine as efficient as it would be on petrol; these would be similar to those used in diesel engines which typically run at a CR of 20:1,] versus about 8-12:1 for petrol engines. In April 2008 the German environmental minister cancelled a proposed 10% ethanol fuel scheme citing technical problems: too many older cars in Germany are unequipped to handle this fuel. Ethanol levels in fuel will remain at 5%.

### **1.4 Utilization of cheese whey and its components**

Annually, approximately 1.2 million tons of lactose and 200,000 tons of milk protein are transferred into whey worldwide, of which less than 60% are utilized for human food and animal feed. Thus, a nutritionally valuable food resource is wasted. Some progress has been made in utilizing whey,

whey solids, and whey protein concentrates in the manufacture of dairy, bakery, and specialized products. However, the potential of whey and whey derivatives is not being fully utilized.

In particular, various avenues of utilizing lactose are open. These possibilities include the conversion of lactose into glucose, galactose and fructose to improve its sweetening power, production of alcohol from lactose, production of single cell proteins from lactose, and synthesis of gums from dairy products. There is a great potential to produce long shelf-life whey-based drinks, but little progress has been made in the widespread commercialization of such products. A need does exist to improve the functional properties of whey proteins. The use of whey proteins to replace non-fat dry milk and casein would require an improvement in their water absorption property. Aside from the development of new processes and products, there is also a great need to economize and commercialize newly developed technology.

## **1.5 Uses**

### **1.5.1 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.

### **1.5.2 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.

### **1.5.3 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.

#### **1.5.4 Cheese whey**

Cheese whey is the liquid remaining following the precipitation and removal of milk casein during cheese-making. This byproduct represents about 85-95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10% of dried extract). Cheese-whey salts are comprised of NaCl and KCl (more than 50%), calcium salts (primarily phosphate) and others.

Two main whey varieties produced are acid (pH <5) and sweet (pH 6-7) whey, according to the procedure used for casein precipitation. Acid wheys typically have higher ash and lower protein contents than sweet wheys, their use in alimentation being more limited precisely because of their acidic flavour and high saline content.

### **1.5.5 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. To recover lactose from cheese whey water using ultrafiltration.
2. To produce cheese whey powder from concentrated cheese whey water.
3. To optimize various parameters such as pH, temperature, inoculum ratio and substrate concentration for ethanol production.

# *LITERATURE REVIEW*

## 2. LITERATURE REVIEW

On account of limited global supply of oil, ethanol has emerged as an alternative for petroleum based liquid fuels. Now a days, its 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. 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 and 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 and Kumar, 1998), sugar beet, beet molasses (Diwany *et al.*, 1992 and Agrawal and Kumar, 1998), Sweet sorghum (Bulawayo *et al.*, 1996) and starchy materials like sweet potato. 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) 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. 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°C employing *Saccharomyces cerevisiae* (Sharma and Tauro., 1986 and Bulawayo *et al.*, 1996). Cane molasses is a complex mixture that varies in composition according to geographical sources, agricultural practices and sugar mill operations. (Yadav *et al.*, 1999) studied the effect of pretreatment of sugarcane molasses for ethanol production by yeast. The effect of pretreatment of molasses with H<sub>2</sub>SO<sub>4</sub> and K<sub>4</sub>Fe(CN)<sub>6</sub> 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.

## 2.2 Organisms

In fermentation, the common ethanol producing micro-organisms are yeast belonging to *Saccharomyces cerevisiae* have been used mostly. (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. (Singh *et al.*, 1998) did a comparative study on ethanol production from molasses using *Saccharomyces cerevisiae* and *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 below lists some of the yeast strains used in distilleries and the amount of alcohol they produce.

**TABLE 2.1:** Different types of ethanol producing strains

Strain	% Ethanol produced
<i>S.cerevisiae</i>	5.8-11.16
<i>Zygosaccharomyces sp</i>	4.2
<i>S.ellipsoids</i>	9.7
<i>S.pombe</i>	8.7
<i>S.mallaeri</i>	7.8

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

## 2.3 Alcoholic fermentation

### 2.3.1 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).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. Fermentation conditions for production of ethanol from sago starch were optimized by (Ratnam *et al.*, 2003).

### **2.3.2 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 °C). Fermentation ceased at 30 °C with 342 g/L sugar in the medium before whole of sugar fermented while 25-30 °C caused a negative effect on survival of *Saccharomyces cerevisiae*. Alcohol yield was higher at lower temperature while at higher temperature secondary metabolites increased. (Phisalaphong *et al.*, 2006) 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 30 °C .The maximum amount of ethanol produced by immobilization VS3 cells using 150, 200 and 250 g/L glucose was 72.5, 93 and 83g ethanol per litre at 30°C after 48h.

Maximum yield was obtained at initial sugar of 20% with fermentation efficiency of 90%. (Converto and Bogni, 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 of the fermenting organism. Fermentation in industries is usually carried out at ambient temperature of 25- 35 °C but temperature exceeds 40 °C 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.*, 1993) 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 marxianus var. marxianus* 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 Ethanol fermentation from cheese whey:**

