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PRODUCTION OF ETHANOL FROM CANE MOLASSES BY USING A WILD YEAST AND BREWERY YEAST

BY:

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List of Abbreviations

Calcium alginate……Ca- alginate
Colony forming unit……CFU
Degree centigrade ……^{0}C
Dinitrosalicylic acid……..DNS
Ethiopian calendar……E.C.
Ethiopian Sugar Development Agency……ESDA
Gram……..g
Gram per liter……g/l
Gram per liter per hour.....g/l/h
Hour……h
Immobilized cell reactor.....ICR
Kilogram.....kg
Kilocalories……..kcals
Liter……..l
Minutes……mins
Milliliter……ml
Species……...sp
'*Tej*' yeast isolate……..TJYI
'*Tella*' yeast isolate……...TAYI
Revolution per minute……....rpm
Weight……wt
Volume……v

Abstract

A fermenting isolate TAYI 4-2 and brewer yeast were utilized for alcoholic fermentation using sugarcane molasses. The fermentation of molasses was optimized with respect to temperature, pH and sugar concentration. Results revealed a pH 4.5 and 11% sugar concentration as optimum for fermentation for both microorganisms studied. The optimum temperature for TAYI 4-2 was 35^0C while for brewer yeast 30 ^{0}C. Under optimized conditions, TAYI 4-2 and brewer yeast produce 50.50 and 48.76 g/l of ethanol, respectively. The time required to produce a maximum alcohol by TAYI4-2 was around 54h and productivity was limited to 1.04 g/l/h. In order to improve the productivity, the immobilization of TAYI 4-2 was simply performed by the enriched cells cultured media harvested at exponential growth phase. Immobilization of yeast cells was carried out by entrapment in 2% calcium alginate and tested for ethanol production. In a batch culture of immobilized cells, the ethanol productivity was improved to 2.12 g/l/h. To further optimize productivity, the immobilized TAYI 4-2 was loaded continuous immobilized cell reactor (ICR). The performance of continuous fermentation system using immobilized TAYI 4-2 in ICR was evaluated in terms of ethanol productivity and fermentation efficiency with varying sugar concentration in the medium, at different initial pH and dilution rate. Ethanol productivity was higher at 11% initial sugar concentration similar to batch fermentation but the productivity was improved to 14.34 g/l/h. High ethanol productivity was achieved with a medium containing 11%(w/v) total sugar concentration at a dilution rate of 0.15 h^{-1}. And it was found that an increase in dilution rate from 0.15 h^{-1} to 0.25 h^{-1} resulted in lowering ethanol concentration in the fermented broth. The dilution rate increased the amount of sugar un-utilized found to increase. The high speed through the continuous system which cause a short residence time in the fermenter which may leads sugars in the medium to pass un-fermented and cause declining in fermentation efficiency. On the basis of the results obtained ,the potential yeast isolate TAYI 4-2 screened from '*tella*' showed the highest fermentation efficiency and productivity when immobilized in continuous system of fermentation within short period of time. This clearly indicates potential ethnologic microbe from '*tella*' was highly effective when it is immobilised and run in continuous system of fermentation at its optimum physico-chemical conditions.

Key words**:** *Brewer yeast, Cane molasses, Ethanol, immobilized cells*

1. INTRODUCTION

1.1 Ethanol

Ethanol (ethyl alcohol, grain alcohol) is an alcohol. The word *alcohol* is derived from Arabic *al-kuhul,* which denotes a fine powder of antimony produced by distilling antimony and used as an eye makeup (Beltize *et al*., 2004). *Alcohol* originally referred to any fine powder, but medieval alchemists later applied the term to the refined products of distillation, and this led to the current usage (Weissermel and Arpe, 2003).

1.1.1 Properties

Ethanol is a clear, colorless liquid with a characteristic, agreeable odor. In dilute aqueous solution, it has somewhat sweet flavor, but in more concentrated solutions it has a burning taste (Stevenson, 1994). Ethanol, CH_3CH_2OH, has been described as one of the most exotic synthetic oxygen-containing organic chemicals (Weissermel and Arpe, 2003). This is 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 (Beltize *et al*., 2004).

Ethanol is a volatile, flammable and a monohydric primary alcohol (Stevenson, 1994). It melts at –114.1°C, boils at 78.5°C and has a density of 0.789 g/ml at 20°C (Beltize *et al*., 2004). Its low freezing point has made it useful as the fluid in thermometers for temperatures below –40°C (Stevenson, 1994).

1.1.2 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 (Atkins *et al*., 1983).While fermentation is the primary method for production of beverage alcohol (Demirbas, 2004).

1.1.2.1 Ethylene hydration

Much ethanol not intended for drinking is now made synthetically, either from acetaldehyde made from acetylene (Gane, 1981), or from ethylene made from petroleum (Robert, 1969). Ethanol can be made from petrochemical feedstocks, typically by acid-catalyzed hydration of ethylene, represented by the chemical equation $C_2H_4 + H_2O \longrightarrow CH_3CH_2OH$ (Atkins *et al*., 1983). The catalyst used most commonly is phosphoric acid, adsorbed onto a porous support such as diatomaceous earth or charcoal (Atkins *et al.*, 1983).

1.1.2.2 Fermentation

Ethanol has been made since ancient times by the fermentation of sugars (Guimarães, 2008). All beverage ethanol and more than half of industrial ethanol is still made by this process (Chaabane, 2006). Yeast metabolizes sugars to produces ethanol and carbon dioxide in the absence of oxygen. An enzyme from yeast, called zymase, changes the simple sugars to ethanol and CO_2 (Marlène, 2006). The overall chemical reaction of the fermentation process represented by the chemical equation $C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2\ CO_2$ (Marlène, 2006).

The ethanol produced by fermentation ranges in concentration from a few percent up to about 14 percent (Munnecke, 1981). Above 14 percent, ethanol concentration by volume destroys the zymase enzyme and fermentation stops (Coutinho, 2005). Ethanol is concentrated using distillation of aqueous solutions, but the composition of the vapor from aqueous ethanol is 96 % ethanol and 4 % water (Kripa, 2006). Therefore, pure ethanol cannot be obtained after distillation (Sharma, 2007). Dehydrating agents can be used to remove the remaining water and produce absolute ethanol (van de Laar, 2007).

1.1.3 Ethanol attributes

There are different attributes of ethanol in day to day life of human beings. Out of the many some of them are: Used as solvent, as fuel for vehicles, as beverage, as liquor, as a means to create decentralized job opportunities and as hard currency saving.

1.1.3.1 As Solvent

Ethanol is miscible in all proportions with water and with most organic solvents. It is useful as a solvent for many substances and in making perfumes, paints, lacquer, and explosives (Hook, 1988).

Most industrial ethanol is denatured to prevent its use as a beverage (Daragan *et al*., 2000). Denatured ethanol contains small amounts commonly, 1 or 2 percent each, of several different unpleasant or poisonous substances (Gerig, 2008). The removal of all these substances would involve a series of treatments more expensive than the federal excise tax on alcoholic beverages .These denaturants render ethanol unfit for some industrial uses also (Ogawa *et al*., 1995).

1.1.3. 2 As a fuel

Another important use of ethanol is as a motor fuel and fuel additive. The largest national fuel ethanol industries exist in Brazil and the United States (Gallagher *et al*., 2006 and Sa´nchez and Cardona, 2008). The Brazilian ethanol industry is based on sugarcane and in the year 2004, Brazil produces 14 billion liters annually, enough to replace about 40% of its gasoline demand (Gallagher *et al*., 2006). Also as a result, the Brazilian have become 80% independent from foreign oil. These days most new cars sold in Brazil are flexible-fuel vehicles that can run on ethanol, gasoline, or any blend of the two (Gallagher *et al*., 2006). The United States fuel ethanol industry is based largely on corn (Dewulf *et al*., 2000 and Sa´nchez and Cardona, 2008)). Canada, Thailand, India, China and Japan also produce ethanol from various feedstocks (Wheals *et al*, 1999; Demirbas, 2005 and Sa´nchez and Cardona, 2008). 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 (Wang *et al*., 1999).

1.1.3.3 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 or as distilled beverages (Macrae *et al*., 1993). 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 (Macrae *et al*., 1993). 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 (Ensminger *et al*., 1994).

1.1.3.4 Social and economic benefits

Energy plantations in rural areas create new employment opportunities and contribute to the social aspects of sustainability. In addition, application of agro- industrial residues in bioprocess not only provides alternative substrates, but it also helps to solve their disposal problems (Hill *et al*., 2006).

Fuel ethanol production, in Brazil, accounts for more than 770,000 direct jobs, in both rural and industrial areas and over 2 million indirect jobs in related industries (Pierce *et al*., 2007). These jobs include qualified, highly trained human power in administrative, chemical (chemists), agricultural (agronomists) and industrial production (engineers) areas. These decentralized jobs are being created away from congested urban centers, saving large sums of scarce public resources required for infrastructure in large cities (Quintero *et al*., 2007).

Fuel ethanol is also used to save hard currency in countries such as Brazil. The use of fuel ethanol in Brazil has saved the country US $35.6 million, until December 1997, in foreign imports of crude oil and gasoline. Considering the interest accrued on Brazil’s

foreign debit, the value of these savings is larger than US $70 billion. The hard currency savings benefit from the use of ethanol is US $75 per barrel of ethanol (Wyman, 1999).

1.2 Microorganisms producing ethanol

Numerous microorganisms are capable of producing ethanol from various feedstocks. Although no one culture is efficient in this conversion, industrially selected microorganisms have certain desirable characteristics such as a high yield of product per unit substrate assimilated, high fermentation ability , substantial ethanol tolerance, the ability to remain viable at higher temperature, stability under adequate fermentation condition and a tolerance to low pH values. Several bacteria, yeast and fungi that optimize these factors are employed in ethanol fermentation.

1.2.1 Yeast

The fermentation of sugar to ethanol by yeast has been an important place among the different process that is used in industry. The organisms of primary interest to industrial operations in fermentation of ethanol include *Saccharomyces cerevisiae*, *S. uvarum*, *Schizosaccharomyces pombe*, and *Kluyueromyces* sp (Kosaric and Vardar-Sukan, 2001; Lin and Tanaka, 2006 and Sa´nchez and Cardona, 2008). Among these yeasts, *S. cerevisiae* which can produce ethanol to give concentration as high as 18% of the fermentation broth is preferred one for most ethanol fermentation (Lin and Tanaka, 2006). This yeast can grow both on simple sugars such as glucose, and on the disaccharide sucrose. *Saccharomyces* is also generally as safe as food additives for human consumptions and is there fore ideal for producing alcoholic beverages and for leavening bread.

1.2.2 Bacteria

A great number of bacteria are capable of ethanol formation (Buchanan and Gibbons, 1974 and Lin and Tanaka, 2006). Many of these microorganisms, however, generate

multiple end products in addition to ethyl alcohol. These include other alcohols (butanol, isopropyl alcohol, 2,3-butanediol), organic acids (acetic, butyric, formic and lactic acid), polyols (arabitol, glycerol, and xylitol), ketones (acetone), or various gases (methane, carbon dioxide, hydrogen).

There are also bacteria which are capable of producing ethanol as the major product. *Zymomonas mobilis* is among such organisms that has several appealing properties. It can tolerate up to 120 g/l ethanol (Lin and Tanaka, 2006). Moreover, its simple nutritional needs and higher ethanol productivities are further benefit that makes it superior than *Saccharomyces* sp. Despite its advantage as an ethanolgen, Zymomonas *mobilis* is not well suited for all of biomass resources conversion because it ferments glucose; fructose and only 50% of the strain can convert sucrose to glucose. The usage of sucrose, also, causes excessive quantities of unwanted by-products during the fermentation and this is a disadvantage of Z. mobilis (Kosaric and Vardar-Sukan, 2001 and Lee and Huang, 2000).

1.2.3 Genetically modified microorganisms

Future progress in ethanol tolerance and the range of possible substrates used in these fermentation systems may be enhanced through genetic recombination techniques. Today, through genetic engineering one can obtain more versatile bacteria that can produce ethanol from cheaper feedstocks and considerably reduce the cost of producing ethanol. The natural form of *Zymomonas mobilis* produces ethanol from the 6-carbon sugars glucose and fructose and the disaccharide sucrose. However, through genetic engineering many bacteria can be made to efficiently ferment all of the sugars present in lignocellulose (Ingram, 1993). This was done by inserting a 106 potential source of energy and chemical products portable, artificial operon containing the *Z. mobilis* genes for alcohol dehydrogenase and pyruvate decarboxylase into other bacteria with a native ability to metabolize different sugars (Ingram, 1993). Organisms have been developed which can ferment xylose, cellobiose, cellotriose, xylobiose, xylotriose, maltose, maltotriose, and other oligomeric sugars (Tao *et al*, 2001 and Walker, 1998). The depolymerization of monomeric sugars prior to fermentation was not required.

1.3. Biomass resource

Ethanol can be made synthetically from petroleum or by microbial conversion of biomass materials through fermentation. In 1995, about 93% of the ethanol in the world produced by the fermentation method and about 7% by the synthetic method (Badger, 2002). Fermentation process from any material that contains sugar could drive ethanol. The varied raw materials used in the manufacture of ethanol via fermentation are conveniently classified in to three main types of materials and are summarized as follows.

1.3.1 Sugar feedstocks

Fermentation involves microorganism that utilize fermentable sugars for food and in the process produces ethanol and other by products. These microorganisms can typically use 6-carbon sugar, one of the most common being glucose. There fore, biomass materials containing high levels of glucose or precursors to glucose are the easiest to convert in to ethanol. Sugars from sugar cane, sugar beets, molasses and fruits are there fore can be directly converted to ethanol. However, since sugar materials are in human food chain, these materials are usually too expensive to use for ethanol production (Badger, 2002).

The most widely used sugary material for ethanol fermentation is molasses which contains about 50 weight (Wt) % of sugar and about 50 Wt % of organic and in organic compounds including water (Lin and Tanaka, 2006). It is thick, dark-colored syrup produced during refinement of sugar. Since molasses contains microorganisms that can disturb the fermentation, the molasses is taken to the sterilizer followed by taking in to fermenter. Then it is diluted with water to the mass fraction of 10 ± 18 % to reduce its viscosity in the pipeline. In addition, very high concentration of sugar can give too much ethanol and results in prolonged fermentation time and an incomplete sugar conversion (Lin and Tanaka, 2006). After the pH of the mash is adjusted to about 4-5 with mineral acid, it is inoculated with yeast or bacteria and the fermentation is carried out non-aseptically at 20-32 ^{o}C for about 1-3 days. Brazil is pioneer in producing ethanol from molasses and nearly all fuel ethanol is being produced from sugar feedstock (de Matta, and de Rocha, 1988).

1.3.2 Starchy feedstocks

Another potential ethanol feedstock is starch. Starch molecules are made up of long chains of glucose molecules. Thus, starchy materials can also be fermented after breaking starch molecules in to simple glucose molecules. Examples of starchy materials commonly used around the world for ethanol production include cereal grains, potato, sweet potato, and cassava.

Fermentation of starch is somewhat more complex than fermentation of sugars because starch must be first hydrolyzed (converted) to sugar and then to ethanol. Starch is first hydrolyzed by adding α-amylase to avoid gelatinization, then cooked at high temperature (140-180^{o}C). Next, the liquefied starch is hydrolyzed to glucose with glucoamylase. The resulting dextrose is fermented to ethanol with the aid of microorganisms producing CO_2 as a co-product. A second co-product of unfermented starch, fiber, protein and ash known as distillers grain (a high protein cattle feed) is produced.

During the process currently employed for industrial scale- ethanol production from starchy materials, high temperature cooking (140-180^{o}C) is very effective for fermentation of starchy materials because it raises saccharification efficiency and achieves high levels of ethanol production under complete sterilization of harmful microorganisms (Lin and Tanaka, 2006). However, production costs are high due to the high energy consumption in the cooking process and the addition of large amounts of amylolytic enzymes (Lin and Tanaka, 2006). So processes to reduce the high production costs are required. Industrial ethanol production has been reported using various starchy materials such as corn and cassava.

1.3.3 Cellulose feedstocks

Like sugar materials, starchy materials are also in the human food chain and are thus expensive. But cellulosic materials from wood, agricultural residues, waste sulfite liquor from pulp and paper mills can be an alternative source for fuel ethanol production. Among the three main types of raw materials, cellulose materials represent the most

abundant global source of biomass and have been largely unutilized (Lin and Tanaka, 2006). They comprised lignin, hemicellulose, and cellulose and are thus some times called lignocellulosic materials. It is considered that lignocellulosic biomass comprises about 50 % of world biomass and its annual production was estimated in 10–50 billion ton (Sa´nchez and Cardona, 2008). However, the effective utilization of the lignocellulosic feedstock is not always practical because of its seasonal availability, scattered stations, and the high cost of transportation and storage of such large amount of organic material (Polman, 1994).

