BRAC UNIVERSITY

Isolation and characterization of Saccharomyces cerevisiae for the production of ethanol from organic sources

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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Microbial production of ethanol is a very popular concept in respect of alleviating energy demand nowadays. In this regard, two fermenting strains of Saccharomyces cerevisiae were isolated from date-juice and grapes and grown in YEPD medium. They are characterized for alcoholic fermentation using sugarcane molasses and organic kitchen-wastes media. The fermentation of molasses was optimized with respect to pH and sugar concentration. Results revealed a temperature of 30□C, pH 6.0 and 6.5% sugar concentration as optimum for fermentation. Stress tolerance tests showed date-juice strain is highly thermo-, pH-, osmo- and ethanol-tolerant. Conway method for estimating percentage of ethanol was employed. Under optimized conditions, S. cerevisiae from date-juice produced 7.75% of ethanol in molasses. Kitchen-wastes resulted in 7.3% ethanol after 48 hours of fermentation by the same yeast cells.

ABSTRACT

Microbial production of ethanol is a very popular concept in respect of alleviating energy demand nowadays. In this regard, two fermenting strains of *Saccharomyces cerevisiae* were isolated from date-juice and grapes and grown in YEPD medium. They are characterized for alcoholic fermentation using sugarcane molasses and organic kitchen-wastes media. The fermentation of molasses was optimized with respect to pH and sugar concentration. Results revealed a temperature of 30➡C, pH 6.0 and 6.5% sugar concentration as optimum for fermentation. Stress tolerance tests showed date-juice strain is highly thermo-, pH-, osmo- and ethanol-tolerant. Conway method for estimating percentage of ethanol was employed. Under optimized conditions, *S. cerevisiae* from date-juice produced 7.75% of ethanol in molasses. Kitchen-wastes resulted in 7.3% ethanol after 48 hours of fermentation by the same yeast cells.

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INTRODUCTION

1.7 Preamble

The continuously increasing population of Earth is dawning a new challenge for biotechnology: supplementation of mankind with commodity products from renewable resources, instead of fossil based ones. Biotechnology producing commodity products should aim to be responsive to societal needs for sustainable resource utilization and improved environmental quality. As raw materials are often dominant factors in determining the price of commodity products, renewable materials available at large scale are required as feedstock. In addition, these renewable sources are geographically more evenly distributed than fossil fuels; therefore the products will be domestic and provide security of supply.

At the beginning of the 20th century, many industrial materials such as dyes, solvents, and synthetic fibers were made from trees and agricultural crops. By the late 1960s, however, many of these bio-based chemical products were replaced by petroleum derivatives which could be produced at lower cost (Ragauskas *et al.*, 2006; van Wyk, 2001).

Over the last few decades, the negative impacts of fossil fuel on the environment and consequent global warming, progressive demand for energy, inevitable depletion of the world's energy supply, and the unstable oil market (such as the energy crisis of the 1970s) have renewed the interest of society in searching for alternative fuels (Himmel *et al.*, 2007; Solomon *et al.*, 2007). The alternative fuels are expected to satisfy several requirements including substantial reduction of greenhouse gas emission, worldwide availability of raw materials, and capability of being produced from renewable feedstocks (Hánh-Hägerdal *et al.*, 2006). Production of fuel ethanol from biomass seems to be an interesting alternative to traditional fossil fuel, which can be utilized as a sole fuel in cars with dedicated engines or in fuel blends. Ethanol is currently produced from sugars, starches and cellulosic materials. The first two groups of raw materials are currently the main resources for ethanol production, but concomitant growth in demand

for human feed similar to energy could make them potentially less competitive and perhaps expensive feedstocks in the near future, leaving the cellulosic materials as the only potential feedstock for production of ethanol (Taherzadeh & Karimi, 2007).

Fuels and energy sources that are made from organic byproducts or naturally occurring, living organisms are known as biomass fuels (Biofuels). Paper and wood waste, grains, and decomposing organic rubbish (e.g. Kitchen wastes) are some of the most popular sources of biofuel. The idea of using biofuel as an alternative to coal energy has existed since the industrial revolution. When Ford designed the Model T, the original fuel source that was supposed to be used was ethanol from biomass (Biofuels).

Kitchen-waste represents a renewable raw material available at large quantity. Generally this term applied to those solid materials which are considered as discards, while preparing foods. Mostly they are organic in nature and degradable through microbial infestation. These feedstocks are mostly built up of lignocelluloses and starch. Their carbohydrate polymer fraction is a brilliant raw material for biotechnology. Utilization of lignocelluloses as feedstock would make new challenges for biotechnology, such as overcoming the recalcitrance of cellulosic biomass and product diversification.

Ethanol from kitchen-waste is an attractive and sustainable energy source for transportation fuel to substitute gasoline. Current ethanol production (so called first generation) using crops such as sugar cane and corn is well established, whereas second generation ethanol production utilizes cheaper and non-food feedstocks like lignocelluloses or municipal solid waste, which could make ethanol more competitive to fossil fuels.