The treatment of whey by fermenting lactose to ethanol has received wide attention to date, and various large-scale procedures have been developed. Several distilleries producing ethanol from whey are in

commercial operation in Ireland, the USA and, particularly, New Zealand, where 50% of the cheese-whey production is used to produce ethanol. Up to now, *Kluyveromyces fragilis* is the microorganism of choice for most commercial plants. In batch fermentation *Kluyveromyces fragilis* utilizes more than 95% of the lactose of unconcentrated whey with a conversion efficiency of 80-85% of the theoretical value of 0.538 kg ethanol/ kg lactose consumed. In general, the production of ethanol from nonconcentrated cheese whey is not economically feasible because the levels of ethanol obtained reach only about 2%, making the distillation process too expensive. Several strains have been selected that are capable of fermenting concentrated lactose solutions and of producing ethanol with more than 90% conversion efficiency. Costs are significantly reduced with the increase of lactose concentration up to about 100-120 g/L lactose. The production of ethanol by continuous fermentation, and with cell-recycling or cell immobilization to increase productivity, has also been explored using both unconcentrated and concentrated whey. Fed-batch fermentations have also been considered. Not only is the number of microorganisms able to metabolize lactose directly to ethanol limited, but also they are inhibited by moderate sugars and ethanol concentrations. *Saccharomyces cerevisiae*, the yeast most utilized in wine and beer fermentations, lacks the lactose permease system (the membrane lactose carrier that controls the entry of sugar into the cells), as well as the intracellular enzyme for lactose hydrolysis,  $\beta$ -galactosidase, thus rendering it unable to ferment lactose directly into ethanol. One interesting alternative consists of the hydrolysis of lactose by  $\beta$ -galactosidase from another microorganism and subsequent fermentation by *Saccharomyces cerevisiae*. This process can be developed in two steps or in only one step with mixed cultures or with the enzyme and

yeast co-immobilized. However, when *S. cerevisiae* uses the mixture of glucose and galactose as a C-source, it manifests diauxic growth and lower yields in ethanol production, even for strains previously adapted to galactose. Moreover, other disadvantages of these processes are the high price of  $\beta$ -galactosidase and the failure of this enzyme to hydrolyze all the lactose, thus leaving the problems associated with effluent disposal unsolved. Another alternative that is currently being intensively studied consists of achieving, by recombinant DNA techniques, the expression of the genes that code for the galactosidase and lactose permease system of *Kluyveromyces lactis* in *Saccharomyces cerevisiae*. In this way, *S. cerevisiae* could be developed directly on cheese whey producing high yields of ethanol or other commercially useful fermentation products. However, the recombinant yeasts elaborated up to this point in time are very slow growing and have reduced genetic stability, so yields are low even when these recombinant yeasts are used in specially designed bioreactors.

## **2.6 Cheese whey:**

Whey is a heavy organic pollutant with high biochemical oxygen demand (40 g/L to 60 g/L) and COD (50 g/L to 80 g/L). Whey also contains appreciable quantities of lactic (0.5 g/L) and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins. Availability of lactose and presence of other essential nutrients for the growth of microorganisms make whey a potential raw material for the production of different bio-products.

Actual market trends point to a gradual increase in cheese production that generates more than  $145 \times 10^6$  ton of liquid whey per year, with  $6 \times 10^6$  ton corresponding to lactose. To make 1 kg of cheese, 9 kg of whey is generated.

Because of its low concentration of milk constituents (whey is only 6-7% dry matter); whey has commonly been considered a waste product.

## **2.7 Recent developments**

Recent research attempts have tried to develop technologies that employ whey as raw material to produce foods or chemicals of added value, and products such as single-cell proteins, lactic, citric and propionic acids, enzymes, glucose, methane, oligosaccharides, ethanol and others have been proposed.

As research in the field of whey utilization continues, a variety of new whey products are currently being developed. The treatment of whey by fermenting lactose to ethanol has received wide attention to date, and various large-scale procedures have been developed. Several distilleries producing ethanol from whey are in commercial operation in Ireland, the USA and, particularly, New Zealand where 50% of the cheese-whey production is used to produce ethanol.

During the last two decades, various research efforts have been done on this theme, and yeasts like *Kluyveromyces fragilis* and *K. marxianus* have been proposed as suitable biocatalysts for this bioprocess to produce an alcoholic beverage by cheese whey fermentation in a large-scale reactor, using a continuous system, which is more advantageous than batch or fed-batch operations, since labour and cleaning costs are lower, equipment size is reduced; product quality is uniform, with high product yield in less time, increasing process productivity. *Kluyveromyces marxianus* grows rapidly even at temperatures above 40 °C, it is very versatile and could be economically explored for a wide range of applications.

## ***MATERIALS AND METHODS***

### 3. MATERIALS AND METHODS

#### 3.1 Microorganism

*Kluyveromyces marxianus var marxianus* (MTCC no.1388) obtained from MTCC, Chandigarh in lyophilized form was used in all experiments.

Growth medium	YEPD
Growth condition	Aerobic
Temperature	25 °C
Incubation time	48 h
Subculture	30 days

#### 3.2 Methods

##### 3.2.1 Maintenance of culture

The yeast cultures were maintained by subculturing them for every 30 days on YEPD agar plates and incubated at 48 hrs at 25 °C thereafter it was stored in a refrigerator at 4 °C for further use.

##### 3.2.2 Composition of YEPD

Appendix 1

##### 3.2.3 Inoculum

A loopful of twenty four hour old culture was inoculated in 250ml flask containing 100ml YEPD broth. The culture was grown in rotary shaker at 150rpm and 25 °C for forty-eight hours.

### **3.3 Optimization of biomass**

Fermentation process carried out by yeast is known to vary with respect to substrate concentration, temperature and pH. It's therefore imperative to optimize the fermentation conditions for yeast cells so that the production efficiency increases. Various factors were investigated affecting ethanol production from cheese waste water.

#### **3.3.1. Effect of sugar concentration**

To study the effect of sugar concentration on ethanol production by *K.marxianus*, the production media was prepared by diluting cheese whey powder to sugar concentration of 5, 10, and 15, 20 and 25 % with distilled water. Fermentation was carried out in 250 ml conical flasks. A forty eight hour old inoculum of yeast was added at the rate of 3 percent to the medium. 1ml of sample from each Samples were withdrawn after every 24-hour interval and estimated for residual sugars as well as ethanol content in the media (Caputi *et al.*, 1968)..