Cellulose molecules consist of long chains of glucose molecules as do starch molecules, but have a different structural configuration. These structural characteristics plus the encapsulation of lignin makes cellulosic materials, more difficult to hydrolyze than starch materials. Recently, the enzymatic hydrolysis of biomass cellulose is considered to be the most promising technology available. However, the industrial scale-up of this process appears to still be hindered by technological issue or by the lack to a biomass refinery approach in which ethanol is one of the several products (Lin and Tanaka, 2006 and Demirbas, 2004).

The biological process for converting the lignocellulose to fuel ethanol requires: delignification to liberate cellulose and hemicelluloses from their complex with lignin, depolymerization of the carbohydrate polymers to produce free sugars, and fermentation of mixed hexose's and pentose sugars to ethanol. Among the key processes described above, the deliginification of lignocellulosic raw materials is the rate limiting and most difficult task to be solved (Lin and Tanaka, 2006 and de Matta, and de Rocha, 1988). Another problem is that the aqueous acid used to hydrolyze the cellulose in wood to glucose and other simple sugars destroys much of the sugars in the processes. One way of making cellulose wastes more susceptible to hydrolysis is by subjecting them to a short burst of high energy electron beam radiation (www.andrew.cmu.edu). An alternative to acid hydrolysis is the use of enzymes. Although they avoid the corrosion problems and loss of fuel product associated with acid hydrolysis, enzymes have their own drawbacks. Enzymatic hydrolysis slows as the glucose product accumulates in a reaction vessel. This

end-product inhibition eventually halts the hydrolysis unless some way is found to draw off the glucose as it is formed. It is expected that the cost of lignocelluloses ethanol can undercut that of starch-based ethanol because of low-value agricultural residue can be used.

1.4. Effect of fermentation parameters

Ethanol fermentation is influenced by different environmental, biological and chemical conditions. The influential parameters are temperature, sugar concentration, available nutrients and vitamins, pH, the amount of oxygen and ethanol concentration.

1.4.1 Temperature

Temperature has an important influence on the growth rate of the microorganisms and rate of ethanol production. Yeasts are capable of utilizing a variety of substrate effetely to yield ethanol at temperature range of 28-35^{0}C (Kosaric and Vardar-Sukan, 2001). Though the initial rate of ethanol production is higher at increased temperatures the overall productivities of the fermentation is decreased due to ethanol inhibition (Jones *et al*, 1983).

Many investigators are attempting to isolate theomorphic bacterial culture which can grow and ferment at elevated temperature (Aiba *et al.*, 1968; Atkinson *et al.*, 1975; Sree *et al.*, 2000 and Limtong et *al.*, 2007). The major advantage is not a higher metabolic rate of ethanol production, but possible lower costs in regard to cooling the fermenter and distilling the fermented broth. Temperature may also influence the ratio of chemicals produced by a bacterial, branched-pathway type of pyruvate metabolism (Munnecke, 1981). Thus, shifts in temperature may vary the amount of pyruvate going to ethanol, organic acids and other alcohols.

1.4.2 Sugar concentration

The concentration of sugar can affect the microbial ethanol fermentation in various ways. The amount of alcohol produced is proportional to the amount of sugar added; thus, high

sugar concentrations are desired. However, sugar concentrations which are too high can inhibit metabolism due to the increased osmotic pressure. Very low levels of sugar, on the other hand, as might be experienced in continuous-flow fermentation processes, may limit the rate of ethanol production. Hence, each fermentation process will have an optimal glucose or equivalent sugar concentration.

Secondly, the concentration of various sugars in mixed substrate fermentation can affect their metabolism. For instance, if glucose is present in a mixture of sugars, some microbes will initially metabolize glucose, and this metabolism represses or blocks the metabolism of the other sugars (catabolite repression). Only when glucose is completely degraded will, for instance, the metabolism of maltose and other sugars derived from starch hydrolysis begin. This sequential sugar metabolism may not present problems in batch fermentation, but may result in lower efficiency of ethanol production in continuous fermentations since one sugar in the feed may delay or repress the metabolism of a second sugar.

1.4.3 Nutrients and Vitamins

Salts are critical for both the growth of ethanol-producing microbes and the production of ethanol. For growth, microorganisms need a proper balance of macronutrients such as nitrogen, phosphorus, potassium, sodium, and sulfur, as well as micronutrients such as zinc, copper, iron, magnesium, and manganese. The macronutrients are required primarily for synthesis of cellular material while the micronutrients are required for coenzymes and as cofactors in enzymatic reactions (Shapouri and Gallagher, 2005). The proper amount of each macro and micronutrient is dependent upon the type of ethanol fermentation process desired (Munnecke, 1981). For instance, by maintaining low levels of nitrogen, less cell mass will be produced and higher levels of ethanol will occur (Swenson, 2005). In some processes, no cellular growth is desired. Therefore, only micronutrient addition is required for glucose conversion into ethanol. The various sugar feedstocks will contain different amounts of inorganic nutrients and the technical problem is how to balance the nutrients for optimum process kinetics (Alfenore, 2004). In many cases, only minor nutrient addition is required.

1.4.4 pH

Another very important factor for cellular growth is external pH .Yeast cultures can grow over a wide pH range from 3 to 8 with an optimum for growth (Munnecke, 1981 and Kosaric and Vardar-Sukan, 2001). Shifts in pH can also affect the final ratio of organic waste products produced by yeast cultures (Munnecke, 1981). Thus, the optimal pH for a fermentation process must support a balance among ethanol production, cellular growth and physiological effects on waste product pathways. Low pH values in yeast fermentation help to inhibit growth of contaminating bacterial cultures. Bacterial cultures generally have a pH optimum around 7-7.5, with less tolerance than yeast to acidic conditions (Abbott, 2005). Therefore fermentation utilizing *Clostridium* or *Bacillus* species are conducted in higher pH buffered media. Some bacteria like *Z. mobilis*, however, have optimum pH around 4.0-5.0 (Abbott, 2005). Since the decrease in pH value is small during ethanol fermentation.

1.4.5 Oxygen

The microorganisms involved in alcoholic fermentations are facultative microbes since they are able to grow with or without the utilization oxygen. Thus two types of different pathways of pyruvate metabolism are available (Abbott, 2005). In the presence of oxygen, more cell biomass is produced from the initial substrate and the growth rate is increased (Alfenore, 2005). Therefore, for inoculum formation, aeration improves the yield of cell mass and its rate of production. However, for ethanol production, oxygen must be restricted from entering the fermenter. But, a small concentration of oxygen must be provided to the fermenting yeast as it is a necessary component in the biosynthesis of polyunsaturated fats and lipids (Cysewaski and Wilke, 1977 and Sa´nchez and Cardona, 2008). According to Kosaric and Vardar-Sukan, (2001) typical amount of O_2 to be maintained in the broth is 0.05-0.10 mm Hg oxygen tension. Any values higher than this will promote cell growth at the expense of ethanol productivity (Kosaric and Vardar-Sukan, 2001). The oxygen concentration which triggers aerobic or anaerobic growth

processes, is however, varies from culture to culture and is dependent on substrate concentration and cell density (Munnecke, 1981).

1.4.6 Ethanol

The concentration of ethanol in the fermentation broth can directly affect the growth rate of the culture and its ability to convert sugars into ethanol. Inhibitory and toxic levels of ethanol vary from culture to culture. With some yeast cultures, 50 % inhibition of growth occurs at 4-6 % ethanol (Najafpour and Lim, 2002), while others are more ethanol tolerant. Generally, maximal ethanol concentrations produced microbiologically range from 11-14 % (Sreenath and Jeffries, 2000). However, some investigators reported that final ethanol concentrations of 20 % are achievable (Hahn-Hagerdal *et al*, 2006). If the ethanol is produced at low rates so that the intracellular ethanol concentration remains low, then higher levels can be tolerated (du Preez, 1994). However, high production rates result in intracellular buildup of ethanol and in an increased sensitivity (Laplace, 1992). Certain other higher alcohols produced as minor waste products can be more inhibitory than ethanol (Jeffnes, 1985). Thus, even though they are produced in much smaller quantities, they may have a significant inhibitory effect (Laplace, 1992).

1.5 By-Products of ethanol fermentation

By-products of ethanol fermentation are waste biomass, stillage, carbon-dioxide and fusel oils. These by-products can be employed in different importance such as; feed or food supplement, pharmaceutical chemical source, as refrigerant and as solvent.

1.5.1 Waste Biomass

Due to the anaerobic nature of the ethanolic fermentation, the overall synthesis of biomass is limited. According to Kosaric and Vardar-Sukan (2001), a 10 % substrate feed with 95% conversion to alcohol will yield 5.0 g /l of dried cell mass. Therefore,

separation of the fermenting organisms for cell recovery may not prove to be economically feasible (Kosaric and Vardar-Sukan, 2001).

For recycle processes, a return of 35-40 % of the total biomass in the broth is all that is required to meet fermentation demands (Kosaric and Vardar-Sukan, 2001). Since the concentration step has been carried out, the remainder may be utilized in by-product markets. After concentration, microbial biomass may be dried and utilized as a high protein food or feed supplement.

1.5.2 Stillage

Stillage is the residue from the first distillation of fermented substrate (corn mash, sugarcane juice, etc.). According to Glazer and Nikaido, (2007) with sugarcane, about 12 l of stillage are produced for each liter of ethanol. Such stillage contains 40 to 65 g of organic matter per liter. Depending on what is done with it, stillage is either a serious water-polluting waste or a source of valuable byproducts (Glazer and Nikaido, 2007).

1.5.3 Carbon Dioxide

According to Kosaric and Vardar-Sukan (2001) for every m^3 of ethanol formed, about 760 kg of CO_2 gas is liberated from the fermentation broth of this total, 70-80 % can be recovered in a closed system. After purification to remove aldehydes and alcohols, the gas may be stored in cylinders or further compressed to solid or liquid form.

The market value of CO_2 is relatively variable. It is not economical to transport it a great distance from the plant. In the gaseous state, it may be used to carbonate soda beverages or to enhance the agricultural productivity of greenhouse plants. Liquid CO_2 is frequently used in fire extinguishers, refrigeration processes, and as a feedstock in the chemical industry. The primary use of solid CO_2 is as a refrigerant.

1.5.4 Fusel Oils

Fusel oils are formed from α-keto acids, derived from or leading to amino acids. The overall composition is found to be an isomeric mixture of primary methyl butanols and methyl propanols, the majority of which is isoamyl alcohol. Yields of up to 20 l may be attained per m^3 of ethanol generated using standard sugar sources; however, this value depends upon the pH in the fermenter (Kosaric and Vardar-Sukan, 2001). Fusel oils are found to come of the distillation tower at relatively high temperatures and must be completely removed. Due to its similarity to the major components in gasoline, the use of this by-product would be as a further fuel extender or as an industrial solvent (Jornvall, 1987).

1.6. Immobilized cell technology

Many processes have been practiced traditionally, embodying the basic principle of microbial conversions offered by cells bound to surfaces. Waste treatment in trickling filters and ethanol oxidation to provide vinegar are few examples of such processes (Bucke, 1983). Immobilization of cells is the attachment of cells or their inclusion in distinct solid phase that permits exchange of substrate, products, inhibitors, etc., but at the same time separates the catalytic cell biomass from the bulk phase containing substrates and products. Thus the deliberate immobilization of cells by human beings for their own convenience does not necessarily place them in an environment foreign and strange to them (Bucke, 1983).

The use of immobilized whole microbial cells and /or organelles eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of intracellular enzymes. It also tends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation. The ease of conversion of batch processes in to continuous mode and maintenance of high cell density without washout condition even at high dilution rates, are few of the many advantage of immobilized cell systems.

1.6.1 Criteria for success in cell immobilization

Many methods are available for immobilization of cells. Their development has intended presumably for commercial use. According to Bucke (1994) and Ramakrishna and Prakasham (2006) to find industrial use an immobilization method must meet various criteria;

1. It must be safe. The process must not harm operators of the plant nor consumers of the products. The huge costs of determining the safety of a product means those novel chemicals will be avoided unless there is no alternative; materials that are already accepted for use in food stuff or food processing will be preferred even if the product is not intended to be a food ingredient.

2. It must be simple. Eventually relatively unskilled personnel must operate a process. The logistics of its use must be simple. Expensive support materials that have to be returned to the manufacturers for regeneration will be avoided unless they are greatly superior in performance to cheaper materials that can be discarded after a single use.

3. It must be gentle. Except where it is intended that only a single enzyme activity be used cell viability or at least integrity of membranes should be retained. Therefore extremes of heat and pH and organic solvents will be avoided.

4. It must be long-lived. An immobilized cell preparation must be resistant to abrasion yet not brittle, it must not be compressed excessively when used in large reactors. It must not be liable to microbial degradation and its activity must be maintained as long as possible since immobilizing them and filling and emptying the reactor is expensive, particularly in labour costs.

5. It must has a high activity.

6. It must be cheap.

1.6.2 Techniques and support for Immobilization

Immobilization of cells can be accompanied with a number of methods and supports which are synthetic or natural with consideration of the nature of enzyme, nature of substrate and its ultimate application. Therefore, it will not be possible to suggest any universal means of immobilization. Techniques for immobilization have been broadly classified into four categories, namely entrapment, covalent binding, cross-linking and adsorption.

1.6.2.1 Entrapment

Entrapment within a gel and absorption to the surface is the most extensively studied method of cell immobilization. This technique approximates to circumstances in which cells might find themselves in nature as adsorbed to surfaces while entrapped within a gel or slime of their own making. Slimes are of little use in industrial processes but entrapment within gels is proving to be probably the most successful means of immobilizing cells (Bucke, 1983).

1.6.2.2 Covalent-binding

The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (cross-linking) agent. For covalent-linking, chemical modification of the surface is necessary to introduce a reactive organic group on support materials. Usually α-amino propyl triethoxy silane has been used as coupling agent between the activated support material and yeast cells. Cell viability is usually lost, which is of no consequence where single enzyme conversions are required and where that enzyme is intracellular and not contacted by the cross-linking

agent. This method has been extensively used for immobilization of enzymes, though it is not a good technique for immobilization of cells.

1.6.2.3 Cross-linking

Microbial cells can be immobilized by cross-linking each other with bi-or multifunctional reagents such as glutaraldehyde. The toxicity of the chemicals used for cross-linking obviously imposes limitation for the general applicability of these procedures. Apart from chemical cross-linking, procedures employing physical process, such as flocculation and pelletization, also benefit the immobilization techniques because of strong mutual adherence forces of some microbial cell cultures (Ramakrishna & Prakasham, 2006).

1.6.2.4 Adsorption

This technique involves the binding of cell surface to support material by ionic or less powerful bonds as it happen naturally growing organisms on surfaces, to both the advantage and the detriment of the human condition. Vinegar production relies on bacteria immobilized on suitable surfaces and many sewage-treatment processes similarly involve microorganisms growing on solid surfaces. Considerable thought and ingenuity has gone in to selection and design of materials with surfaces excellent for adsorption of cells (Bucke, 1983).

Immobilization by adsorption is based on the electrostatic interactions (Van der walls forces) between the charged support and microbial cell. This technique for whole cell immobilization is gaining a considerable importance because of its elimination or reduction of the mass transfer problems associated with the commonly used gel entrapment methods. It often results in the immobilization of cells in available form for use in heterogeneous fermentations. On the other hand, treating the cells or the support material with trivalent metal ions like Al^{3+} or Fe^{3+} charged colloidal particles will result in the immobilization of cell in non- viable form that allows to be used as an enzyme source for simple chemical conversions.

1.6.3 Ethanol production by immobilized cells.

Ethanol fermentation using immobilized cells of yeast is one of widely studied systems. Infact, almost all the methods of immobilization, namely, gel entrapment, adsorption on the surface of various carriers, and cross linking were tried for alcohol production. Yeast immobilized on these carriers has been also used as effective model system for evaluating the methods for alcoholic production. However, among many of these methods, only one or two methods have been effectively utilized for industrial production (scale) and the remaining are confined within laboratory investigations (D'souza, 2006).

One of the earliest publications about the immobilization of cells in calcium alginate cells (Kierstan and Bucke, 1977) described the production of ethanol by *Saccharomyces cerevisiae* cells supplied only with glucose or sucrose. These investigators found that the productivity of the cells was low, but the immobilized cell-complex survived for several months by keeping producing ethanol.