In this study, fermentation has been carried out both in molasses and kitchen waste to compare the efficacy of the fermentation media. And to find out if a wild-type microorganism has the ability to ferment both glucose and cellulose efficiently, to ensure a cheap source of raw material for the production of biofuel. For a growing economy like Bangladesh a new source of energy can be very helpful to meet the rising energy demand.

1.8 Bioethanol and its application as a fuel

Ethanol or ethyl alcohol (CH_3CH_2OH) is one of the most versatile oxygen containing organic chemicals. Ethanol act as a solvent, a germicide, a beverage, an antifreeze, a fuel, a depressant and as a chemical intermediate for other organic chemical. The use of fermentation-derived ethanol or so called bioethanol as an automotive fuel additive to enhance octane and reduce emissions has seen an explosive growth over the last 12 years worldwide (Logsdon, 1994).

Ethanol, also known as ethyl alcohol with the chemical formula C_2H_5OH, is a flammable, clear, colorless and slightly toxic chemical compound with acceptable odor. It can be produced either from petrochemical feedstocks by the acid-catalyzed hydration of ethene, or from biomass feedstocks through fermentation. On a global scale, synthetic ethanol accounts for about 3-4% of total production while the rest is produced from fermentation of biomass – mainly sugar crops, e.g. cane and beet, and of grains (mainly corn) (Licht, 2006).

Ethanol as a neat fuel or even in the blended form with gasoline has a long history as automotive fuel. In 1860, German inventor Nicholas Otto used ethanol as a fuel in an early prototype of an internal combustion engine because it was widely available throughout Europe for use in spirit lamps. A few years later, Henry Ford built his first automobile with an engine that could run on ethanol. In 1908, Ford unveiled his Model T engine equipped with carburetors that could be adjusted to use alcohol, gasoline or a mixture of both fuels (Solomon *et al.*, 2007). Ethyl alcohol as "the fuel of the future" was presented by him for the first time. In 1925, he told the New York Times: “The fuel of the future is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust – almost anything… There is fuel in every bit of vegetable matter that can be fermented. There's enough alcohol in one year's yield of an acre of potatoes to drive the machinery necessary to cultivate the fields for a hundred years.” However, fossil fuels were predominantly used for automobile transportation throughout the last century, obviously due to their lower production cost*. As an automotive fuel, hydrous ethanol can be used as a substitute for gasoline in dedicated engines. Anhydrous ethanol, on

the other hand, is an effective octane booster when mixed in blends of 5 to 30% with no engine modification requirement (Licht, 2006).

$$C_6H_{12}O_6 + 2ADP + 2Pi \xrightarrow{Yeast} 2\,C_2H_5OH + 2\,CO_2 + 2ATP + 2H_2O$$

Glucose Ethanol

1.9 Objectives

The overall objective of this project is to meet the demand for an inexpensive and highly efficient integrated anaerobic *Saccharomyces* sp. fermentation process to produce ethanol as an energy source directly from insoluble lignocellulosic substrate (kitchen-waste):

- Isolation and Characterization of wild yeast strain capable of ethanol production.
- Identification of isolated strain by DNA sequencing.
- Study of Thermo-tolerance, pH-tolerance, osmo-tolerance and ethanol-tolerance of the yeast strain.
- Study fermentation kinetics of ethanol production at laboratory level.
- To economically produce ethanol by using available and cheap raw materials.
- Improvement of ethanol production by genetic mutation.

1.10 Hypothesis

A wild-type strain of yeast can be used as a potential candidate for industrial ethanol producing organism. After proper isolation, identification and characterization of stress tolerance (thermo-, ethanol-, pH-, osmo- & sugar tolerance), detailed characterization and optimization of physiochemical parameters for ethanol production and after genetic mutation (if needed) the strain can be dubbed as an industrial strain.
Ethanol can be obtained from cheap and available medium, such as Kitchen-waste.

1.11 Scope and limitation of the study

Selected wild-type (Date-juice, grapes) yeast strains were screened for thermo-tolerance, pH-tolerance, osmo-tolerance and ethanol tolerance. The yeast strain was characterized using morphological, physiological and biochemical characteristics. Genomic analysis of the selected isolate was also performed. The optimization of some ethanol production conditions was also investigated.

This experiment is not designed for downstream processing. The production of Ethanol may be observed by the detection method, but purified ethanol cannot be harvested.

1.12 Expected results

A high efficient stress (thermo-, pH-, osmo- and ethanol-) tolerant yeast strain for ethanol production would be obtained. The yeast strain could be useful for the ethanol industry.