#### **3.3.2 Effect of pH**

pH of 6.0, 7.0 and 8.0 were tested for fermentation using cheese whey with 20 % sugar concentration (best of previous experiment) and temperature of 28 °C.

#### **3.3.3 Effect of temperature on fermentation of cheese whey**

To optimize the fermentation temperature, fermentation was carried out at 25 °C, 30 °C and 35 °C. Cheese whey was diluted to 20% sugars and used as production media .The periodic samples were analyzed for reducing sugars and ethanol content.

### **3.4. Estimation of reducing sugars**

The DNS method was used to estimate reducing sugars.

## Appendix 2

### **3.5 Separation of lactose from cheese whey water**

#### **3.5.1 Ultrafiltration**

Ultrafiltration (UF) is a variety of membrane filtration in which hydrostatic pressure forces a liquid against a semipermeable membrane. Suspended solids and solutes of high molecular weight are retained, while water and low molecular weight solutes pass through the membrane. This separation process is used in industry and research for purifying and concentrating macromolecular (10<sup>3</sup> - 10<sup>6</sup> Da) solutions, especially protein solutions. Ultrafiltration is not fundamentally different from microfiltration or nanofiltration, except in terms of the size of the molecules it retains. The molecular weight cut-off of 50 kDa was used for the filtration purpose. Carefully the permeate was collected and checked for the lactose concentration.

### **3.6 Production of cheese whey powder**

#### **3.6.1 Flash evaporation**

Cheese whey water was taken, it is then poured in round bottom flask of flash evaporator(laborota 4000-Efficient, Heidolph , Germany) and maintain the temperature at 65 °C and undergo evaporation. After flash

evaporation cheese whey water will be converted into concentrated cheese whey water.

### **3.6.2 Freezing**

Larger crystals are easier to freeze-dry. To produce larger crystals, the product should be frozen slowly or can be cycled up and down in temperature. This cycling process is called annealing. Usually, the freezing temperatures are between - 50 °C and - 80 °C. The freezing phase is the most critical in the whole freeze-drying process, because the product can be spoiled if badly done.

### **3.6.3 Lyophilization**

In this step, it is important to cool the material below its eutectic point, the lowest temperature at which the solid and liquid phases of the material can coexist. This ensures that sublimation rather than melting will occur. During the primary drying phase, the pressure is lowered (to the range of a few millibars), and enough heat is supplied to the material for the water to sublimate. The amount of heat necessary can be calculated using the sublimating molecules' latent heat of sublimation. In this initial drying phase, about 95 % of the water in the material is sublimated. The secondary drying phase aims to remove unfrozen water molecules, since the ice was removed in the primary drying phase. This part of the freeze-drying process is governed by the material's adsorption isotherms. In this phase, the temperature is raised higher than in the primary drying phase, and can even be above 0 °C, to break any physico-chemical interactions that have formed between the water molecules and the frozen material, finally freeze-dried product was obtained (Lark innovative, Chennai).

### **3.7 Ethanol determination**

#### **Appendix 3**

## ***RESULTS AND DISCUSSION***

#### 4. RESULTS AND DISCUSSION

**Table 4.1:** Chemical composition of cheese waste water

<b>Composition of cheese waste water</b>	
Total COD	48,750.17 mg/l
Total soluble COD	47,250.17 mg/l
Total solids	45.833 mg/l
Total volatile solids	42.233 mg/l
Total fixed solids	3.6 mg/l
Total suspended solids	0.866 mg/l
Lactose	50g/l
pH	7
Temperature	25 °C

## **4.1 Optimization studies on fermentation of cheese whey by *Kluyveromyces marxianus*.**

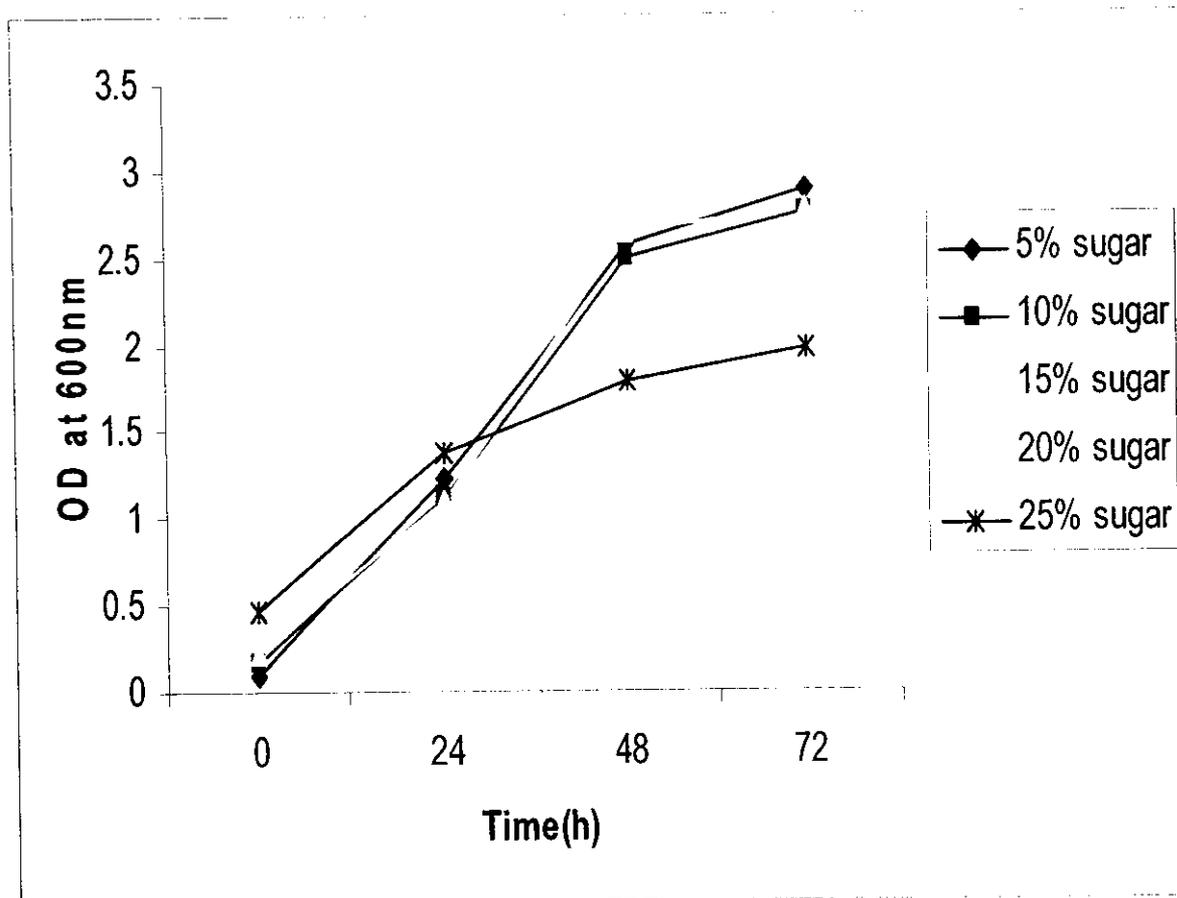
### **4.1.1 Growth studies and effect of sugar concentration**