A study of Sheoran *et al.,* (1998) aimed at continuous production of ethanol. They immobilized *Saccharomyces cerevisiae* strain HAU-1 in 1.5 % calcium alginate and these yeast beads were employed for ethanol production at various initial sugar concentrations and dilution rate in a vertical column reactor. High ethanol productivity was achieved with medium containing 10 % w/v sucrose at a dilution rate of $0.2h^{-1}$. Other researchers; Najafpour *et al.,* (2004) have studied the effect a different concentrations of alginate (1.5 %, 2 %, 3 % and 6 %). Beads with low alginate (1.5 %) were found to be soft and easily breakable and also faced problems such as overgrowth and expansion of beads diameter when grow in sugar solution. On the other hand, the high alginate beads (6 %) were hard and almost unbreakable by pressing manually. Therefore the suitable alginate concentration, as they concluded, based on the activity of the beads for ethanol production was 2 %. In addition, their research has shown that high sugar concentrations (150 g/l) in immobilized continuous reactor column were successfully converted to ethanol, which is promising for scale up production.

The ability of immobilized cells to grow within beads of *K*- carrageenan was studied by Wada *et al.*, (1979). *S.cerevisiae* cells were immobilized at a density of 3×10^6 cells ml^{-1} after incubation in a growth medium, the cell density reached 5×10^9 cells ml^{-1} by forming a new cells layer near the surface of the *K*-Carrageenan bead. Later such preparations were used to produce ethanol continuously from medium containing glucose at $100gl^{-1}$. A stable steady state maintained was for over 3 months and the yield of ethanol was close to theoretical maximum.

Apart from calcium alginate and *K*-Carrageenan, Joekes and his co-workers (1998) immobilized *S.cerevisiae* (CCT3174 and commercial baker's yeast) by adsorption onto chrysotile. The adsorbed yeast was easily washed out, but cells grown *in situ* were strongly attached by entrapment by chrysotile micro fibres. They also came with conclusion that, for immobilized CCT3174, the final ethanol yield was 26 % higher than that with free cells. In another work of Alegre *et al*., (2003) has evaluated the catalytic role of *S.cerevisiae* adsorbed on chrysotile support for alcoholic fermentation under-aseptic conditions. The fermentation medium employed consisted only of diluted sugar-cane molasses. In a batch fermentation process, the initial rate of CO_2 production increased roughly 27 % during the first 30 minute, compared to the system containing no chrysotile. A study of continuous alcoholic fermentation with chrysotile in the reactor bed showed a higher ethanol production rate at different dilution rates investigated compared to similar fermentation without chrysotile (Alegre *et al.*, 2003).

Vasconcelos *et al.*, (2004) immobilized yeast cells on 2.0 cm long sugar cane stalks envisaging ethanol production. The operational stability of the immobilized yeast, the efficiency and the stability of the process as well as the best dilution rate were evaluated .The yeast-stalk system was shown to be stable for 60 days period at extremely variable dilution rate ranging from $0.05h^{-1}$ to 3 h^{-1}. Ethanol yield and efficiency were 29.64 g/l.h and 86.40% respectively, and the total reducing sugar conversion was 74.61 % at dilution rate of $0.83h^{-1}$. They also found out that the concentration of immobilized cell reached around 10^9 cells/gram of dry sugar cane stalk when the fermenter was operating at the highest dilution rate (3.00 h^{-1}).

In view of testing and finding a low cost support material, Plessas *et al.,* (2007) prepared a biocatalyst by immobilizing a commercial *S. cerevisiae* strain (baker's yeast) on orange peel pieces for use in alcoholic fermentation and for fermented food applications. Cell immobilization was shown by electron microscopy (Fig.1) and by the efficiency of the immobilized biocatalyst for alcoholic fermentation of various carbohydrate substrates (glucose, molasses, raisin extracts) and at various temperatures (30–15 °C). Fermentation times in all cases were low (5–15 h) and ethanol productivities were high showing good operational stability of the biocatalyst and suitability for commercial applications. Reasonable amounts of volatile by-products were produced at all the temperatures studied, revealing potential application of the proposed biocatalyst in fermented food applications, to improve productivities and quality (Plessas *et al.,* 2007).

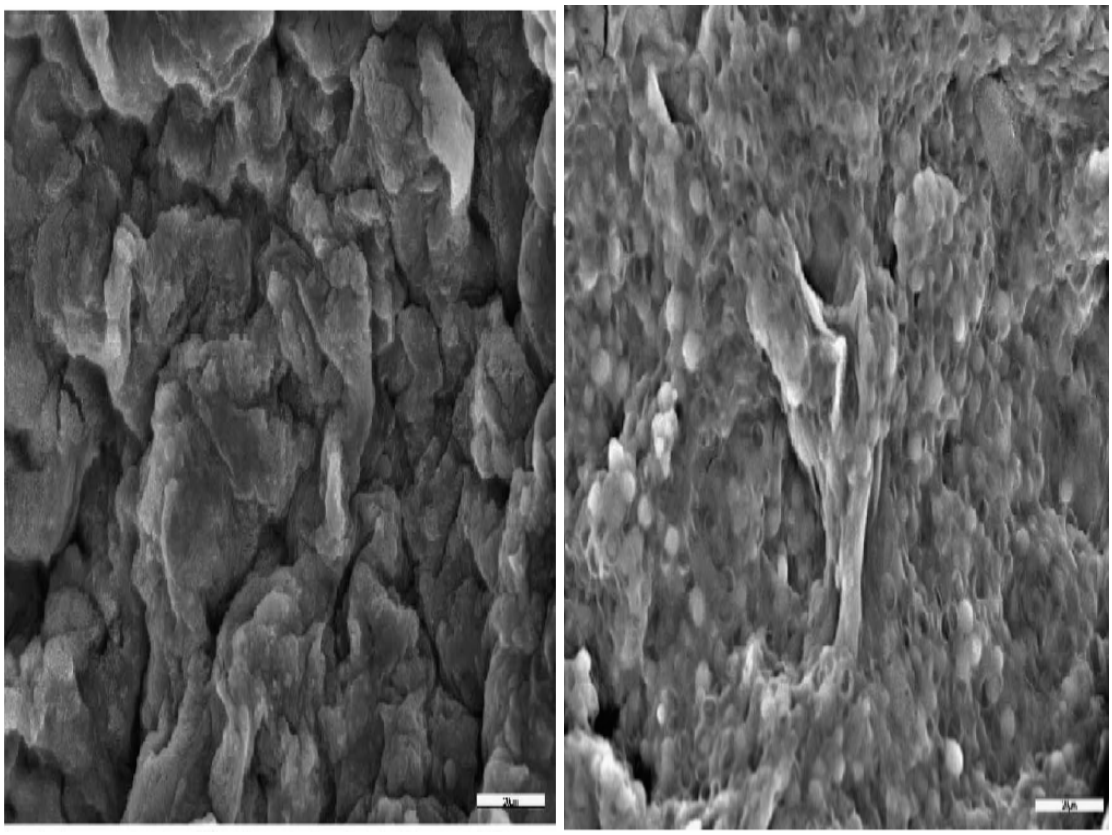


Figure 1. Electron micrographs (£1200) showing the surface of orange peel, before (left) and after (right) yeast immobilization. (Adapted from Plessas *et al*., 2007)

Apart from *S. cerevisiae*, Chibata and Tosa (1981) investigated the alcoholic fermentative capacity of *Saccharomyces carlsbergensis* by immobilizing on carrageenan. Pre-cultured broth of *Saccharomyces carlsbergensis* was directly mixed with carrageenan solution and made into immobilized cells of bead type. These gels containing small amounts of cells (3.5×10^6 cells/ml of gel) were incubated in nutritional medium on a rotary shaker at 30 °C. After 60 h incubation, the numbers of living cells in gel increased by 1000-fold (5.4×10^9 cells/ml of gel). The number of living cells in unit volume of gel was about 10 times higher than that of full growth of free cells in ordinary culture, and formed a thin layer of condensed yeast cells near the surface of the beads. The cell layer is formed as a result of selection of suitable environmental conditions by microbial cells. Therefore, those immobilized cells forming a condensed thin layer near the surface of the gel bead were expected to show efficient catalytic activities in enzyme reactions.

1.7 Current status of ethanol production in Ethiopia

With inevitable depletion of the world's energy supply and with the gradual increase in the price of all fossil fuels, there has been an increasing worldwide interest in alternative sources of energy. It is now understood that it is important to use biomass energy as means of providing modern energy to the billions who lack it. It would complement solar, wind and other intermittent energy in the renewable energy mix of the future (Gallagher *et al*, 2006).

Ethanol has emerged as an alternative for petroleum based liquid fuels. Now a days, its use in automobiles as an alternative fuel has attracted many countries including Ethiopia for its production on a large scale and has led to setting up of number of ethanol plants and co-generations along with sugar mill plants. Currently in Ethiopia, there is only one sugar mill producing ethanol and few distilleries participating in down stream chemicals from alcohol. Among molasses derived products, ethanol takes the largest part, but its utilization must attract the attention of the government policy makers in order to utilize as a bioethanol. Bioethanol or biofuel is ethanol-based products that can be

processed into liquid fuels for either transport or heating purposes. With the coming into being of the sugar sector expansion and modernization in the country, implementation of the different domestic measures for bioethanol fuels utilization has to take place. At present there was about 5.6 million liter annual production of ethanol, but there are projects towards increasing the product to over 142000 cubic meter (Ethiopian sugar development agency (ESDA), 2005).

An efficient ethanol production requires four components: fermentable carbohydrates, an efficient yeast strain, a few nutrients and simple culture conditions. Among the widely used substrates for ethanol production are molasses of sugarcane and sugar beet are out of them. This is because they are ready for conversion with limited pre-treatments as compared with starchy or cellulosic materials.

Sugarcane resource can be used to produce a variety of commercial products that can be marketed domestically, regionally and internationally. In economic and environmental terms, the three products that have special significance are sugar, ethanol, and electricity (ESDA, 2005). When compared to others, there was less molasses utilization in Ethiopia at present mainly due to low technological development and low market availability. The use of power alcohol from molasses source for vehicles increases the demand of molasses in most other countries and there is a promising move towards production and use of power alcohol in Ethiopia also (ESDA, 2005).

Ethiopia through its potential in developing large sugarcane production can play a pro-active role in mitigating sugar, ethanol and electricity demands of the country. Molasses the non-crystallizable residue remaining after crystallizing sucrose, has additional advantage; it is relatively inexpensive raw material, readily available and already in use for industrial ethanol production. However, 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. Studies on isolation of new potent strain and improvement of the available strain towards

higher productivity were necessary for the newly emerging ethanol industries of the country.

1.8 Objective of the study

- To isolate and evaluate an ethanol producing isolate from Ethiopian traditional beverages; '*tella*' and '*tej*'
- To optimize certain environmental factors (pH, temperature, additives)under batch fermentation system
- In order to evaluate ethanol production by immobilized yeast cells under continuous ethanol fermentation system by using diluted sugar-cane molasses as source of carbon.

2. Materials and Methods

2.1 Sampling sites and sample collection

The samples of '*Tella*' and '*Tej*' were collected from traditional beverage suppliers found in Addis Ababa using plastic bottles and transported to Applied Microbiology Laboratory of Addis Ababa University where they kept in refrigerator until use.

2.2 Microorganism isolation

The yeast isolates used in these studies were isolated from the '*Tej*' and '*Tella*' samples after dilution followed by plating aliquots of appropriate dilutions of the samples on yeast extract peptone dextrose agar (YPDA) medium and incubating at 30^0C for a day. The composition of YPDA per liter was as follows: yeast extracts 10 g, peptone 20 g, dextrose 20 g and agar, 20 g. Each of the isolates' culture was maintained on YPDA medium by transferring every month to fresh medium and it was stored at - 4^0C.

2.3 Substrate for fermentation

Sugar cane molasses was used throughout the study. It was supplied by Wonji Showa Sugar Factory. The average molasses sample consisted of 52% total sugars of which 14% was reducing sugars.

2.4 Selection of an isolate for ethanol production

Selection of ethanol producing microorganisms was according to a method followed by (Huang *et al*., 1996). A total of 50 isolates of yeast were tested for their capacity to ferment molasses as sole source of carbon and energy. Each of the representative colonies were transferred to YPD broth and incubated at 30 ^{0}C for 16 h. About 10 ml of 16 h yeast culture was inoculated in molasses media containing 10 % total sugar concentration and was incubated at 30 ^{0}C for 72 h. The amount of ethanol produced was analyzed at the end of fermentation. This was carried out in order to compare the ethanol fermentative capacity of the isolated microorganisms. Out of the 50 yeast isolates from '*tella*' and

'*tej*', one was found to produce higher alcohol amount than the others. The isolate was named as ''*tella*' yeast isolate 4-2' or (TAYI 4-2). In addition to the wild isolated yeast, brewer yeast was also brought from '*Meta-Abo*' brewery to compare its ethanol fermentative capacity.

2.5 Inoculum preparation

The inoculum used in this study was prepared in two stage growth process. In the first stage, the microorganism was cultured in 250 ml Erlenmeyer flasks containing 100 ml of the following medium (g/l): glucose 50, yeast extract 5, KH_2PO_4 5, NH_4Cl 1.5, $MgSO_4$ 0.7, KCl 1.7 (Alegre *et al*., 2003). The Erlenmeyer flasks were incubated at temperature 30 ^{0}C for 16 hours on rotary shaker at 120 revolutions per minute (rpm). Afterwards the medium was transferred to larger volume Erlenmeyer flask containing the same medium, except the glucose is replaced by sucrose and incubated following the above-mentioned conditions. The inoculum prepared was used for studies on process optimization for the production of ethanol by different fermentation methods.

2.6 Free- cells batch fermentation

Simple batch reactor was constructed from 120 ml flasks. The working volume of the system was restricted to 100 ml only. This was to ensure the height of the medium would always never exceed the side sampling probe of the system; hence the accumulation of the medium inside the sampling tube could never happen. The top unoccupied, empty space of the reactor was connected by plastic tubing to a 250 ml cone flask which was filled with 100 ml sterile distilled water. One end of the tubing was completely immersed in to distilled water, which functioned as a channel to remove all carbon dioxide and other waste gases produced by the microbe during the fermentation period. All these waste gases would be dissolved in distilled water. Tubes and other heat labile materials were disinfected by using 70% ethanol for 30 minute and washed twice with sterilized distilled water. The experimental set-up of batch fermentation was as it is indicated on Fig. 2

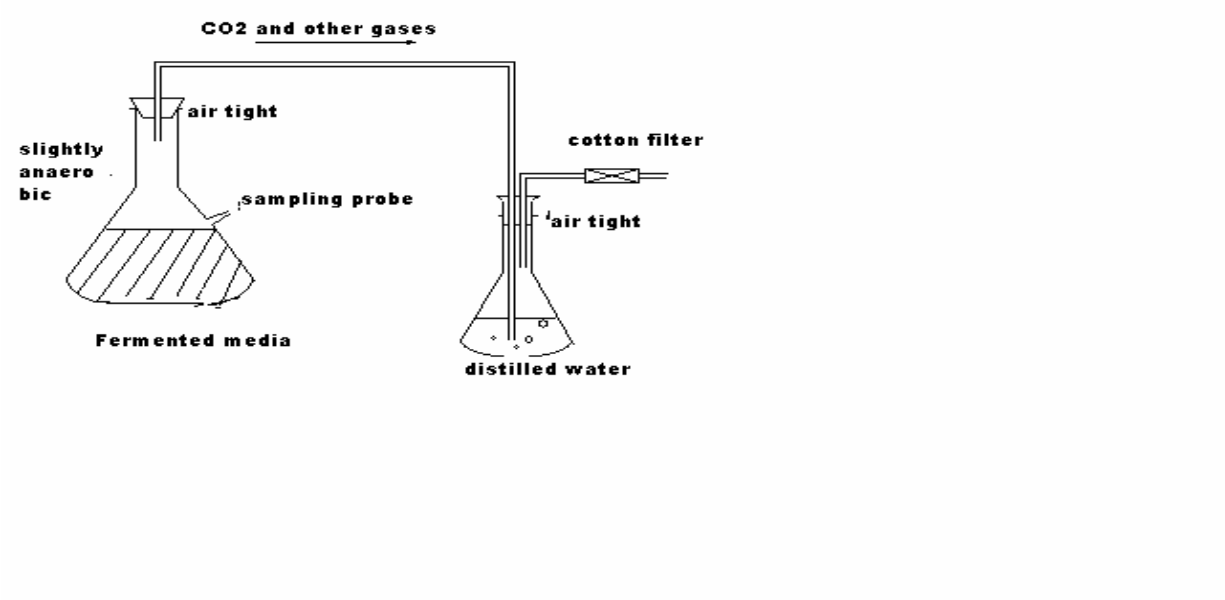


Figure 2 Experimental set up of batch fermentation

2.6.1. Optimization of batch fermentation

To undergo fermentation in batch fermentation system, different parameters were optimized first for the selected isolate of yeast from '*tella*' (TAYI 4-2) and for the yeast strain from '*Meta*-A*bo*' brewery. The optimized parameters were molasses sugar concentration, temperature, pH and additives.