CHAPTER II
LITERATURE SURVEY

2.1 History

Fermentation is a process of chemical change caused by organisms or their parts, usually producing effervescence and heat. Fermentation is the slow decomposition of the large organic molecules (such as starch) into smaller molecules by micro-organisms. The microbial production of ethanol was first reported by Pasteur in 1861 (Moreira, 1983). Ethanol fermentation can be described as the biochemical process by which sugar such as glucose; fructose and sucrose are converted into cellular energy thereby producing ethanol and carbon dioxide as metabolic waste products. Yeasts carry out ethanol fermentation on sugar in the absence of oxygen. Because the process does not require oxygen, fermentation is classified as anaerobic (Ibeto *et al.*, 2011).

Several studies have shown that sugarcane-based ethanol reduces greenhouse gases by 86 to 90% if there is no significant land use change (Isaias *et al.*, 2004; Goettemoeller & Goettemoeller, 2007).Sugar cane also contains parts that cannot yet be profitably processed into ethanol. The bagasse is the name given to the biomass that remains from the sugar stalk after it has been crushed and the sugar and garapa (juices) have been extracted. Although, it is not yet commercially converted to ethanol, however research works are on-going to profitably convert the bagasse into ethanol. The bagasse is also useful in the production process. Many sugar mills have utilized the bagasse for cogeneration of both heat and electric energy production to power the mills. Like other parts of the plant, the bagasse does not add to net atmospheric carbon dioxide, because any carbon dioxide released will be consumed by another cane plant. Bioethanol production from sugarcane was started in Brazil and the United States in the early 1970's (Chatanta *et al.*, 2008).

2.2Microorganism

Cell Type	Eukaryotic
Cell Membrane	Sterols present

Cell wall	Glucans; mannans; chitin (no peptidoglycan)
Spores	Sexual and asexual reproductive spores
Metabolism	Limited to heterotrophic; aerobic, facultativelyanaerobic

Table 2.1: Features of Fungi (Tortora *et al.*, 2010)

Yeasts are eukaryotic micro-organisms classified in the kingdom Fungi, with about 1,500species currently described (Kurtzman & Fell, 2006). They dominate fungal diversity in the oceans (Bass *et al*., 2007). They are ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission, and that form sexual states which are not enclosed in a fruiting body (Boekhout & Kurtzman, 1996). The yeast species are all characterized by a similar set of features, both morphological and physiological. This type of description, in which physiological characters are important, distinguishes yeast taxonomy from other fungal taxonomy (Kreger-Van Rij, 1984).

In fermentation, of the various ethanol producing micro-organisms (Bhatt *et al.,*1987; Laplace *et al.,*1992 and 1993) yeast belonging to *Saccharomyce scerevisiae* have been used most commonly. Ok and Hashinanga (1997) isolated yeast from spoiled high sugar foods.

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

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

Most of the distilleries in Bangladesh operate at a low efficiency because the yeast strains used are not of good quality. Fermentation efficiencies less than 90% are quite common while it should be 95% on an average. Secondly, exact conditions of temperature, pH and nutrients, which are essential for yeast fermentation, are not

vigorously maintained. The Table 2.2 below lists some of the yeast strains used in distilleries and the amount of alcohol they produce.

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

Table 2.2: Different types of ethanol producing strains

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

Saccharomyces uvarum (Detroy *et al.,* 2004), *Schizosaccharomyces pombe* (Jong-Gubbels *et al.,* 1996), and *Kluyueromyces sp*. (Morikawa *et al.,* 2004), *Pachysolen tannophilus, Candida Shehatae, Pichiastipitis,* and especially *Saccharomyces cerevisiae,* are the major yeast species currently used for industrial ethanol fermentation processes (Matsushika *et al.,* 2008). For yeast-mediated ethanol production, small amount of oxygen (0.05 – 0.10 mm Hg) is reqired during the Embden-Meyerhof pathway. Besides the oxygen, nutrients are also required as other microbial bio-fuel process to maintain the cell-related reactions (Visser *et al.,* 1990).

Saccharomyces cerevisiae is budding yeast that can tolerate up to 20% v/v ethanol during fermentation which makes this strain appealing to ethanol production industry (Morais *et al.,* 1996). In addition, better than bacterial fermentations, yeast fermentations don't usually result in various by-products such as acetic acid (Figure 2.1). However, this strain only utilizes hexoses, and no sufficient crystalline cellulose degradation has been reported for this strain (Xu *et al.,* 2009). The other drawback of using yeast in bio-ethanol production is the acid sensitivity of yeasts. Acidic hydrolysates, even diluted hydrolysates, are reported to inhibit the fermentation process (Singha *et al.,* 2011). Thus, modifications for gaining xylose-utilizing and arabinose-utilizing functions and higher acid tolerance are developed (Bettiga *et al.,* 2009).