The growth of *Kluyveromyces marxianus* in gradually increasing concentrations of sugar showed an increase in optical density upto 20% sugar concentration in YEPD medium. However on increasing the sugar concentration beyond 20%, the growth was inhibited as shown by the optical density measured. Samples were taken every 24 hours for the study of growth kinetics. The growth was measured at 600nm. 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%.

**Table 4.2:** Effect of increasing sugar concentration on growth of *Kluyveromyces marxianus* at pH 6 and 30 °C temperature

Time(h)	Sugar concentration (%)				
	5	10	15	20	25
0	0.09	0.16	0.23	0.39	0.46
24	1.23	1.12	1.09	1.52	1.38
48	2.56	2.49	2.65	2.89	1.78
72	2.89	2.76	2.78	3.01	1.98

**Figure 4.1:** Effect of increasing sugar concentration on growth of *Kluyveromyces marxianus* at pH 6 and 30 °C temperature

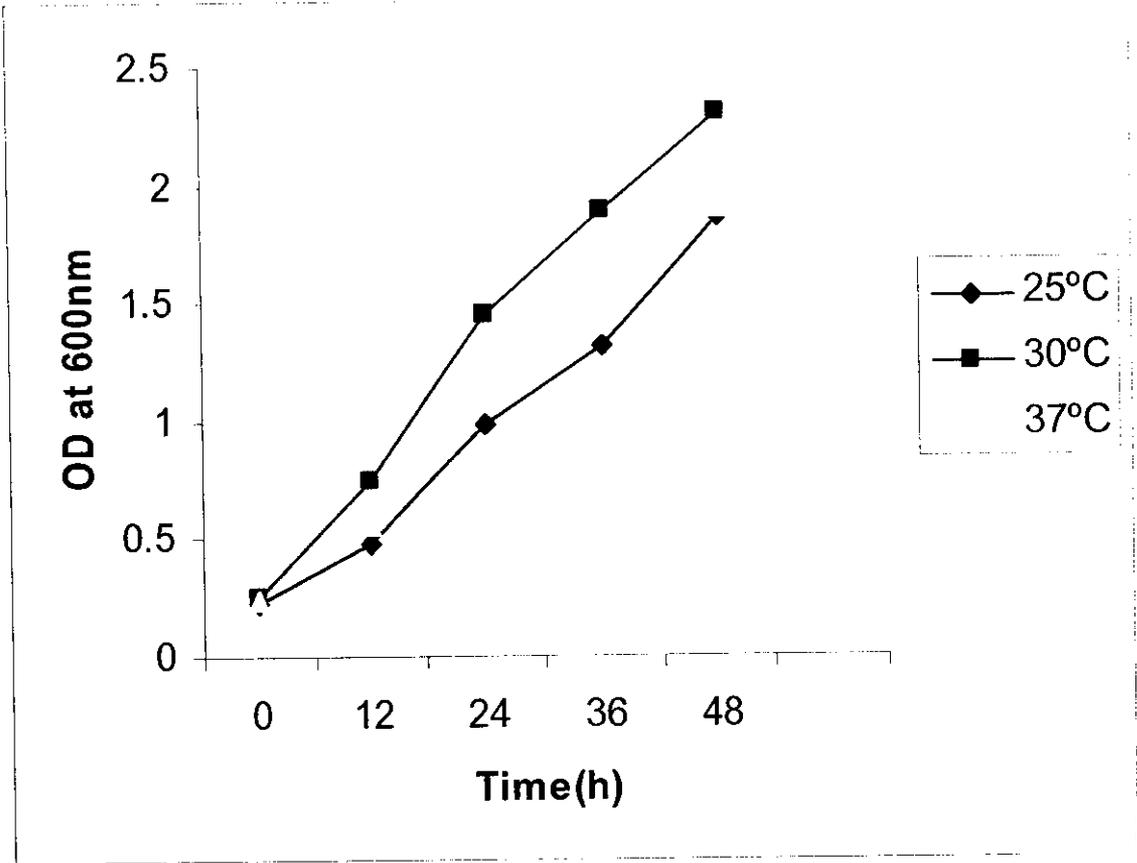


**4.2.2 Effect of temperature on growth of *Kluyveromyces marxianus* at pH 6 and 20% concentration.**

**Table 4.3:** Effect of temperature on growth of *Kluyveromyces marxianus* at pH 6 and 20% concentration.

Time(h)	Growth (in OD)		
	25 °C	30 °C	37 °C
0	0.23	0.25	0.24
12	0.48	0.74	0.56
24	0.98	1.45	1.23
36	1.32	1.89	1.56
48	1.86	2.3	1.9