2.6.1.1 Effect of molasses sugar concentration

To evaluate the effect of molasses sugar concentration on ethanol yield the following experiment was carried out. Aliquots of wet cells (equivalent to 3g dry weight/l) were dispensed into 120 ml flasks and the fermentation medium added to make up to a final volume of 100 ml containing 80 ,110,130,150 and 200 g/1 of total sugar. The pH was initially adjusted at 4.5 with mineral acids for both microorganisms. Flasks were incubated at 35 ^{0}C and 30 ^{0}C for isolate TAYI 4-2 and brewer yeast respectively. After 54 h fermentation was hindered inorder to measure residual sugar and produced ethanol amount.

2.6.1.2 Temperature effect

The effect of temperature on ethanol yield of the two yeast isolates was tested. The process of fermentation at various temperatures ranging from 25-40 ^{o}C with 5 ^{o}C interval was undertaken using cane molasses containing 110 g/1 of glucose equivalent of total sugars. It was followed by fermentation over a period of 54 h under conditions similar to those stated above at section 2.6.1.1.

2.6.1.3 Effect of pH

The effect of pH on ethanol yield by the yeast isolates was tested as follows. Cane molasses containing 11% total sugars at pH values of (3.5-5) with 0.5 interval were prepared and inoculated with 3g/l yeast inoculum and fed to batch reactor. The temperature was controlled at 35 ^{o}C and 30 ^{o}C for yeast isolates TAYI 4-2 and brewer yeast respectively. The system was allowed to ferment for about 54 h. Analysis of residual sugar and ethanol was carried out at the end of fermentation.

2.6.1.4 Effect of supplement for ethanol production

The effect of supplement for ethanol production was tested as follows. Cane molasses containing 11% total sugars at pH 4.5 was prepared and supplemented with different additives such as ammonium nitrate (1g/l), ammonium phosphate (1g/l), yeasts extract (1g/l) and urea (5 g/l). The effect of these supplement on ethanol yield was evaluated after measuring the ethanol content of the fermented broth.

2.6.2 Time course of ethanol production

To determine the time for maximum ethanol production during fermentation process, yeast isolate TAYI 4-2 was cultured in molasses media with pH 4.5 and with 11% total sugar concentration. It was incubated at 35 ^{o}C. Samples were taken every 6 h interval, and analyzed for ethanol and residual sugar content.

2.7 Anaerobic repeated batch fermentations

In order to run anaerobic repeated batch fermentation the following steps were carried out. An amount of 125 g of the alginate immobilized-yeast cells and 120 ml of molasses media containing 110 g/l of total sugars were added in a 250 ml glass bottle and anaerobic repeated batch fermentations were carried out at 35 ^{0}C and pH 4.5 for one day. The fermented liquid was decanted after the end of each fermentation batch. And the immobilized cells were washed with 200 ml of fresh molasses medium and then fresh medium was added for the next fermentation batch. Samples of the fermented liquids were collected and analyzed for ethanol and residual sugar amount.

2.8 Continuous ethanol fermentation

Continuous ethanol fermentation system was run after preparing a fermenter from locally available equipments and immobilizing yeast TAYI 4-2. In this system the effect of different parameters on the ethanol yield was evaluated. The evaluated parameters were: initial sugar concentration in molasses media, initial pH and dilution rate.

2.8.1 Cell immobilization

Cell immobilization was carried out in this study according to the method followed by (Kierstan and Bucke, 1977 and Najafpour *et al*, 2004). Sodium alginate was prepared by dissolving 10 g of powder in 500 ml of distilled water. A separate solution of calcium chloride ($CaCl_2$) was prepared by dissolving 120g of $CaCl_2$ in 2 l distilled water. Sodium alginate and calcium chloride solution were autoclaved at 121 ^{o}C; at a pressure of 15 lb for about 15 min. And it was allowed to cool. The sterilized sodium alginate solution and the high cell density of the grown seed culture were thoroughly mixed. A 100 ml aliquot of alginate-cell suspension was added drop wise to 1000 ml $CaCl_2$ solution with a peristaltic pump. Alginate drops solidified upon contact with $CaCl_2$, forming beads and entrapping the yeast cells. The beads were allowed to harden for 30 min. The beads were

then washed with sterile saline solution (0.85 % $NaCl_2$) to remove excess calcium ions and cells. The cell density of seed culture for bead preparation was 3 g/l.

2.8.2 Equipment and experimental procedures

Continuous ethanol fermentation experiments were carried out according to a method followed by (Ksungur and Zorlu, 2001 and Najafpour *et al.*, 2004) using a continuous fermenter designed from a plastic bottle, a flask, a 20 cm length tube and a peristaltic pump. Prior to use, the bioreactor was disinfected with ethyl alcohol and then filled with Ca-alginate beads. The sterile molasses solution was fed to the bottom of the fermenter continuously by means of a peristaltic pump through sterile silicon tubing. Effluent liquid overflowed from an outlet port at the top of the bioreactor, maintaining a constant level inside the column, and collected in a 2 l-Erlenmeyer flask serving as product reservoir. The beads were trapped inside the bioreactor with a metal mesh filter covered by a plastic barrier. A flow breaker was installed between the column and feed pump, which prevented the growth of microorganism and contamination of feed line and feed tank. The samples from the ICR (immobilized cell reactor) column were taken from the outlet compartments of the column. The experimental set-up of ICR was as is shown in Fig. 3.

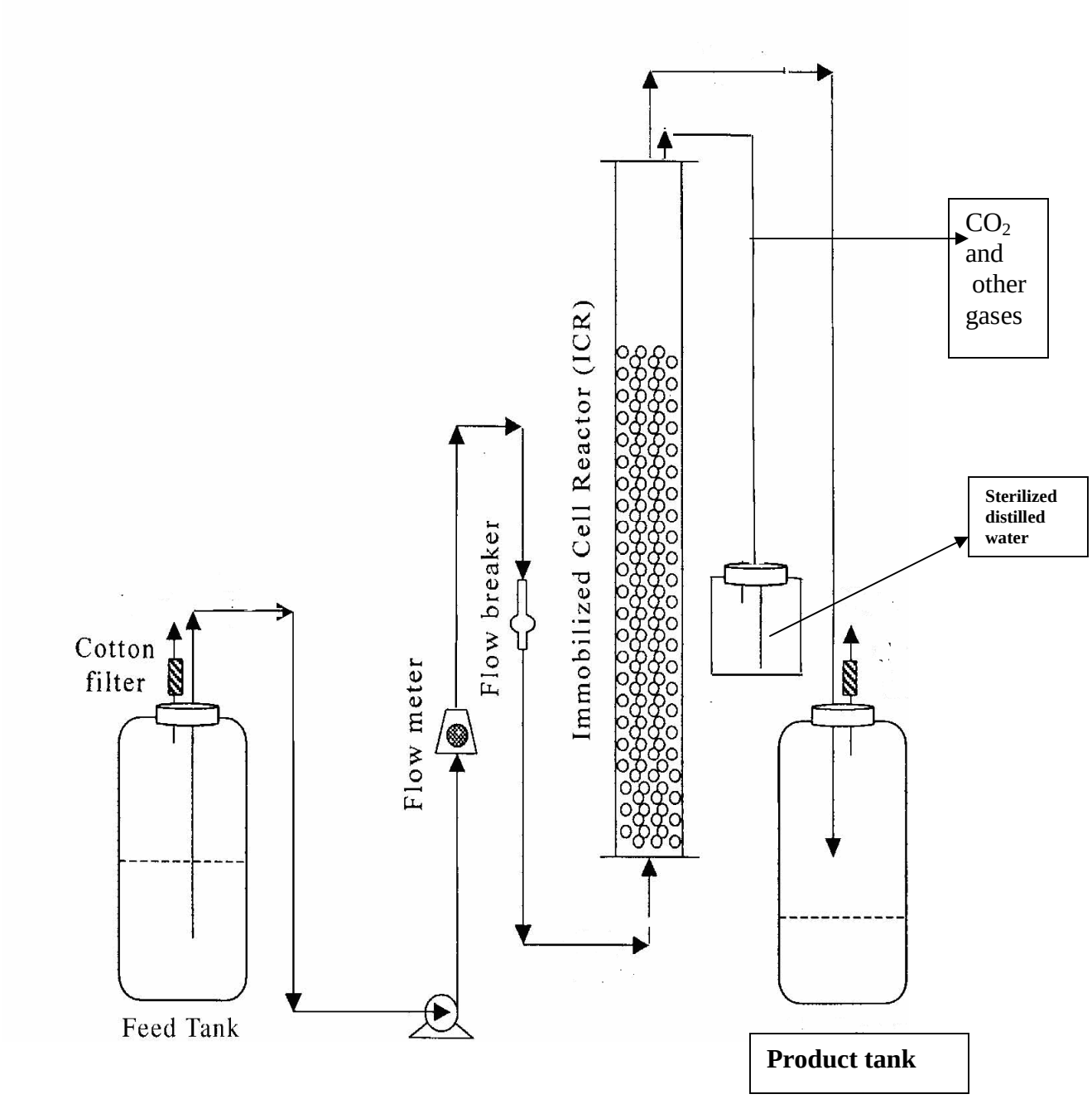


Figure 3. Experimental set up immobilized cell reactor

2.8.3 Effect of initial sugar concentration

In order to determine the effect of initial sugar concentration on continuous ethanol production by Ca-alginate immobilized yeast cells, diluted molasses containing 8, 11, 150 and 20 % of total sugars were prepared. The pH of the medium and dilution rate was 4.5 and 0.15h^{-1}, respectively. The temperature was maintained at 35 ^{o}C. Samples were taken every 12 h for analysis. Before increasing the sugar concentration of the feed, 2% $CaCl_2$ solution was passed through the column to prevent deformation and to maintain the integrity of the beads.

2.8.4 Effect of initial pH

To evaluate the effect of initial pH of the molasses media, experiments were carried out at different pH values. Diluted cane molasses with 11% total sugars was prepared at different pH values from 3.5 to 5 with 0.5 intervals. The prepared media were fed into the ICR continuously at different fermentation periods. The dilution rate was 0.15 h^{-1} and the temperature was maintained at 35 ^{o}C.

2.8.5 Effect of dilution rate

The effect of dilution rate or the speed of addition of feed into the bioreactor was evaluated in this study. Diluted cane molasses containing 11% total sugars with pH values 4.5 was prepared. This was then fed to ICR continuously at different dilution rates: 0.1, 0.15, 0.2 and 0.25 h^{-1}. The temperature was maintained at 35 ^{o}C. Experiments were run for one week to test each of the dilution rates.

2.9 Analytical methods

Different analytical methods were employed in this study. The employed methods were used to measure cell biomass, total sugar concentration, ethanol amount, dilution rate and fermentation efficiency of the different fermentation systems.

2.9.1 Cell biomass

To measure cell biomass of free cell batch system, sample was taken from fermentation systems and centrifuged at 10,000 rpm for 20 minutes (mins.). It was then washed twice with sterilized distilled water and dried at 60 ^{o}C following measuring the weight continuously till getting constant dry weight.

To measure the entrapped cell population in ICR, the following steps were carried out. Liquefaction of Ca-alginate beads was performed by dissolving 1 g of beads in 20 ml, 1% (w/v) sodium citrate solution (pH=6.0) with continuous stirring for 30 mins. at room temperature. To determine the concentration of viable cells entrapped in Ca-alginate beads, counting of yeast was undertaken by plating appropriate dilutions of liquefied beads on YPDA and incubating plates at 35 °C for about 48 h.

2.9.2 Total sugar

Total sugars were determined by Dinitro salicylic acid (DNS) method (Miller, 1959) after hydrolyzing the samples. For hydrolysis, 5.0 ml of distilled water and 1.0 ml of concentrated HCl acid was added to 5.0 ml of the samples. The contents were boiled for one minute only, followed by cooling, neutralizing with 4.4 ml of 2.5 N NaOH and diluted appropriately (Sheoran *et al.*,1998). The DNS solution was prepared by dissolving sodium sulfite 0.5 g, phenol 2 g, sodium hydroxide 10 g, potassium-sodium tartarate 200 g, and 3-5, dinitro-salicylic-acid 10 g in a liter of distilled water.

The standard curve for glucose was prepared using the standard proportion of glucose followed by (Miller, 1959). The standard proportion for glucose was prepared by adjusting the final concentration to 0.2 mg/ml as 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14,0.16,0.18,0.2 where the actual proportion of glucose solution to water was 0:1000, 20:980, 40:960, 60:940, 80:920, 100:900, 120:880,140:860 160:840,180:820 and 200:800. Then 2 ml of DNS solution was added into each proportion. This was followed by boiling for 5 minus. and finally reading of the absorbance at 540 nm after cooling at

room temperature. The standard formula, Y= aX + b was developed based on the result of standard curve where:

X= the amount of glucose equivalent and

Y= absorbance value.

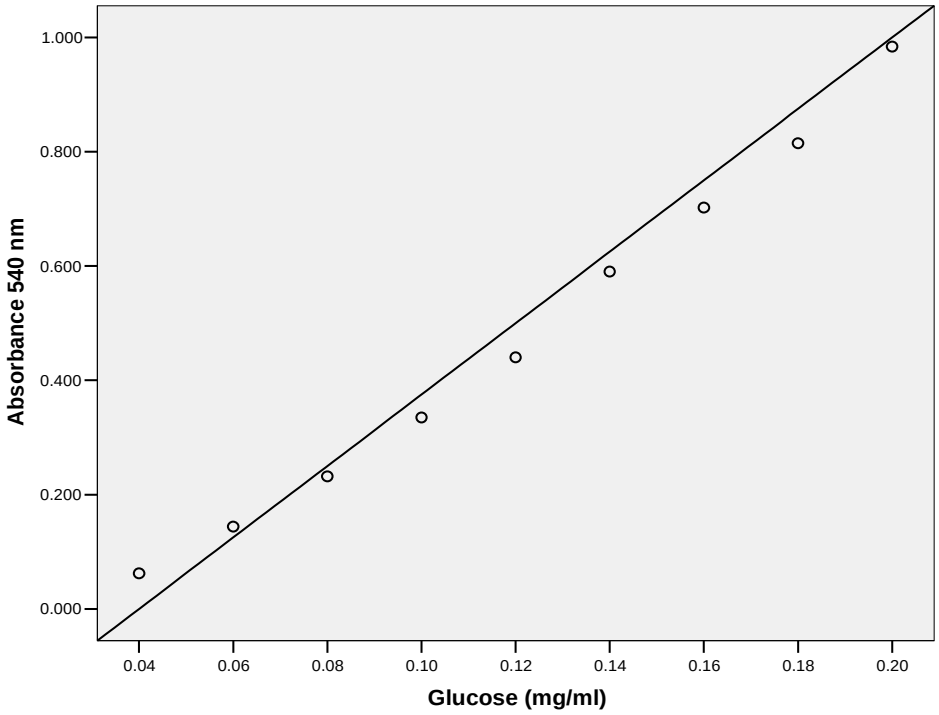


Figure 4. The standard curve used to estimate total sugar. It was developed by varying the proportion of glucose to the final concentration of 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2 and following the DNS method (Miller, 1959) where the respective average values of spectrophotometer readings are laid on Y axis.

The standard curve was used to estimate the amount of glucose equivalent in cane molasses during fermentation studies. The standard formula obtained was Y=OD x 0.18 + 0.33 based on the result of standard curve (Fig. 2.3).

2.9.3 Ethanol measurement

The amount of alcohol in fermented product was measured by specific gravity method after distillation (Bunting and Stockwell, 1978). The specific gravity bottle was filled with 30 ml distillate of fermented product and temperature at the moment of measurement was measured by a thermometer from ZELCO, England. The weight of the distillate and the bottle together; weight of empty bottle and weight of 30 ml distilled water with the bottle were measured.