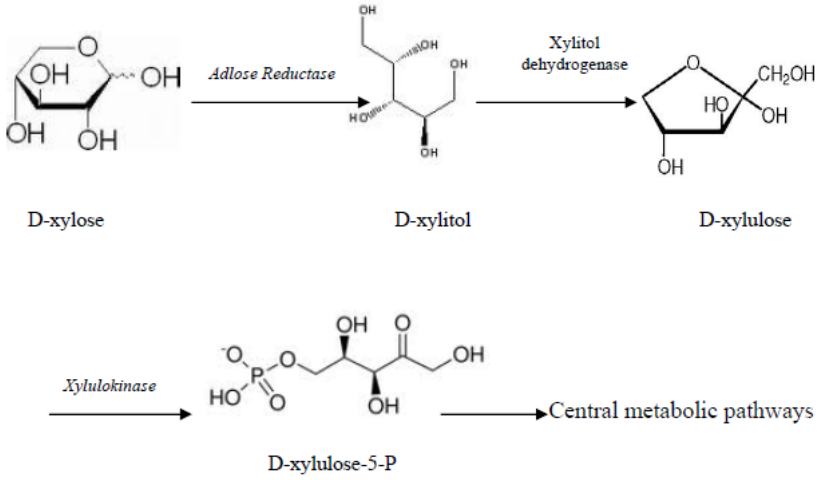


Figure 2.1: Yeast xylose utilization

Application	Yeast species
Ale fermentation	*Saccharomyces cerevisiae*
Bread and dough leavening	*S. cerevisiae, S. exiguus, S. rosei*
D- Arabitol (sweetener)	*Candida diddensiae*
Emulsifier	*C. lipolytica*
Ethanol fermentation	*S. cerevisiae*
Fish and poultry feeds	*Phaffia rhodozyma*
Fodder and single cell protein	*C. utilis*
Lactose and milk fermentation	*C. pseudotropicalis, Kluyveromyces fragilis, K. lactis*
Lager beer fermentation	*S. carlsbergensis*
Mannitol (humectant)	*Torulopsis manitofaciens*

Shoyu, Miso	*Zygosaccharomyces rouxii*
Wine fermentation	*S. cerevisiae*
Xylitol (sweetener)	*T. candida*
D-Xylose fermentation	*C. shehatae, Pachysolen tannophilus, Pichia stipis*

Table 2.3: Some present and potential uses of yeasts in the food, beverage and fermentation industries (Jacobson & jolly, 1989)

2.2.1 Classification and identification of yeasts

Yeasts are classified on the basis of the microscopic appearance of the cells, the mode of sexual reproduction, certain physiological features (especially metabolic capabilities and nutritional requirements) and biochemical features. The physiological features, that distinguish different yeasts, include the range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can use as a source of carbon and energy under semi-anaerobic and aerobic condition, the relative ability to grow in the presence of 50-60% (w/v) D-glucose or 10% (w/v) sodium chloride plus 5% (w/v) glucose (a measure of osmo-tolerance) and the relative ability to hydrolyze and utilize lipids. These properties help investigators determine which yeast strains merit investigation for a particular application (Glazer & Nikido, 1995).

Yeasts, which form one of the important subclasses of fungi, are rather more complex and usually larger than bacteria. They are distinguished from most fungi by their usual existence as single ovoid cells about 8 µm long and 5 µm in diameter, doubling every 1-3 hours in favorable media (Wayman & Parekh, 1990).

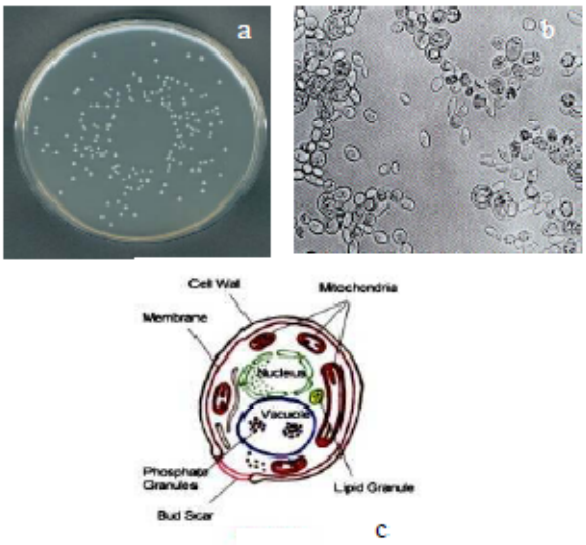


Figure 2.2: Yeast cell; a) colonies of *S. cerevisiae* on agar plate (www, 2005), b) *S. cerevisiae* under microscrope (400x) (www, 2006), c) Yeast cell composition (www, 2002)

Individually yeast cells appear colorless, but when grown on artificial solid media they produce colonies which may be white, cream colored, or tinged with brownish pigments. Colony characteristics are useful in the taxonomy of yeasts, very difficult group to classify (Figure 2.2). Physiological characteristics are also used to a great extent in determining yeast species (Alexopoulos, 1962). Yeasts may reproduce asexually or sexually (Wayman & Parekh, 1990).