**Figure 4.2:** Effect of temperature on growth of *Kluyveromyces marxianus* at pH 6 and 20% concentration.

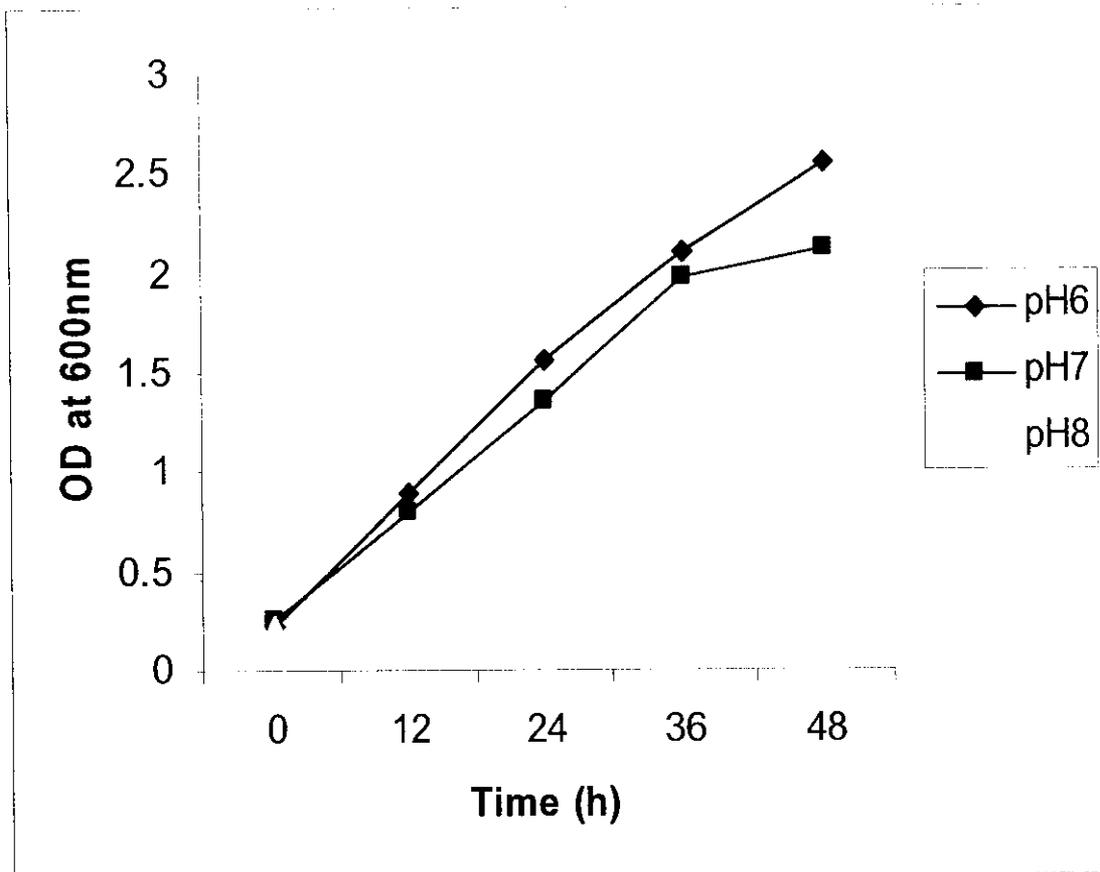


**4.2.3 Effect of pH on growth of *Kluyveromyces marxianus* at 20% concentration and 30°C temperature.**

**Table 4.4:** Effect of pH on growth of *Kluyveromyces marxianus* at 20% concentration and 30 °C temperature.

Time(h)	Growth(in OD)		
	pH 6	pH 7	pH 8
0	0.23	0.26	0.23
12	0.89	0.59	0.46
24	1.56	1.06	0.98
36	2.1	1.48	1.32
48	2.56	1.92	1.56