Then specific gravity was measured by a formula W_2-W_3/W_1-W_3, where W_1 is weight of distilled water and bottle, W_2 is weight of distillate and bottle and W_3 is weight of specific gravity bottle alone. The resulted specific gravity was measured by Indian standard table of alcoholometry by pyknometer of gravity conversion table with corresponding alcohol content volume/volume and corresponding temperature.

2.9.4 Dilution rate

The dilution rate (D, h^{-1}) was calculated by dividing the flow rate of the medium by the working volume of the bioreactor.

$$D=F/V_f \quad \text{.....Eq 1}$$

Where,

D= dilution rate

F = Flow rate

V_f =The working volume of the reactor

2.9.5 Fermentation efficiency

Considering only the ethanol present in the medium aqueous phase, the fermentation efficiency (η) is defined by equation 2, where M_S is the glucose initial mass, M_E is the ethanol final mass in the aqueous phase, and 0.511 is the stoichiometric ethanol yield factor.

$$\eta = \frac{M_E 100}{(0.511 * M_S)} \quad \text{----------Eq. 2}$$

2.9.6 Calculation of productivity

Productivity (P) was calculated using formula

$$P_f = \frac{F \; X \; C}{V_f} \text{----------Eq.3}$$

Where,
P_f is the productivities ($gl^{-1}h^{-1}$) with respect to fermenter working volume
F = Flow rate
C = Steady state alcohol concentration (gl^{-1})
V_f= The working volume of the reactor (l)

3. RESULTS

3.1 Isolation TAYI 4-2

The wild yeast isolate used in this study was obtained from local '*tella'* after screening has been done through three times. As shown in the Fig 5, it was possible to get pure colonies that can be used in fermentation experiments. The colonies were whitish with convex appearance.

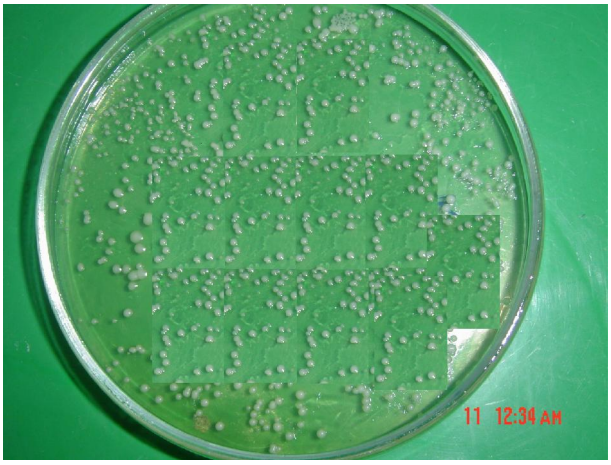


Figure 5. Isolation of TAYI 4-2 on YPDA medium. Pure colonies were obtained after three time's successive screening

3.2 Selection of an isolate for ethanol production

A total of 50 isolates were obtained from Ethiopian traditional beverages ('*tella*' and '*tej*'). The alcoholic fermentative capacity varied between 1.18- 5.07 (v/v) (Table 1). Among the tested isolates, 38% were able to produce alcohol in the range of 3.1-4 (v/v), 30% in the range of 4.1-5, and 22 % in the range of 2.1-3, 8% in the range of 1-2 and only 2% in the range of 5-6 % (Fig 6).

Table 1 Ethanol yield of the 50 isolate by batch fermentation using cane molasses medium. About 10ml of 16 h yeast inoculum of each isolates were inoculated in flask containing 10% total sugar and incubated at 30 ^{0}C.

Isolate	Ethanol yield (v/v)	Isolate	Ethanol yield (v/v)	Isolate	Ethanol yield (v/v)	Isolate	Ethanol yield (v/v)	Isolate	Ethanol yield (v/v)
TAYI 1-1	2.90	TAYI 6-1	2.37	TAYI 11	1.85	TAYI 21	3.43	TJYI 31	3.59
TAYI 1-2	3.43	TAYI 6-2	1.87	TAYI 12	3.24	TAYI 22	3.95	TJYI 32	4.88
TAYI 2-1	3.07	TAYI 7-1	3.24	TAYI -13	1.18	TAYI 23	3.79	TJYI 33	4.70
TAYI 2-2	3.59	TAYI 7-2	4.13	TAYI -14	1.18	TAYI 24	4.33	TJYI 34	3.79
TAYI 3-1	2.55	TAYI 8-1	3.98	TAYI 15	2.39	TAYI 25	4.32	TJYI 35	4.88
TAYI-3-2	3.95	TAYI 8-2	2.55	TAYI 16	3.95	TAYI 26	4.13	TJYI 36	3.08
TAYI 4-1	3.75	TAYI 9-1	4.33	TAYI 17	4.51	TAYI 27	4.51	TJYI 37	4.70
TAYI 4-2	5.17	TAYI 9-2	3.25	TAYI 18	3.25	TAYI 28	4.51	TJYI 38	2.75
TAYI 5-1	2.55	TAYI 10-1	4.15	TAYI -19	4.33	TAYI 29	3.95	TJYI 39	2.90
TAYI 5-2	2.37	TAYI 10-2	3.24	TAYI 20	4.33	TAYI-30	2.55	TJYI 40	2.20

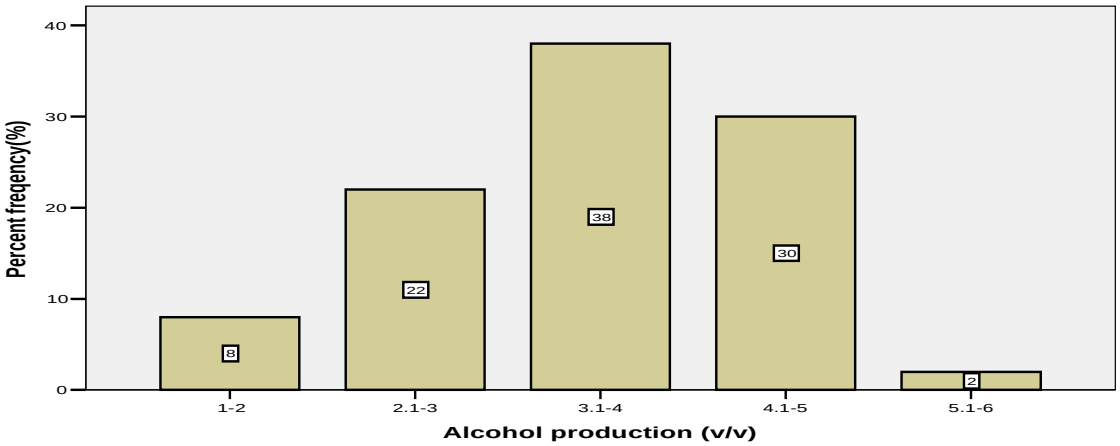


Figure 6. Summary of alcohol percentage produced by 50 isolates of yeast from '*tella*' and '*tej*'.

3.3 Optimization studies of fermentation of molasses

3.3.1 The effect of initial sugar concentration

The initial sugar in fermentation medium has a direct impact on final ethanol concentration. As shown in (Fig 7) below, the amount of ethanol produced increased from 34.16 g/l to 52.54 g/l as sugar in medium increased from 80 g/l to 130 g/l. This happened for brewer yeast and for yeast isolates TAYI 4-2; however, the increase was shown only up to 110 g/l. A further increase in initial sugar has found to hinder fermentation as shown by low ethanol yield at 200 g/l. Brewer yeast gave the lowest ethanol yield (10.57 g/l) at 200 g/l initial sugar concentration. At 110 g/l initial sugar, yeast isolate 4-2 was efficient 89.83 % and at 130 g/l brewer yeast was efficient only 79.10 %. The fermentation efficiency was shown to decline beyond an initial sugar of 110 g/l for both microorganisms studied (Fig 7).

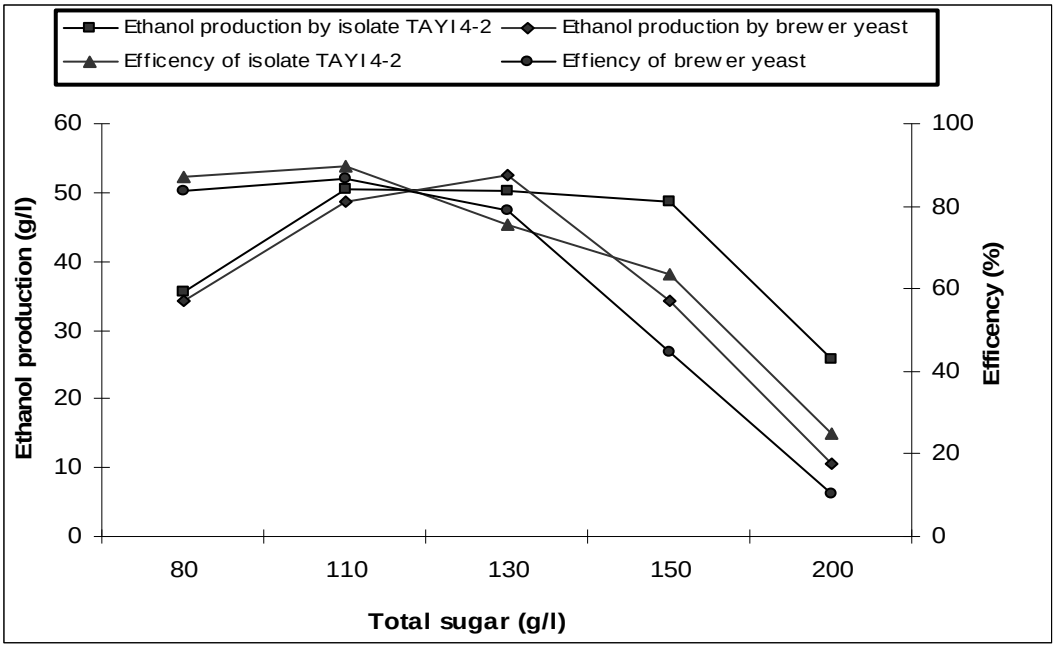


Figure 7. Illustration of the effect of initial sugar concentration of molasses medium on the alcoholic yield and efficiency of the yeast isolate TAYI 4-2 and brewer yeast.

3.3.2 The effect of temperature

Temperature is one of the major constraints that determine the ethanol production. To know the optimum temperature for ethanol fermentation, the broths were kept at 25, 30, 35 and 40°C with 11% initial sugar concentration. A low ethanol yield of 32.59 g/l and 6.63 g /l was observed at 40°C for isolate TAYI 4-2 and brewer yeast respectively in 54 hours (Fig. 8). The ethanol concentration was increased from 35.58 g/l to 50.50 g /l when temperature was raised from 25 °C to 35 °C for isolate 4-2 (Fig 8). At 35 °C ethanol yield was maximum and turned out to be 50.50 g /l. However, increasing the temperature above 35°C the concentration of alcohol decreased. Brewer yeast on the other hand had an optimum activity to yield ethanol at temperature of 30^{o}C. Up on increasing the temperature above 30°C the concentration of alcohol decreased and the decrease was pronounced at 40°C. At optimum temperature, isolate TAYI 4-2 was found to be efficient 89.8% while brewer yeast 85.62%.

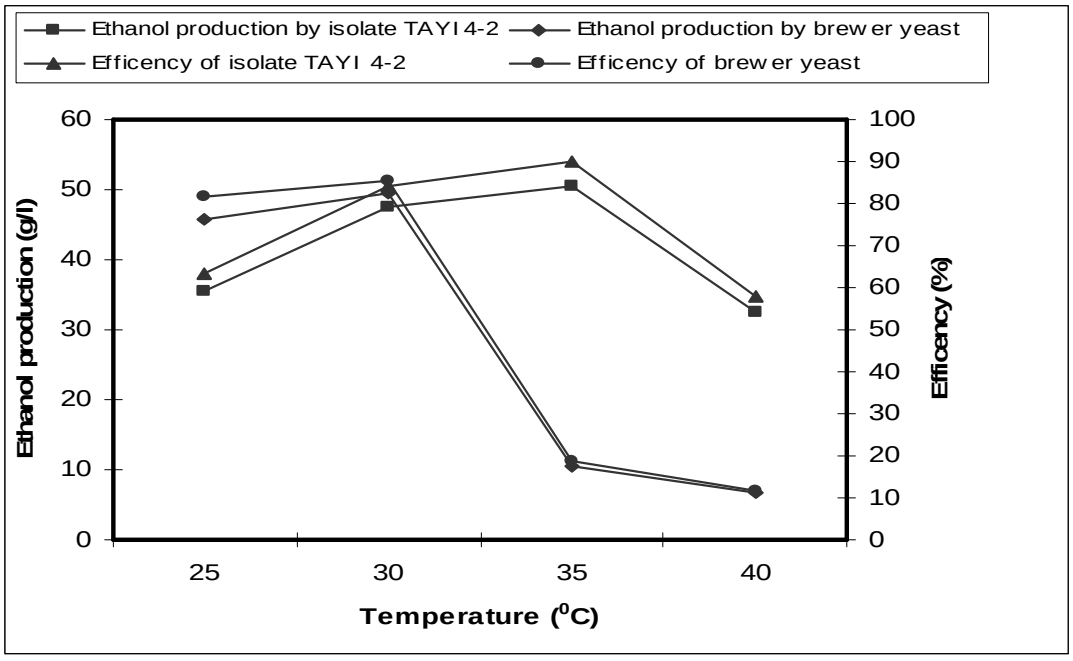


Figure 8. Ethanol yield and efficiency against temperature profile of fermentation process using yeast isolate TAYI 4-2 and brewery yeast. Incubation temperature was from 25-40 ^{o}C with 5 ^{o}C intervals.

3.3.3 The Effect of pH on ethanol yield

Initial sugar concentration of 11% and optimum temperature of 35 ^{o}C for isolate TAYI 4-2 and 30 ^{o}C for brewer yeast was selected for further studies and subjected to initial pH of treatments 3.5, 4, 4.5 and 5. The results are shown in Fig 9. At pH 3.5, fermentation took place but it gave low ethanol content. Brewer yeast at pH 3.5 gives the lowest ethanol yield of 7.72 g/l. Best results were obtained at pH 4.5 where maximum ethanol production was noticed for both yeast isolates. However yeast isolate TAYI 4-2, the ethanol production from molasses was not much affected by pH in the range of 4-5 as indicated by having fermentation efficiency above 80%. A decrease in pH from 4.5 to 3.5 was found to hinder the fermentation of cane molasses by brewer yeast (Fig 9).

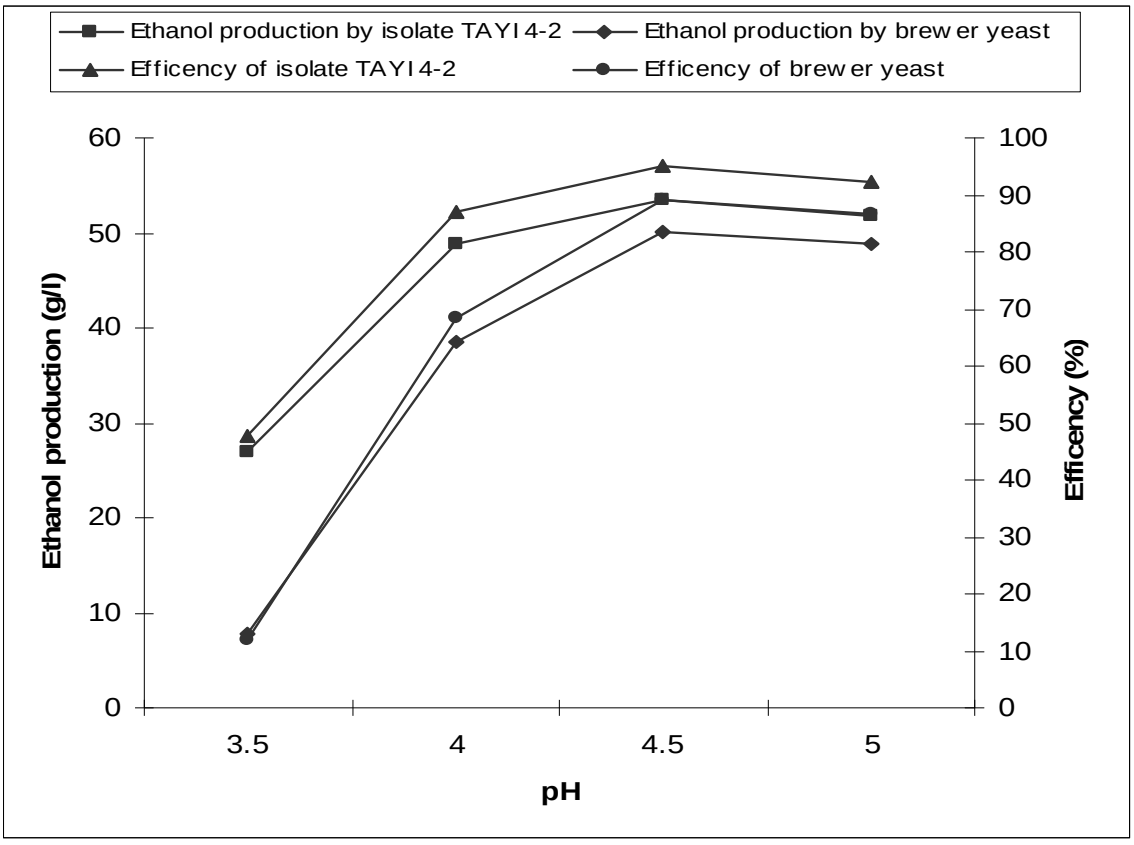


Figure 9. Ethanol yield and efficiency against pH profile of fermentation process using yeast isolate TAYI 4-2 and brewery yeast. Initial pH was at values 3.5-5 with 0.5 ^{o}C interval.