2.2.2 Yeast reproduction

a) Asexual reproduction

Alexopoulos (1962) classified yeasts into the budding yeasts and the fission yeasts, depending on their types of asexual reproduction. The budding yeasts reproduce by budding, in this process the protoplasm of the cell, covered by a thin membrane, pushes out of the cell wall in the form of a bud and forms daughter cells. The bud enlarges until it is separated from the mother cell by a constriction at the base. The arrow signs indicate a bud and forms a daughter cell under microscopic observation are shown in Figure 2.3.

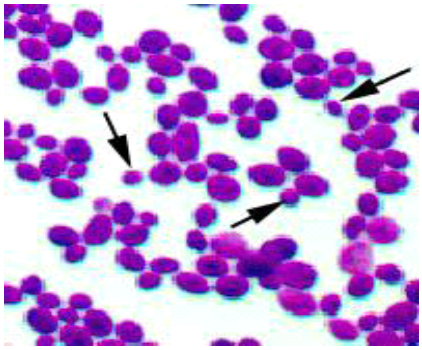


Figure 2.3: Microscopic observation of buds and daughter cells

b) Sexual reproduction

Sexual union in the yeasts takes place either between two somatic cells or between two ascospores which assume the function of copulating gametangia, unite and form a zygote cell. Eventually an ascus forms which contains ascospores, their number depending on the number of nuclear divisions which take place and on the subsequent development of the nuclei. Four or eight ascospores per ascus are the usual number, but other numbers may also be encountered. The reproduction of yeast, proceeding by the formation of buds on the cell surface, but sexual reproduction can be inducedunder special condition. In the sexual cycle, a normal diploid cell divides by meiosis, and sporulation gives rise to asci, or spore cells, that usually contain four haploid ascospores. The ascospores are of two mating types; a and α. Each type can develop by budding into order haploid cells. The mating of an a haploid cell and an α haploid cell yields a normal α diploid cell.

Haploid cells of the same sex also unite occasionally to form abnormal diploid cells (a/a or α/ α) that can reproduce only asexually, by budding in the usual way. The majority of industrial yeasts reproduce by budding (Glazer & Nikido, 1995) (Figure 2.4).

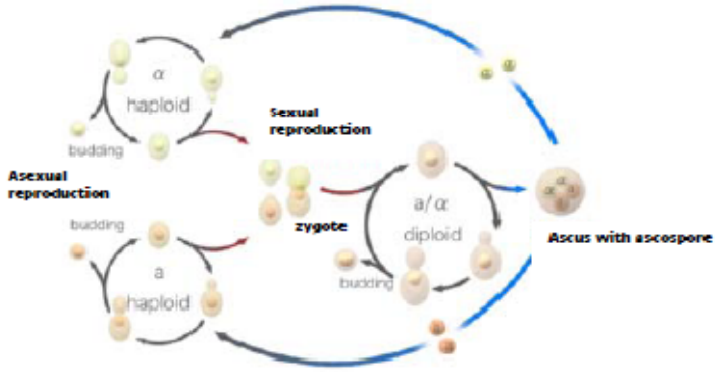


Figure 2.4: The reproduction of yeast by sexual and asexual reproduction (www, 2005)

Ascospores formed by yeasts are often globose or ovoid, as in *Debaryomyces, Saccharomyces, Schizosaccharomyces*, and *Saccharomycodes*. Other yeasts form different types of ascospores. Thus, in *Pichia* and some species of *Hansenula*, the ascospores are hat-shaped; in other species of *Hansenula* they may behemispherical of shaped like the planet Saturn. Release of ascospores may occur when the ascus wall deliquesces; this is the usual method of release in species with hat- or Saturn-shaped spores. In other species the germinating spores bud or form germ tubes, which results in bursting of the persistent ascus wall (Alexopoulos *et al*., 1996). Miller (1989) pointed out that yeast ascospores are much more durable than somatic cells and have the ability to withstand even snail gut enzyme, a distinct advantage in their natural environment.

2.3 "Green Energy" Ethanol

Ethanol has been used by humans since prehistory as the intoxicating ingredient in alcoholic beverages. Dried residues on 9000-year-old pottery found in northern China imply the use of alcoholic beverages even among Neolithic peoples. Its isolation as a relatively pure compound was first achieved by Islamic alchemists. Even the word *alcohol* is derived from Arabic *al-kuhl.*

Antoine Lavoisier described ethanol as a compound of carbon, hydrogen, and oxygen, and in 1808, Nicolas-Theodore de Saussure determined ethanol's chemical formula. Ethanol was first prepared synthetically in 1826, through the independent efforts of Henry Hennel in Britain and S.G. Serullas in France.