**Figure 4.3:** Effect of pH on growth of *Kluyveromyces marxianus* at 20% concentration and 30 °C temperature.

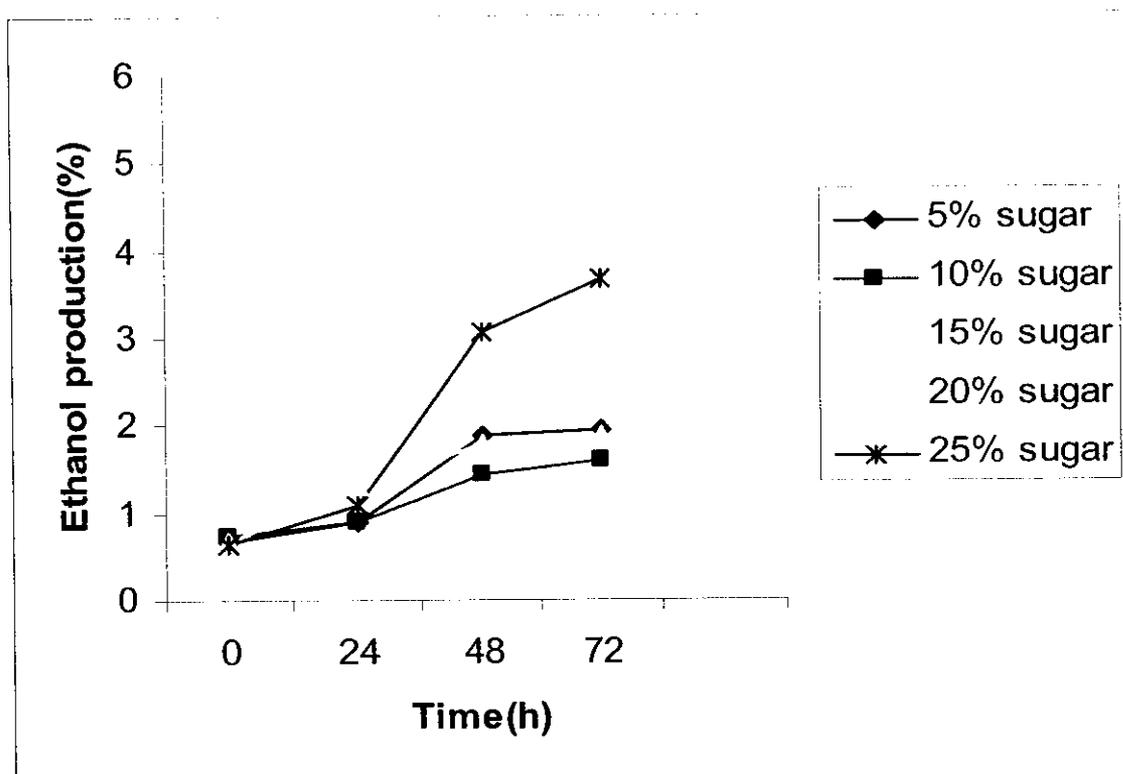


#### 4.2.4 Effect of sugar concentration on ethanol yield

**Table 4.5:** Effect of sugar concentration on ethanol yield

Time(h)	Alcohol production (% v/v)				
	5	10	15	20	25
0	0.666	0.726	0.666	0.654	0.624
24	0.888	0.888	1.296	1.4	1.086
48	1.88	1.434	1.776	4.41	3.072
72	1.944	1.608	1.872	5.358	3.672

**Figure 4.4:** Effect of sugar concentration on alcohol yield



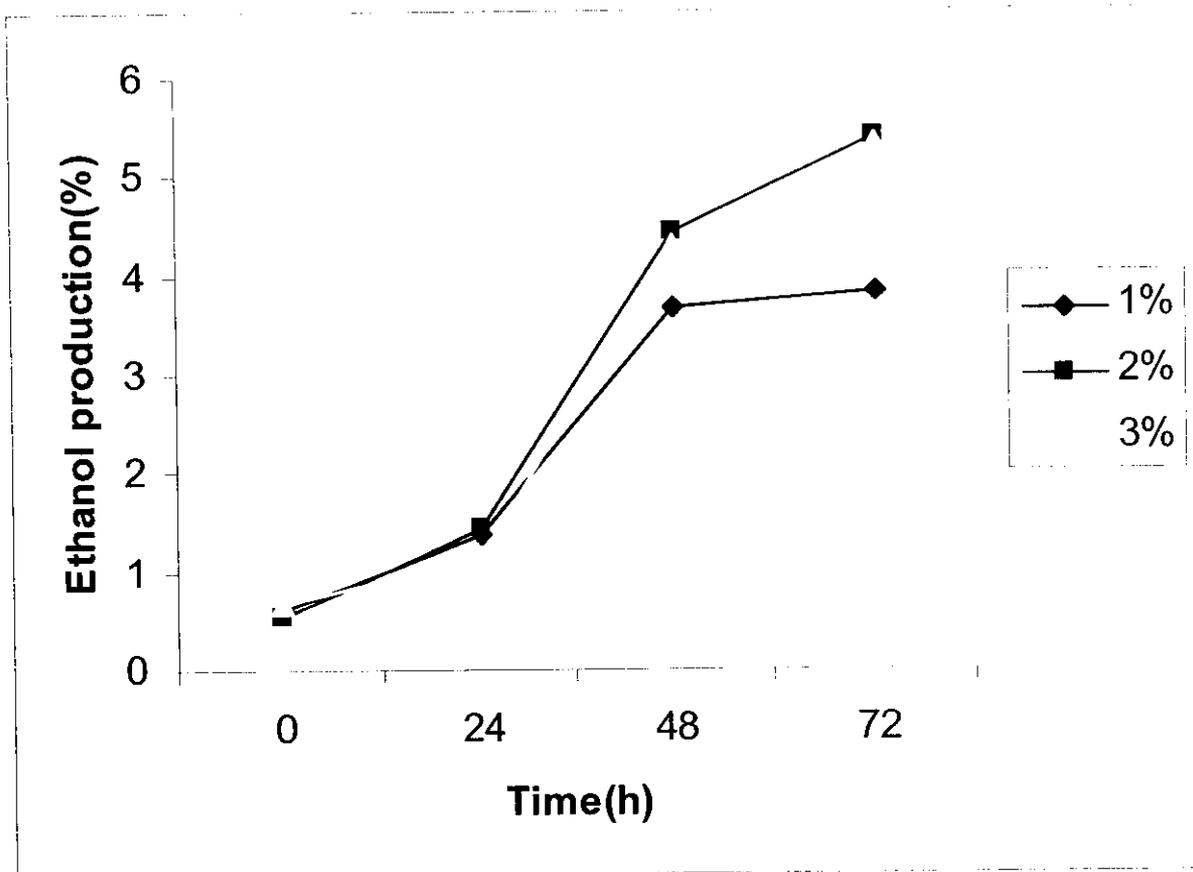
#### **4.2.5 Effect of inoculum concentration on ethanol yield**