3.3.4 The effect of supplements on ethanol yield

The effect of additives in molasses media on ethanol yield was evaluated. Compared to the control (molasses medium with out any additive), ethanol yield with the supplements effect was shown on (Fig 10). The additives were urea, yeast extract, ammonium phosphate and ammonium nitrate. The supplemented molasses did show a slight increase in yield in all supplementation cases compared to the control (Fig.10). The molasses medium supplemented with $(NH_3)_2PO_4$ showed the best ethanol yield increase than the other supplemented media compared to the control. The least ethanol yield was observed in the media supplemented with yeast extract when compared to the other supplemented media.

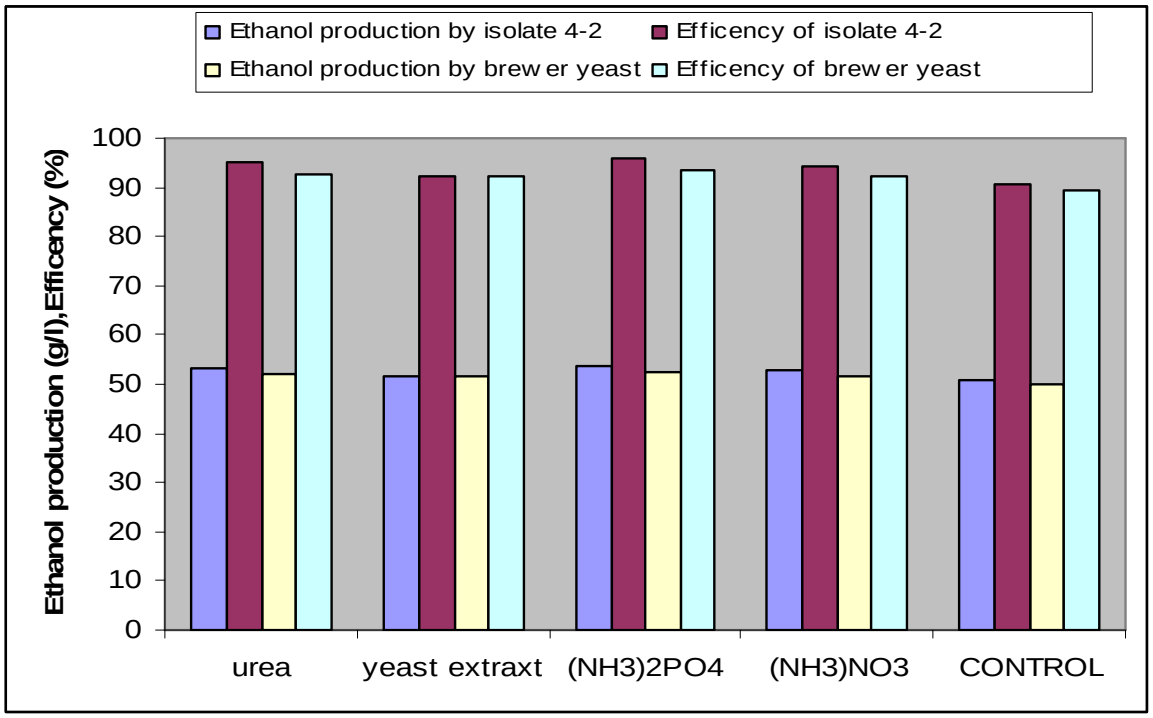


Figure 10. Ethanol yield against additives in fermentation process using yeast isolate TAYI 4-2 and brewery yeast. The additives were urea, yeast extract, ammonium phosphate and ammonium nitrate.

3.3.5 Time course of ethanol fermentation

Ethanol production by isolate TAYI 4-2 was started before 6 h and continued to increase for 48 h where ethanol yield was found to be maximum afterwards (Fig 11). A sharp decrease in total sugar concentration was noticed during this period and the sugar consumption profile appeared to be consumed at low rate towards the end of fermentation. Accumulation of alcohol during fermentation was accompanied by a progressive decrease of rate of sugar conversion to ethanol (Fig 11).

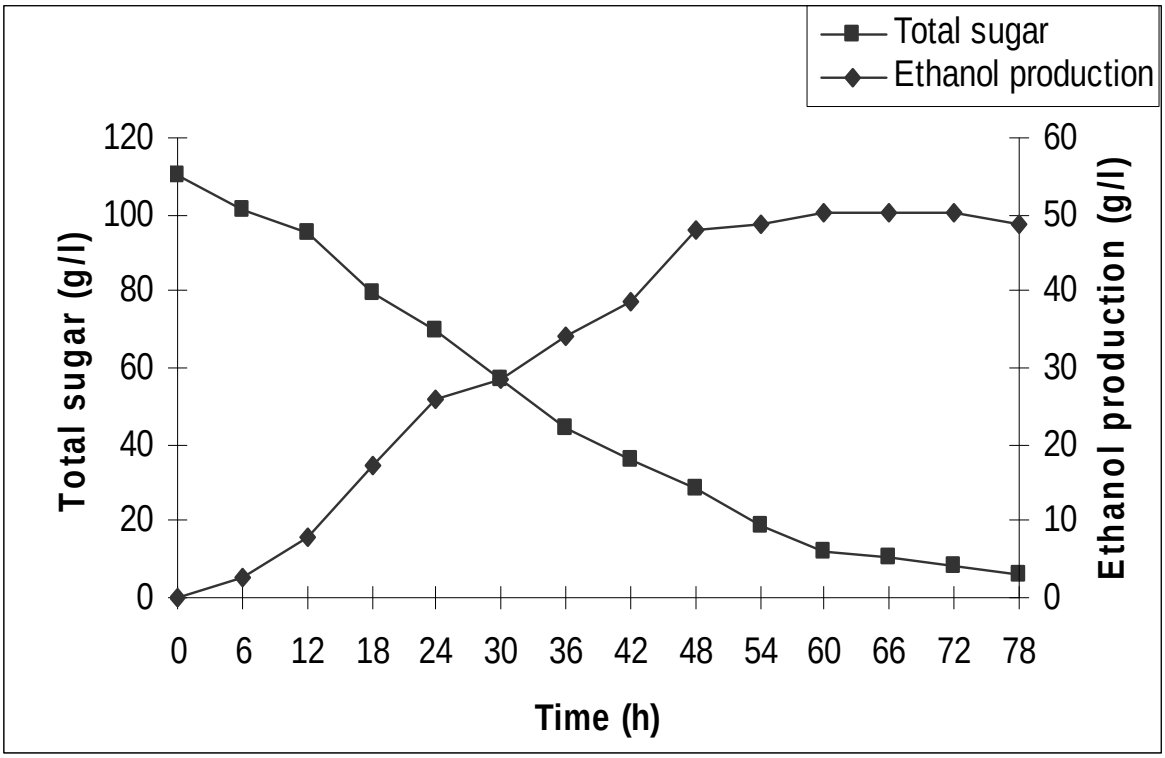


Figure 11. Residual sugar concentration and production of ethanol in batch fermentation with initial sugar concentration of 110 g/l versus time. About 10 ml of a 16 h yeast culture was used to inoculate a 100 ml molasses media containing 110 g/l initial total sugar. Samples were taken every 6 h for analysis of residual sugar and ethanol content. The graph shows the declining trend of total sugar as fermentation time goes on while ethanol production increases.

3.3.6 Biomass yield

Yeast biomass required to produce a specific amount of ethanol in fermentation medium was found to increase slightly during early stage of fermentation. And it was observed to continue to increase till 48 h where the ethanol production turns to maximum afterwards (Fig 12). The maximum cell density of TAYI 4-2 in batch fermentation was 12.65 g/l with 110 g/l initial sugar concentration. A slight decrease was noticed in yeast biomass after 54 h as indicated by a drop from 12.65 g/l to 11.875 g/l.

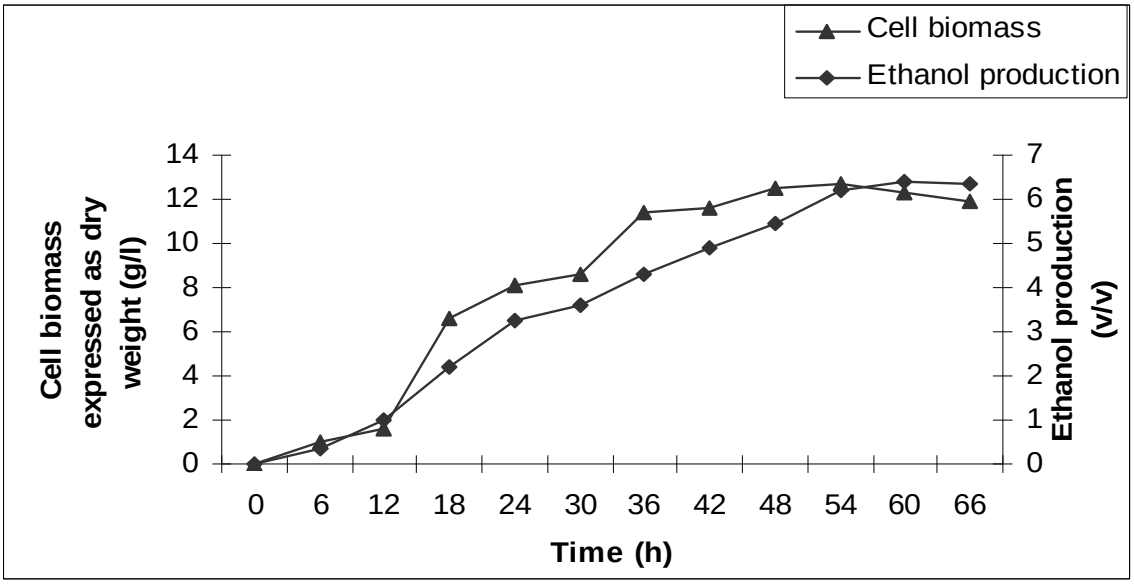


Figure 12. Yeast biomass expressed as dry weight and ethanol yield measured against time during ethanol fermentation in molasses medium using yeast isolate TAYI 4-2.

3.4 Anaerobic repeated batch fermentation of molasses

Ethanol production by Ca-alginate immobilized yeast cells was studied by running ten consecutive batches. Every 24 h the original culture was removed and the beds washed and re-suspended with fresh molasses medium having a sugar concentration of 110 g/l. An increasing trend of ethanol production was observed till the third batch and turned out to constant thereafter (Fig. 13). Repeated batch production could be performed by maintaining high conversions and the average conversion was calculated to be 90.85%.

Through out the whole cultivation cycle the beads were stable without any sign of rapture.

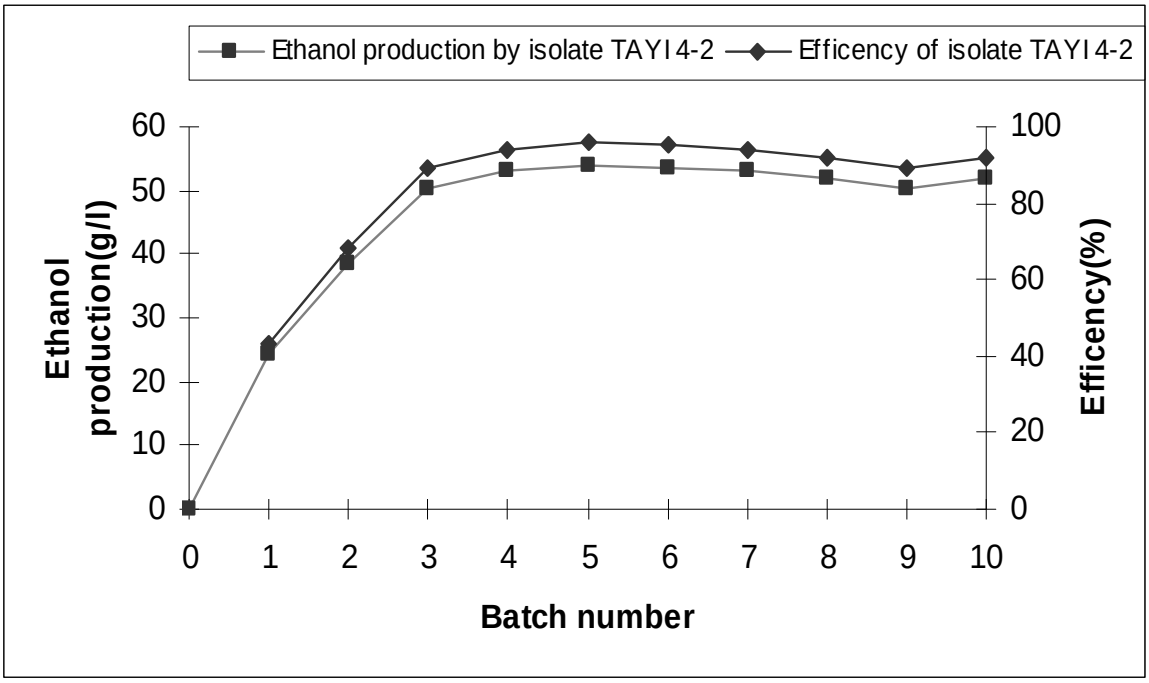


Figure 13. Repeated batch ethanol production by TAYI 4-2 immobilized by entrapment in Ca-alginate. TAYI 4-2 was immobilized on 2% alginate and fed to the reactor. Every day the fermented media was removed and the resulted beads were washed twice with fresh autoclaved molasses media. The samples were distilled to measure the alcohol content.

3.5 Continuous ethanol fermentation

3.5.1 Characteristics of alginate beads

Yeast cells were immobilized using Ca –alginate (Fig. 14). The average bead diameter used in this study was 2 mm having a moisture content of 89 %. The beads were stable for more than a month during fermentation experiments. In addition the amount of cell in a gram of bead immediately after preparation was 4×10^7 CFU/g (Table 2).