With the advent of distillation, which appears to have been discovered first in ancient Arabia, people were able to obtain beverages with higher ethanol content. In its strictest sense, **fermentation** (formerly called **zymosis**) is the anaerobic metabolic breakdown of a nutrient molecule, such as glucose, without net oxidation. Depending on which organism it is taking place in, fermentation may yield lactate, acetic acid, ethanol or other reduced metabolites. Normal fermentation processes typically cease when a beverage has achieved an alcohol content of 10 to 15 percent. Distillation is the process by which ethanol is boiled from the fermented mixture and captured, producing a liquid with a much higher concentration of alcohol.

Carbon dioxide, a greenhouse gas, is emitted during fermentation and combustion. However, this is canceled out by the greater uptake of carbon dioxide by the plants as they grow to produce the biomass [Source: oregon.gov, biomass forum] When compared to gasoline, depending on the production method, ethanol releases less greenhouse gases (Wang *et al*., 2009; Wang, 2009).

Compared with conventional unleaded gasoline, ethanol is a particulate-free burning fuel source that combusts with oxygen to form carbon dioxide, water and aldehydes. Gasoline produces 2.44 CO_2 equivalent kg/l and ethanol 1.94 (Popa, 2009). Since ethanol contains 2/3 of the energy per volume as gasoline, ethanol produces 19% more CO_2 than gasoline for the same energy.

2.3.1 Properties

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

Ethanol, also known as **ethyl alcohol** or grain alcohol, is a volatile, flammable, colorless chemical compound. It is a monohydric primary alcohol and it boils at78.5°C. It is miscible (i.e., mixes without separation) with water in all proportions and is separated from water only with difficulty; ethanol that is completely free of water is called absolute ethanol. Ethanol forms a constant-boiling mixture, orazeotrope, with water that contains

95% ethanol and 5% water and that boils at78.15°C. Ethanol is a psychoactive agent and it produces a variety of physiological and behavioral effects.

2.3.2 Production routes

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

2.3.2.1 Ethylene hydration

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

$$C_2H_4 + H_2O \rightarrow CH_3CH_2OH$$

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

2.3.2.2 Fermentation

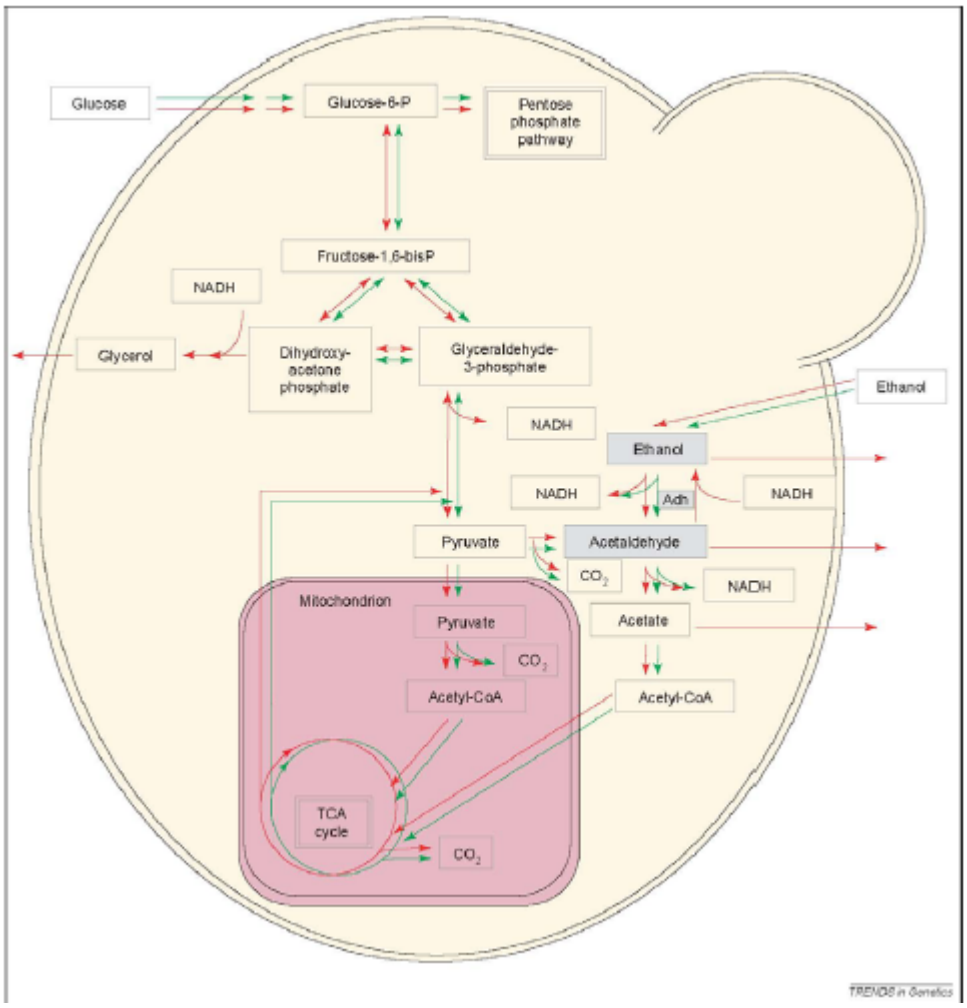


Figure 2.5: A scheme of the pathways

The overall metabolic pathways in a yeast cell explicit the characteristics of a perfect eukaryotic cell (Figure 2.5).