Inoculum concentration is also one of the constraint that determines the ethanol production. To know the optimum inoculum concentration for ethanol fermentation, the solutions were kept at 30 °C and 20% initial sugar concentration with 1%, 2% and 3% inoculum concentration respectively. The cheese whey were diluted upto 20% sugars and fermentation was carried out in 100 ml flasks. Samples were withdrawn every 24 hours and the fermentation was carried out for 72 hours. A low ethanol yield of 3.82% was observed at 1% inoculum concentration in 72 hours. As shown in Table at 2% inoculum concentration, ethanol yield was maximum and turned out to be 5.38%. However increasing the inoculum concentration beyond 2% the concentration of alcohol decreased. Hence, 2% inoculum concentration was selected as optimum inoculum concentration for ethanol production.

**Table 4.6:** Effect of inoculum concentration on ethanol yield

<b>Time(h)</b>	<b>Alcohol production (% v/v)</b>		
	<b>1</b>	<b>2</b>	<b>3</b>
0	0.6	0.54	0.66
24	1.368	1.44	1.152
48	3.672	4.44	4.32
72	3.828	5.388	5.34

**Figure 4.5:** Effect of inoculum concentration on ethanol yield



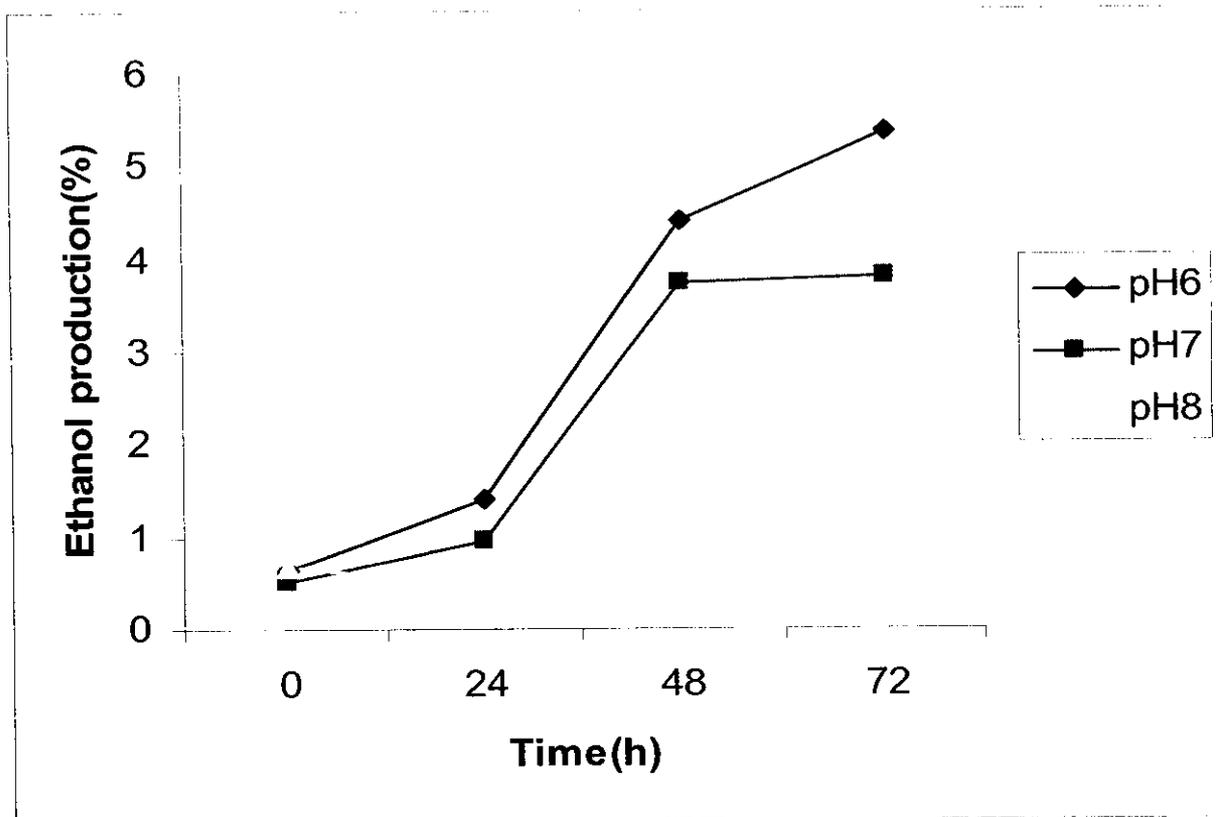
#### **4.2.6 Effect of pH on ethanol yield:**

Initial sugar concentration of 20% and optimum temperature of 30°C was selected for further studies and subjected to pH treatments 6, 7 and 8. The results are shown in table. At pH 7 and 8, fermentation took place but it gave low ethanol content. Best results were obtained at pH 6 where maximum ethanol production was noticed.

**Table 4.7:** Effect of pH on ethanol yield

<b>Time(h)</b>	<b>Alcohol production (% v/v)</b>		
	<b>pH 6</b>	<b>pH 7</b>	<b>pH 8</b>
0	0.654	0.504	0.66
24	1.4	0.954	0.474
48	4.41	3.726	1.326
72	5.358	3.792	1.872

**Figure 4.6:** Effect of pH on ethanol yield



***CONCLUSION***

## 5. CONCLUSION

- Lactose (5%) from cheese waste water was recovered by ultrafiltration using 50 kDa membrane.
- Cheese whey powder (12.81g) was obtained from cheese waste water using flash evaporator and lyophilizer.
- The fermentation of cheese whey using *Kluyveromyces marxianus* under optimized conditions i.e. pH 6, 20% sugar concentration, 2% inoculum concentration and temperature 30°C revealed an increase in ethanol production.