Table 2. Physical properties and appearance of yeast isolate TAYI 4-2 beads

Beads diameter	2mm
Beads moisture content	89 %
Physical appearance	Flexible
Stability	More than a month
Number of CFU/g of beads	4×10^7

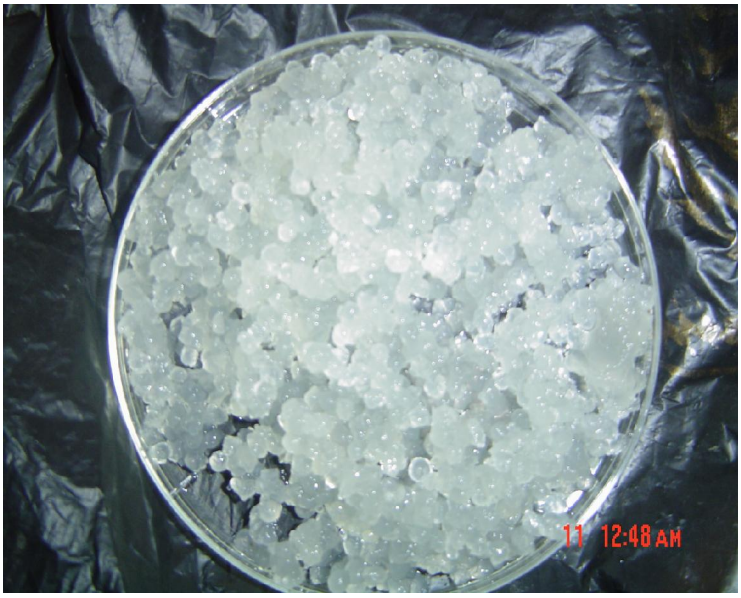


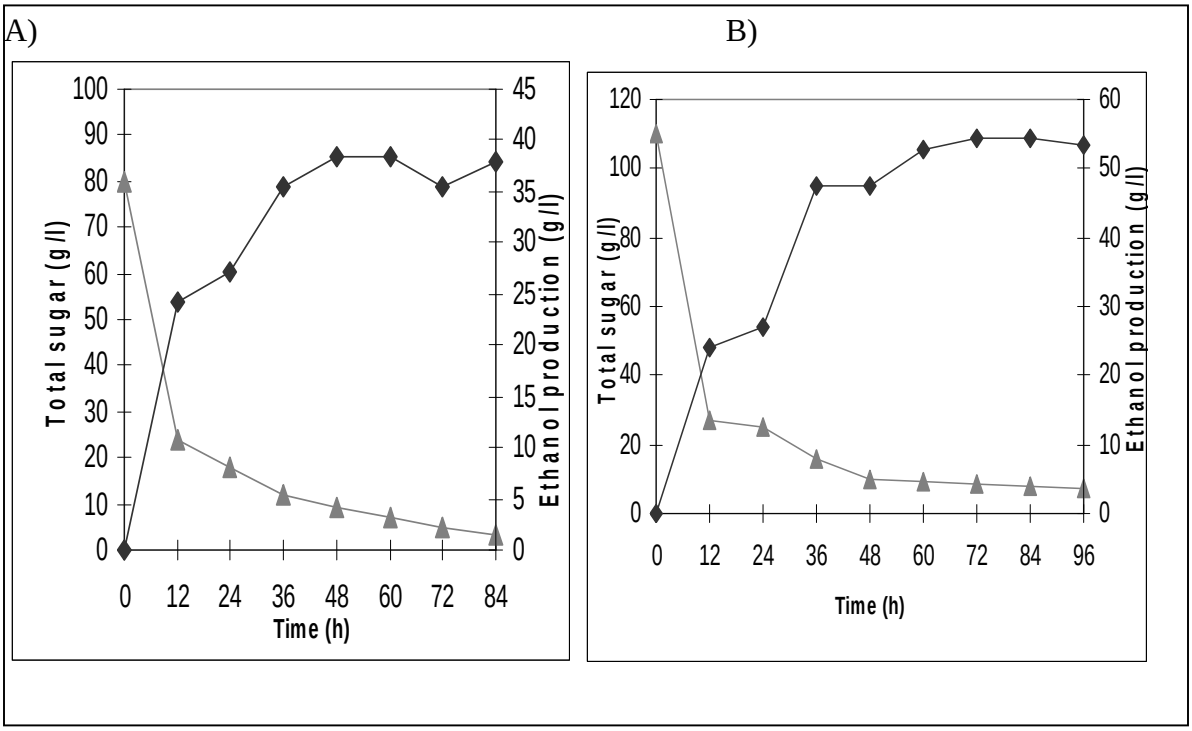
Figure 14 Ca –alginate beads entrapped with yeast isolate TAYI 4-2

3.5.2 Effect of initial sugar concentration

To determine the effect of sugar concentration on continuous ethanol production, Ca-alginate immobilized yeast cells and diluted molasses containing 8 %, 11 %, 15 % and 20 % of total sugar were used. The pH of the medium and dilution rate was 4.5 and 0.15 h^{-1},

respectively. The sugar consumption trends of various sugar concentrations were similar, with rapid reduction of substrate occurring within the first 12 h. A very high sugar concentration in the medium leads to incomplete sugar conversion as it is indicated on Fig. 15.The continuous cultivation of TAYI 4-2 on molasses media with a sugar feed concentration of 110 g/l achieve stable ethanol concentrations of 52.77 g/l (Fig.15). The ethanol concentrations were 37.60, 52.44 and 28.99 g/l for 80, 150 and 200 g/l of initial sugar concentration respectively.

Maximum ethanol productivity (14.34 $g1^{-1}$ h^{-1}) was achieved at 11% initial sugar concentration. At 8%, 15% and 20% of initial sugar concentrations, 10.03, 14.25 and 7.914.34 g l^{-1} h^{-1} ethanol productivity values were obtained, respectively (Fig. 16).



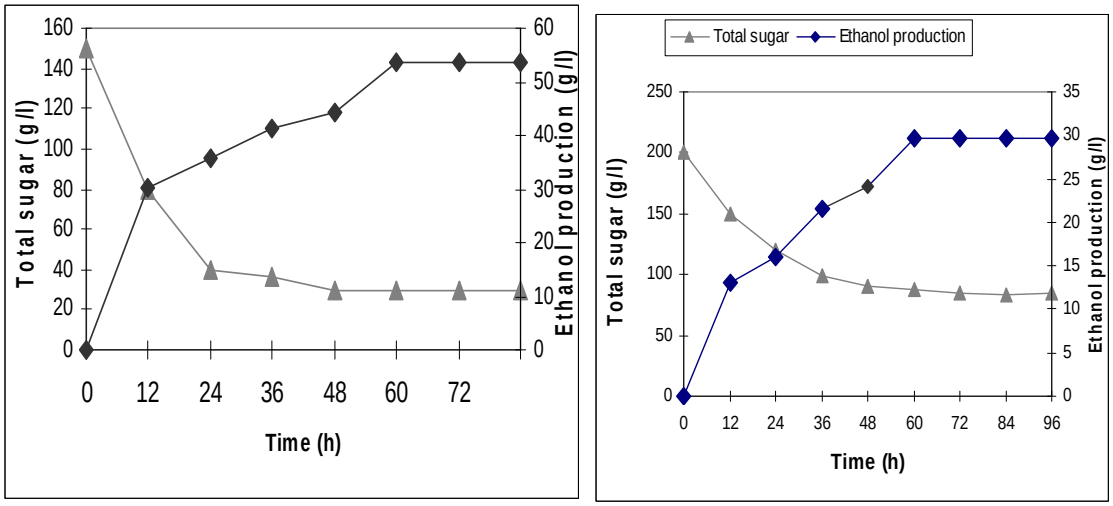


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Figure 15 The effects of initial sugar concentration on ethanol production from cane molasses by immobilized yeast isolate TAYI 4-2 at pH 4.5 and 0.15 h^{-1} dilution. A) The initial concentration of 80g/l was used ICR and analysis of residual sugar and ethanol production was done every 12 h. B) The initial concentration of 110 g/l was used ICR and analysis of residual sugar and ethanol production was done every 12 h. C) The initial concentration of 150 g/l was used ICR and analysis of residual sugar and ethanol production was done every 12 h. D) The initial concentration of 200 g/l was used ICR and analysis of residual sugar and ethanol production was done every 12 h.

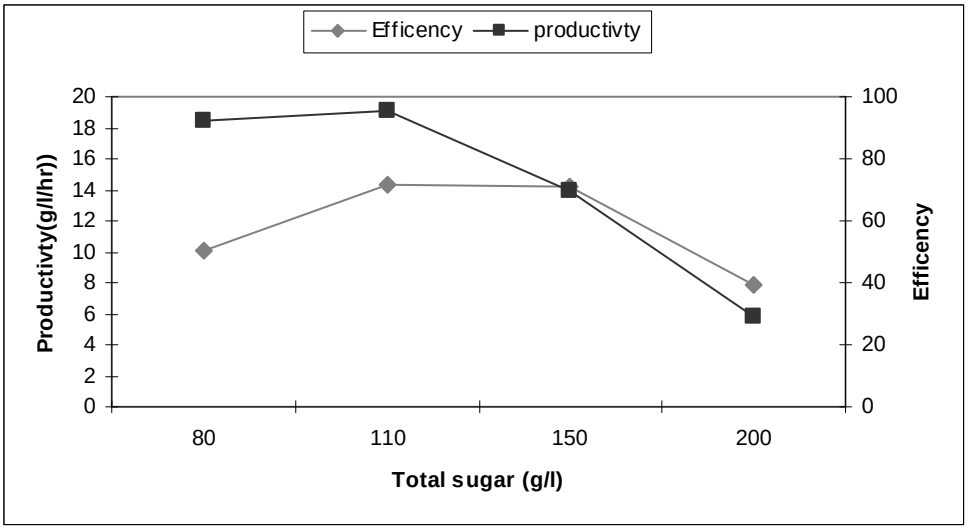

Figure 16 Ethanol productivity and fermentation efficiency as function of initial sugar concentration at pH 4.5 and 35^0C incubation temperature.

3.5.3 Effect of initial pH

In order to evaluate the effect of initial pH on ethanol yield diluted cane molasses containing 11% total sugars at different pH values were prepared and fed into the immobilized cell reactor continuously. The dilution rate was $0.15h^{-1}$ and the temperature was controlled at 35 ^{0}C. Maximum ethanol concentration (52.1 g/l) and theoretical yield (92.68 %) were obtained at pH 4.5 (Fig. 3.13). A pH decrease from 4.5 to 3.5 was found to reduce ethanol productivity from 14.25 g/l/h to 5.72 g/l/h (Fig.18). The ethanol production at pH 5 and 4 was also lower than pH 4.5.

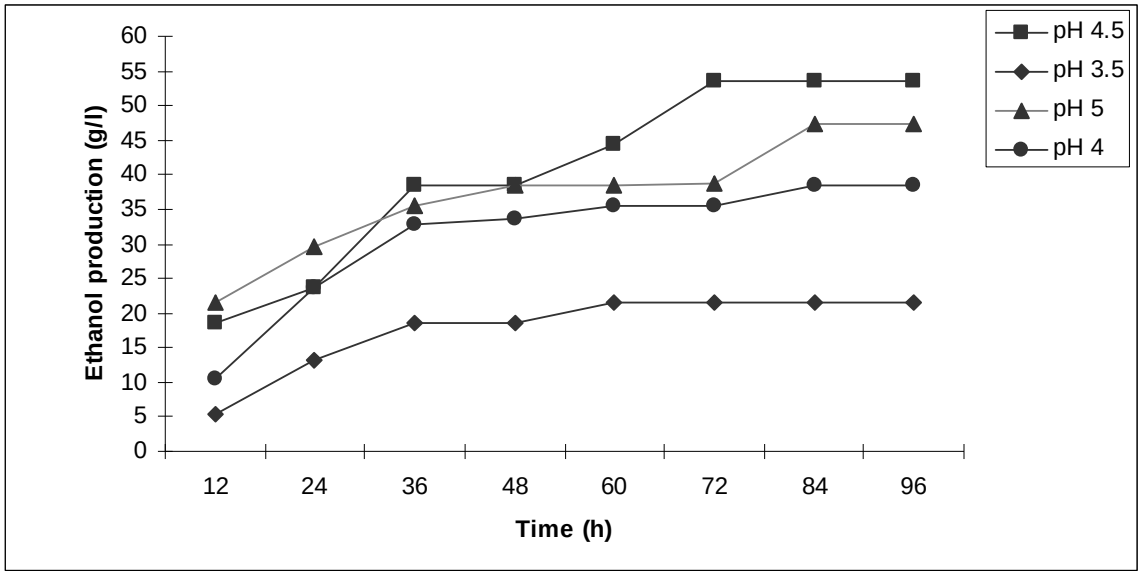

Figure 17 The effect of initial pH of medium on ethanol yield from cane molasses by immobilized yeast isolate TAYI 4-2 cells in ICR (T=35^{0}C, initial substrate concentration=11%).

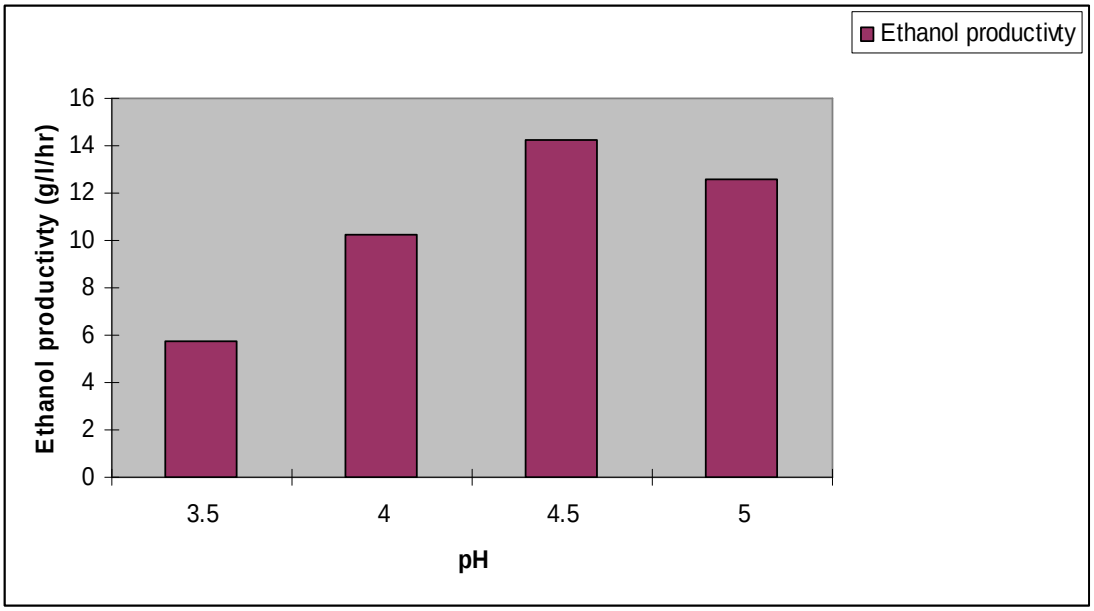

Figure 18 Ethanol yield in relation with initial pH of molasses medium in immobilized continuous cell reactor using yeast isolate TAYI 4-2.

3.5.4 Effect of dilution rate

To determine the effect of dilution rate in continuous ethanol fermentation, the immobilized cell reactor using molasses as fermentation medium was run continuously at four different dilution rates for a period of 7 days for each dilution rates. The results indicate that an increase in dilution rate from 0.15 h^{-1} to 0.25 h^{-1} resulted in lowering ethanol concentration in the fermented broth. On comparing the performance of the system at different dilution rate, the highest ethanol yield was observed at 0.15 h^{-1} (Fig. 19). The fermentation efficiency was also lowered with increase in dilution rate and also at 0.25h^{-1}, a marked decrease was observed in ethanol concentration and in fermentation efficiency. Higher concentration of ethanol was obtained at a lower dilution rate (0.15 h^{-1}) and residual sugar left was less than 2 g/l. The amount sugar un-utilized found to increase as higher dilution rate were employed ICR.

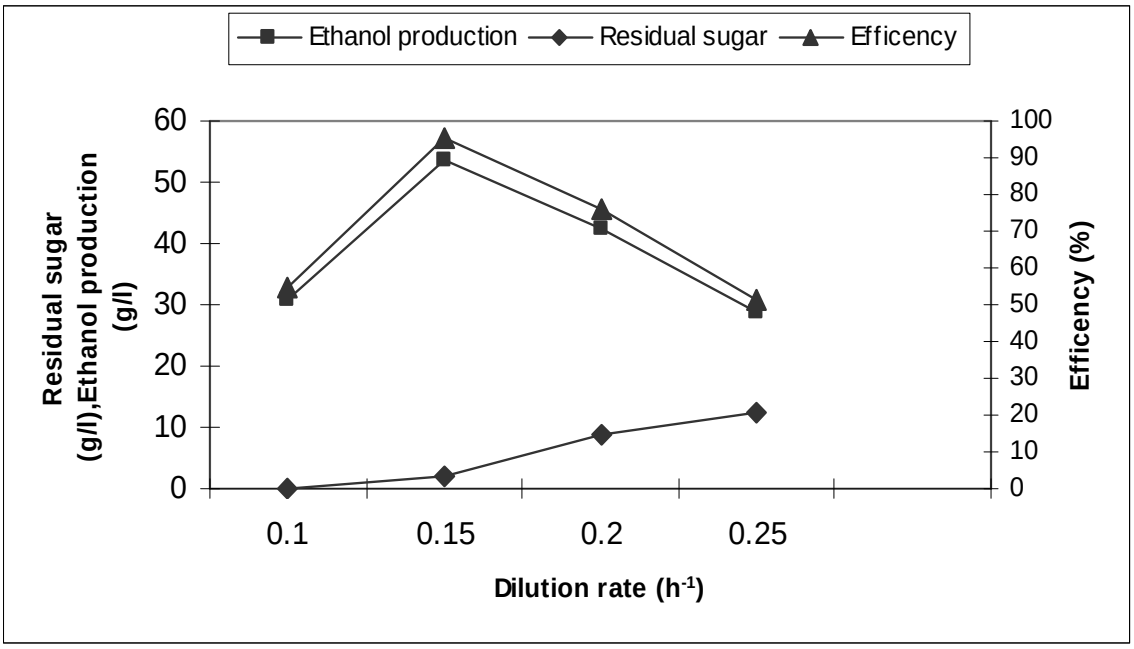

Figure 19 Comparison of parameters (residual sugar concentration g/l, efficiency and ethanol yield) in immobilized continuous system by TAYI 4-2 against dilution rate.