Ethanol for use in alcoholic beverages, and the vast majority of ethanol for use as fuel, is produced by fermentation: when certain species of yeast (most importantly, *Saccharomyces cerevisiae*) metabolize sugar in the absence of oxygen, they produce ethanol and carbon dioxide (Figure 2.6). The overall chemical reaction conducted by the yeast may be represented by the chemical equation

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

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

concentrated ethanol solutions are toxic to yeast. The most ethanol-tolerant strains of yeast can survive up to about 25% ethanol (by volume).

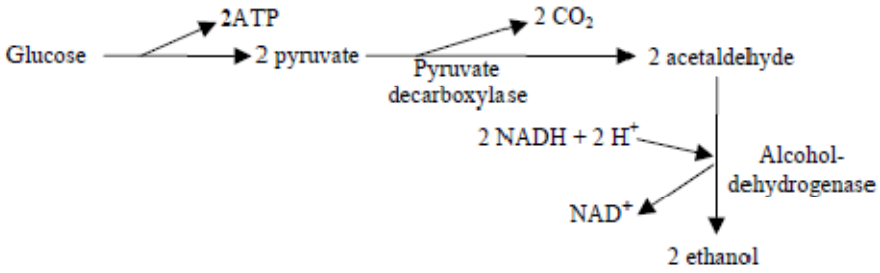


Figure 2.6: The ethanol fermentation pathway results in the formation of ethanol and carbon dioxide (Norr *et al.*, 2003)

In order to produce ethanol from starchy materials such as cereal grains, the starch must first be broken down into sugars.

2.3.3Principles involved in Ethanol production

There are different types of fermentations among which the batch and continuous fermentation are the most common. The batch fermentation process is the oldest and conventional method used for the production of ethanol from molasses. In this process, several fomenters are usually operated in staggered intervals to provide a continuous feed to the distillation columns. As this method is not only laborious, time-consuming (36-72 hrs) but also less efficient (80%) and yields less alcohol and productivity (2.2 g/t/h) Continuous fermentation is among the several processes that have been developed for improvement. The inherent problems in the batch process are, low cell density product and substrate inhibition. The productivity of continuous fermentation can be greatly enhanced by yeast cell recycle by the use of a centrifuge and the yeast cell density is increased to four-fold to 50% g/t in a laboratory trial. The residence time for completion of conversion of 10% glucose feed was reduced to 1.6 hr with a corresponding productivity of 30 g/t/h. Simple cell settling systems using flocculating yeasts with essentially no equipment (centrifuge) cost could be developed (Bassapa, 1989).

No matter which process is chosen (free cells, flocculent cells, or immobilized cells); as a general solution to reduce production costs and improve process efficiency and ethanol yield; the continuous fermentation method is the most appropriate. When compared with processes using immobilized cells, alcoholic fermentation using free cells offers some advantages: the larger area of contact between cells and nutrient medium and the management of current technology. However, disadvantages include the higher costs of microbial recycling and installation, high contamination risks, susceptibility to environmental variations, and the limitations of the dilution rate in continuous fermentation due to wash out (Vasconcelos *et. al.*, 2004).

2.3.4 Bio-ethanol potential and global atmosphere

The liquid energy carriers produced biologically are considered to be promising alternative energy carries because of the well established storage, logistics, and applications methods (Karakashev *et al.*, 2007). Available liquid energy carriers are mainly ethanol, butanol, mixture of ethanol, butanol and acetone (ABE), as well as biodiesel.

Among all of these energy carriers, ethanol is beneficial in many aspects. First of all, the production of bio-ethanol is the most established process as compared to the processes used for other energy carriers (Claassen *et al*., 1999). Especially, ethanol production from agronomic plants is well established. 12.5 billion liters of ethanol are produced in Brazil every year from sucrose (Mielenz, 2001). Almost one fourth of the cars in Brazil run on the alternative fuel called gasohol, which is the mixture of ethanol and petroleum (Lachke, 2002). In the US, corn is the major substrate for bio-ethanol production. Every year, about 5 billion liters of ethanol are produced in the US from corn kernels (Mielenz, 2001).

World ethanol production for transport fuel tripled between 2000 and 2007 from 17 billion to more than 52 billion liters. From 2007 to 2008, the share of ethanol in global gasoline type fuel use increased from 3.7% to 5.4%. In 2011 worldwide ethanol fuel production reached 22.36 billion U.S. liquid gallons (bg) (84.6 billion liters), with the United States as the top producer with 13.9 bg (52.6 billion liters), accounting for 62.2%

of global production, followed by Brazil with 5.6 bg (21.1 billion liters).Ethanol fuel has a "gasoline gallon equivalency" (GGE) value of 1.5 US gallons (5.7 L), which means 1.5 gallons of ethanol produce the energy of one gallon of gasoline.