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## 7. APPENDICES

### Appendix 1

#### COMPOSITION OF YEPD MEDIUM:

Malt extract	3.0g
Yeast extract	3.0g
Peptone	5.0g
Glucose	10.0g
Distilled water	1.0L
Agar	20.0g

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

## **Appendix 2**

### **ESTIMATION OF REDUCING SUGARS**

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

#### **REAGENTS**

##### **SUBSTRATE SOLUTION:**

Standard solution of 1000  $\mu$  g/ml concentration was prepared by dissolving 100 mg of glucose in 100ml of distilled water.

##### **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.

##### **POTASSIUM SODIUM TARTARATE (ROCHELLE SALT):**

40 g of potassium sodium tartarate was dissolved in distilled water and the volume was made to 100ml.

##### **PROCEDURE:**

One ml of 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 $\mu$ g concentration prepared in distilled water. Unknown concentration of sample was found using standard graph.

### **Appendix 3**

#### **ETHANOL DETERMINATION BY CHROMIC ACID METHOD**

##### **REAGENTS:**

##### **CHROMIC ACID SOLUTION:**

25% chromic acid in 0.01% H<sub>2</sub>SO<sub>4</sub>

##### **ETHANOL:**

60% ethanol

##### **PROCEDURE:**

One ml of sample was taken in a test tube and made up the volume of the solutions to 4ml using distilled water. Finally 2.2ml of chromic acid was added and measured the OD at 550nm. A standard curve of chromic acid was prepared using 0.5, 1.0, 1.5, 2.0 and 2.5ml of 60% ethanol. Unknown concentration of sample was found using standard graph.

## Appendix 4

### TOTAL SUSPENDED SOLIDS

1. A clean gooch crucible is ignited in a muffle furnace and after partial cooling in the air, cool in a dessicator and weigh ( $W_1$ )
2. Pour 100ml of well mix sample on gooch crucible or glass fibre filter, which is kept on filter flask and apply suction
3. Wash the gooch crucible with 100ml distilled water to remove all soluble salts.
4. Carefully remove the glass fibre filter paper or gooch crucible and dry in an oven at 105 °C for one hour.
5. Cool in dessicator and weigh ( $W_2$ )
6. Ignite gooch crucible in a muffle furnace at 600 °C.
7. Cool the crucible partially in air until most of heat has been dissipated and then in dessiccator and record final weight ( $W_3$ ).

#### Calculations

Total suspended solids (mg/l) =  $(W_2 - W_1) * 1000 / \text{ml of sample taken}$

Suspended volatile solids =  $(W_2 - W_3) * 1000 / \text{ml of sample taken}$

$W_1$ ,  $W_2$  and  $W_3$  are recorded weights in mg.

## Appendix 5

### TOTAL FIXED AND VOLATILE SOLIDS

1. A clean porcelain dish is ignited in a muffle furnace and after partial cooling in air, it is cooling in a dessicator and weigh ( $W_1$ )
2. A 100ml of well mixed sample (graduated cylinder in rinsed to ensure transfer of all suspended matter) is placed in the dish and evaporated at 100°C on water bath, followed by drying in oven at 103 °C for 1 hour.
3. Dry to a constant weight at 103 °C, cool in a dessicator and weigh ( $W_2$ )
4. Ignite the residue on evaporation at 600 °C in the muffle furnace to constant weight in 10 to 15 minutes.
5. Allow the dish to cool and moisten the ash with a few drops distilled water.
6. Dry to a constant weight at 103 °C, cool in a dessictor and weigh ( $W_3$ )

#### Calculations

- a. Total solids(mg/l)=  $(W_2 - W_1) * 1000 / \text{ml of sample}$
- b. Total volatile solids =  $(W_3 - W_2) * 1000 / \text{ml of sample}$
- c. Total fixed solids = (a)-(b)

## Appendix 6

### CHEMICAL OXYGEN DEMAND

1. Place 0.4g of  $\text{HgSO}_4$  in the reflux flask.
2. Add 20ml of sample (or an aliquot diluted to 20ml)
3. 10ml of more concentrated potassium dichromate solution are placed into flask together with glass beads
4. Add slowly 30ml  $\text{H}_2\text{SO}_4$  containing  $\text{Ag}_2\text{SO}_4$  and mix thoroughly.
5. Connect the flask to condenser. Mix the contents thoroughly before heating improper mixing results in bumping and the sample may be blown out.
6. Reflux for a minimum period of 2 hours, cool and wash down the condenser with distilled water.
7. Dilute the sample and make upto 150ml and cool it.
8. Titrate excess  $\text{K}_2\text{Cr}_2\text{O}_7$  with 0.1N  $\text{Fe}(\text{NH}_4)_2\text{SO}_4$  using ferroin indicators; sharp colour change from blue green to wine red indicates the end point.
9. Reflux the blank in the same manner using distilled water instead of sample.

Calculations:

Quantity of  $\text{Fe}(\text{NH}_4)_2\text{SO}_4$  added for blank = A ml

Quantity of  $\text{Fe}(\text{NH}_4)_2\text{SO}_4$  added for the sample = B ml

$\text{COD} = (\text{A}-\text{B}) \times \text{normality } \text{Fe}(\text{NH}_4)_2(\text{SO}_4) \times 8 \times 1000 / \text{quantity of sample.}$