3.6 Ethanol production by yeast isolate TAYI 4-2 in different fermentation method

Comparison of the amount of ethanol production in different systems was carried out using a yeast isolate screened from '*tella*' (TAYI 4-2). The yeast was immobilized in alginate gel beads and allowing the yeast to ferment in continuous fermentation, repeated batch and by free batch method. The amount of initial sugar was the same for all systems. The efficiency and productivity of continuous immobilized method of ethanol fermentation was 92 and 14.34 g/l/h respectively (Table 3). This system was found to be with highest ethanol productivity within short period of time when compared to free batch and immobilized batch systems. In continuous immobilized system, it was recorded that the yeast produced about 52.14 (g/l) of ethanol from 110 (g/l) initial sugar concentrations within 12 h and the residual sugar concentration was recorded to be 2 (g/l). Rather in immobilized batch system, the efficiency and productivity was 90 and 2.12 respectively (Table 3). And the produced ethanol amount was about 51.2 (g/l) .The

residual sugar amount in this system was 0.1 (g/l). The least ethanol yield was observed in free batch system, 50 (g/l). The efficiency and productivity of ethanol in this system was 1.04 and 89 respectively. The residual sugar left was 2 (g/l).

Table 3. Comparison of ethanol production capabilities of TAYI 4-2 when immobilized in alginate gel beads in continuous fermentation and repeated batch and by free batch method.

Fermentation conditions	Batch	Immobilized batch	Continuous (immobilized yeast)
Fermentation time (h)	48	24	12
Initial sugar concentration(g/l)	110	110	110
Residual sugar (g/l)	2	0.1	2
Final ethanol concentration (g/l)	50.08	51.2	52.14
Efficiency	89	90.07	92
Productivity (g/l/h)	1.04	2.12	14.34

3.7 Ethanol production potential of Ethiopia from cane molasses

Currently in Ethiopia there are three factories engaging in the processing of sugar cane to sugar and other by products. Only one factory, Fincha Sugar Factory, engaged in ethanol production from molasses. The annual molasses produced by each factory based on the average of the last three years (1997-1999 E.C.) was indicated in the Table 4. Although the technology used to produce ethanol from cane molasses source determines the efficiency of a distillery plant; mostly high ethanol yield depends on quality of molasses used for fermentation.

In the anaerobic pathway, every mole of glucose converted into two moles of ethanol, two moles of CO_2 and two moles of ATP along with 56 Kcals of heat. In another words, every gram of glucose converted yield 0.511 gram ethanol. The ATP produced used in biosynthesis or cell maintenance. Molasses contains about 50 total sugars, of which 30-

33% was sucrose and the rest was reducing sugars. During the fermentation, yeast strains convert sugar present in the molasses such as sucrose or glucose to alcohol.

$$\text{Glucose} \longrightarrow \text{Ethyl alcohol} + \text{Carbondioxide}$$

Thus, 110 gm of sugars on reaction give 55.5 gm of alcohol. Therefore, one tone of sugar gives 511.1 Kg of alcohol. The specific gravity of alcohol is 0.7934. Therefore, 511.1 Kg of alcohol is equivalent to 644 liters of alcohol. During fermentation other by-products like glycerin, succinine acid etc. are also formed from sugars. And the fermentation efficiency TAYI4-2 under the studied conditions was 90%. Thus, one tone of sugar will give only 644 x 0.90 = 579.6 liters of alcohol. One tone molasses containing about 50.4% fermentable sugars gives an alcoholic yield of 290 liters per tones. There fore, Ethiopia has a potential of producing 28,045,165 liters of alcohol per year. Considering the current market price of alcohol, the gross earnings can be estimated as 28,045,165 X 7 =196,316,151 birr/yr. The average production cost based on the last three years rate was 1.3 birr/yr. Therefore, to produce 28,045,165 liters of alcohol per year it will require 28,045,165 X1.3=36,458,714 birr/yr. There is also income tax 35% on additional income, 196,316,151 birr/yr X .35=68,710,654. The net benefit from ethanol production will be around 91,146,786 birr per year.

Table 3. Summary of the annual molasses production potential of the three factories
(Ethiopian sugar cooperation sales report)

Sugar factory	Molasses production(ton per year)
Metahara	39058
Wonji	24375.5
Fincha	33229
Total	96662.5

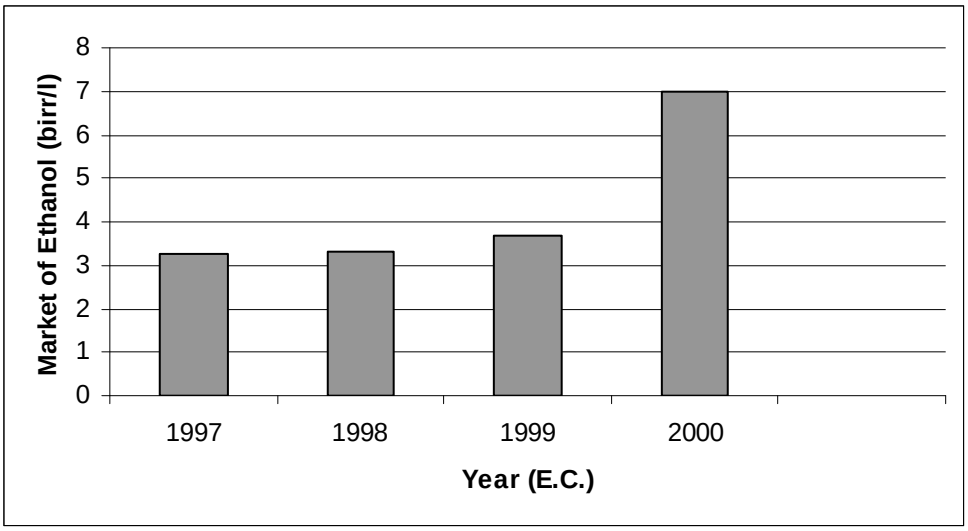

Figure 20. The trend of ethanol market price for the last four years in Ethiopia (Ethiopian sugar cooperation sales report)

Table 4. Summary of the total ethanol production costs and benefits.

Molasses production	96662.5 ton/yr
Average sugar %	50.04
Ethanol production	290 l/ton
Total Ethanol production	28,045,165 l/yr
Total revenue from Ethanol production	196,316,151 birr/yr
Total production costs	36,458,714 birr/yr
Net benefit from ethanol production	91,146,786 birr /yr

4. DISCUSSION

Fuel alcohol production via fermentation from biomass resource is now getting attention by many countries so as to reduce the dependency on foreign fossil fuels. Many countries including Ethiopia developed policies and strategies that enable them to exploit their potentials in this sector. A country with abundant agricultural residues, by-product of agro-industries, lingo-cellulosic biomass etc… can have a potential of producing bioethanol. Bioethanol production, in addition to biomass resources, requires an efficient yeast strain, a few nutrients and simple culture conditions.

Ethanol production requires an efficient strain with high ethanol yield and alcohol tolerance. Thus selection of the right strain for fermentation can have significant impact on the over all efficiency and productivity of the fermentation process. Ethiopian traditional alcoholic beverages such as '*tella*' and '*tej*' are fermented by different strains of yeast and other microorganisms, and many of them expected to differ in their alcohol productivity and tolerance. In this study several yeast isolates were isolated from '*tella*' and '*tej* 'and many of them differ in their alcohol productivity (Table 1). One yeast isolate, isolate TAYI 4-2, isolated from *'tella'* and showed superior quality in terms of fermenting molasses. This indicates that traditional fermented alcoholic beverages may serve as sources of high ethanol yielding yeast strains.

In addition to using efficient strains, optimization of physico-chemical parameters for maximum alcohol yield can have significant economic importance. Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation (Munnecke, 1981). In this study ethanol production by TAYI 4-2 was higher at 11% initial sugar concentration with fermentation efficiency of 90%. For brewer yeast on the other hand the ethanol yield increased as the sugar concentration increased from 8 % to 13%. At 13 % sugar concentration it produced 52 g/l of ethanol with an efficiency of 85%. The yield attained in practical fermentations, however, does not usually exceed 90-95% of the theoretical value and many fermentation exhibit an efficiency of 80-85% (Kosaric and Vardar-Sukan, 2001). This is because some nutrient is utilized for the

synthesis of new biomass and for maintenance. Side reactions may also occur in the fermentation (usually to glycerol and succinate) which may consume up to 4-5% of the total substrate (Kosaric and Vardar-Sukan, 2001). If these reactions could be eliminated, an additional 2.7% yield of ethanol from carbohydrate would result (Oura, 1977).The fermentation efficiency and the ethanol yield of TAYI 4-2 was in the acceptable range. The new isolate was more tolerant to the presence of high initial sugar in the medium than the brewer yeast.

Ability of yeast strains to grow and ferment high sugar concentration is very important for it could lead to higher final ethanol concentration and allow reduction of distillation costs. In addition at high sugar concentration growth of osmotic sensitive contaminants could be suppressed. However, in most yeast strains high sugar concentration leads to osmotic stress and alter their metabolism (Vasconcelos *et al*, 2004). In this study the yeast strain isolated from '*tella'* TAYI 4-2 performed better at high sugar concentration than brewer yeast indicating the potential of traditional alcoholic beverages as source of osmotolerant yeast strains. Increasing the initial sugar concentration to 20% resulted in a severe reduction in fermentation efficiency as well as ethanol yield for both yeast strains. But the effect was more pronounced for brewer yeast than for TAYI 4-2. Previous studies have shown that direct substrate inhibition of fermentative ability becomes significant somewhere between 15 and 25% sugar concentration (van Uden, 1989). The inhibitory effect of sugar can be avoided by adding it in small amounts in staggered intervals. When the substrate is introduced in several batches ethanol yields were reported to be higher (Casey and Ingledew, 1986 and D'Amore and Stewart, 1987).

The optimum temperature for alcohol production by TAYI 4-2 was at 35 ^{o}C while the optimum for brewer yeast was at 30^{o}C. The fact that TAYI 4-2 optimally grow at higher temperature may have some economic advantage. During fermentation there is always heat evolution which requires cooling. Thus tolerance to even a slight temperature rise could lead to significant reduction in cooling cost.

Another important factor in ethanol fermentation is pH. Yeasts are able to grow and efficiently ferment ethanol at pH values of 3.5-6.0 (Kosaric and Vardar-Sukan, 2001). TAYI 4-2 grew and efficiently ferment cane molasses in the pH range of 4-5 with an optimum initial pH of 4.5. Brewer yeast had an optimum pH of around 4.5 and also exhibited an efficiency of 86.74% at pH 5. Ethanol fermentation was found to decrease at pH 3.5 for both yeast strains studied. Alcohol production and fermentation efficiency of *Saccharomyces* yeasts is known to maximum in the pH range of 4.0 – 5.0 (Gokisungur and Zorlu, 2001). Thus isolate TAYI 4-2 was more acid tolerant than brewer yeast, this quality indicates harsh environmental condition resistant ability of the isolate.

The time required to produce a maximum alcohol by TAYI 4-2 was around 54 h. During this period, the concentratration of residual sugar was gradually decreased while the cell density and ethanol production increased. Accumulation of alcohol during fermentation is accompanied by a progressive decrease on the rate of sugar conversion to ethanol and represses the microorganisms' growth. According to van Uden (1989) inhibition of yeast growth is evident when the alcohol concentration of the fermenting medium reach about 4%. This inhibition of fermentation is usually attributed to an indirect effect of ethanol through repression of enzymes of glycolysis (Doelle *et al*., 1993 and Hobley and Pammet, 1994). Thus ethanol inhibition is the principal factor restricting fermentation rate and the concentration of ethanol obtained in ethanol production processes. Therefore, the effect is of major economic significance (van Uden 1989).

Productivity of TAYI 4-2 through batch fermentation was 1.04 g/l/h. To make the process of ethanol fermentation economical it is important to reduce production costs and improves process efficiency and ethanol yield. In this regard continuous fermentation using free, flocculent, or immobilized cells is the most preferred method. Compared to free cells, immobilized cells exhibit many advantages for ethanol fermentation. This includes relative ease for product separation, high volumetric productivity, improved process control, and reduction in susceptibility of cells to contamination. In this study TAYI 4-2 was immobilized by entrapping with Ca-alginate beads and used for ethanol fermentation.

In a batch culture of immobilized cells, the ethanol productivity was improved. Despite the presence of unproductive time during charging and discharging of the substrate in to the fermenter, the system was able to ferment molasses at higher fermentation rate for ten consecutive batches while maintaining the integrity of the Ca-alginate beads.

The performance of continuous fermentation system using immobilized TAYI 4-2 in ICR was evaluated in terms of ethanol productivity and fermentation efficiency with varying sugar concentration in the medium, at different initial pH, and dilution rate. Ethanol productivity for immobilized cells was higher at 11% initial sugar concentration, similar to free cell fermentation, but the productivity was improved by up to 14 fold (from 1.04 g/l/h for the free cells to 14.34 g/l/h for immobilized cells). Ethanol productivities of continuous system with immobilized yeast cells were high. The high productivity of this system decreases both the investment costs (low capacity of bioreactors) and operation costs in ethanol fermentation processes (Busche *et al*., 1992; Quenesi and Mamderson, 1995).

The sugar consumption profile at different initial sugar was similar where there was a rapid consumption initially and decreases with time. Ethanol concentration, productivity and theoretical yield values decreased as initial sugar concentration of the medium increased. This decrease in the overall performance of the fermentation process may be caused through product and/or substrate inhibition.

Ethanol production using immobilized cell reactor decreased and residual sugar increased as the dilution rate increase from 0.15 h^{-1} to 0.25 h^{-1}. At high dilution rate the added sugars has short residence time and pass through the column unfermented, and thus decreasing the conversion efficiency.

For efficient ethanol production, the microbial and engineering processes require two opposing situations. Microbial processes such as growth and conversion of sugars into ethanol proceed best at 0% ethanol and are increasingly inhibited as the alcohol concentration rises (Munnecke, 1981). Yet, for efficient distillation, high concentrations

(above 6%) are desired. Attempts to surmount this problem have involved screening programs for the isolation of ethanol tolerant microorganisms and genetic studies to improve ethanol tolerance.

The yeast isolate TAYI 4-2 screened from '*tella*' showed the highest fermentation efficiency and productivity when immobilized in continuous system of fermentation within short period of time. This clearly indicates immobilization of and continuous operation of the fermentation process for ethanologic microbe could significantly increase fermentation efficiency.

With the planned expansion of the sugar sector in Ethiopia, it is important to incorporate ethanol producing plants which can produce ethanol that can be marketed domestically and /or internationally. The current demand of ethanol in the country and in world can be a further driving force to set up ethanol plants. Molasses which is a by product of sugar industries can be used as substrate for fermentation. Currently, about 96,662.5 ton of molasses is produced per year from the three factories. Thus, Ethiopia can have an estimated potential of 28,045,165 liter of ethanol per year. Considering the current market price of alcohol, the gross earnings can be estimated as 28,045,165 X 7 =196,316,151 birr/yr. The average production cost based on the last three years rate was 1.3 birr/yr. Therefore, to produce 28,045,165 liters of alcohol per year it will require 28,045,165 X1.3=36,458,714 birr/yr.

5. CONCLUSION

- The optimum physiological conditions for TAYI 4-2 were pH 4.5, temperature 35 ^{0}C and 11 % initial sugar concentration. The fermentation of cane molasses using TAYI 4-2 under optimized conditions revealed an increase in ethanol production with good fermentation efficiency.

- Brewer yeast efficiently ferment cane molasses with an initial sugar concentration 11 % at pH 4.5 and temperature 30 ^{0}C.

- Immobilization of TAYI 4-2 was carried out in batch and continuous process yielding more alcohol than free batch method.

- On the basis of the results obtained in above experiments it can be concluded that the immobilized yeast in a simple reactor can be employed for the continuous production of ethanol from a cheap sugar industry by-product sugarcane molasses.

- A wild isolate of yeast screened from '*tella*' showed a great potential in ethanol fermentation over the other isolates of '*tella'* and '*tej'*. Thus, Ethiopian traditional alcoholic beverages can be one of the potential sources of an ethanolgenic microorganism.

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DECLARATION

I the undersigned person declare that this thesis is my original work and that all sources of material used for the thesis have been genuinely acknowledged.

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