Furthermore, bio-ethanol is a cleaner fuel than fossil fuel. Burning ethanol made from plants is estimated to reduce greenhouse gas emissions by 86% (Wang *et al*., 2007). In addition, ethanol, as a petroleum gasoline additive, is safer than the methyl tertiary butyl ether (MTBE) which is currently used for cleaner combustion (Sun & Cheng, 2002). MTBE is reported to be toxic and has a potential to contaminate ground water (Sun & Cheng, 2002).

However, for bio-ethanol production, the transport cost per energy unit is still higher than for petroleum. Moreover, water needed for feeding the feedstock, as well as the fluctuations of feedstock availability due to weather conditions all influence the cost of bio-ethanol.

In Brazil and in the United States ethanol fuel is widely used, and together both countries were responsible for 86 percent of the world's ethanol fuel production in 2009 (Licht, 2010). Most cars on the road today in the U.S. can run on blends of up to 10% ethanol [Source: Worldwatch Inst., 2006], and the use of 10% ethanol gasoline is mandated in some U.S. states and cities. Since 1976 the Brazilian government has made it mandatory to blend ethanol with gasoline, and since 2007 the legal blend is around 25% ethanol and 75% gasoline (E25) [Source: Ministério da Agricultura, Pecuária e Abastecimento, 2007]. In addition, by December 2010 Brazil had a fleet of 12 million flex-fuel automobiles and light trucks and over 500 thousand flex-fuel motorcycles regularly using neat ethanol fuel (known as E100) [Source: ANFAVEA, 2010; ABRACICLO, 2010; UNICA, 2010].

British Airways has announced (February 2010 and May 2011) it will develop a facility in East London to convert 500000 tonnes of waste per annum to 16 million gallons of jet fuel. The plant will use Solena's Plasma Gasification (SPG) technology, which involves a combination of plasma arc gasification and the Fischer-Tropsch process to convert waste into biofuel.

California Energy Commission has recently allocated $26m for biomethane production projects, including a $4.5m project to demonstrate production of biomethane from MSW.

In December 2011, CHO Power SAS (a subsidiary of Europlasma) and Sunrise Renewables announced plans to build 4 high temperature plasma gasification facilities at UK docks to convert waste wood into clean syngas. The Syngas will be cleaned further and the tar removed, prior to power production via gas engine generators. The company is also developing a demonstration facility in Morcenx, France that will gasify 37,000 tonnes of ordinary industrial waste and 15,000 tonnes wood chips per annum, generating power for EDF.

2.3.5 Bangladesh scenario

Ethanol has emerged as an alternative for petroleum based liquid fuels. Nowadays, its use in automobiles as an alternative fuel has attracted many countries including Bangladesh. Currently in Bangladesh, there is only one sugar mill producing ethanol and few distilleries participating in downstream chemicals from ethanol. Among molasses driven products, ethanol takes the largest part, but its utilization must attract the attention of government policy makers in order to utilize it as a biofuel. With the present trend in sugar sector expansion and modernization in the country, implementation of different domestic measures for bioethanol production and utilization has to be taken into consideration. An efficient ethanol production requires four components: cheap fermentable carbohydrate, an efficient yeast strain, a few nutrients and simple culture conditions. Among the widely used substrates for ethanol production molasses of sugar cane and molasses of sugar beet are the most important sources. This is because they are ready for conversion with limited pre-treatments as compared with starchy or cellulosic materials (Yadav *et al.*, 1997).

Bangladesh through its potential in developing large scale sugar cane production can play a productive role in mitigating sugar, ethanol and electricity demands in the country. Molasses non-crystallizable residue remaining after crystallizing sucrose has additional advantage. It is relatively inexpensive, readily available and use for industrial ethanol production. Immobilization offers advantages of modern technique of continuous fermentation along with low cost design and optimization of available expertise. So studies on isolation of new potent strain and improvement of the available

strain to increase higher productivity are necessary for the existing and newly emerging ethanol industries of the country.

It is unfortunate that Bangladesh, a producer of sugar and sugar cane and also of molasses, the cheapest feed stock for ethanol has lagged far behind in exploiting these natural renewable resources. There is a wide scope of ethanol fuel production in Bangladesh. Bangladesh at present produces, on an average, about 80,000 tons molasses in 14 sugar mills under Bangladesh Sugar and Food Industries Corporate (BSFIC) against the molasses production capacity of about 1 lac tons. Three to four more raw sugar processing factory will go on production within one to two years. Then molasses production in the country will be increased many folds which will result in sufficient feed stock available for ethanol, fuel production. At present ethanol is produced in Carew's distillery under the management of BSFIC to meet the demand for beverage alcohol (foreign liqure).

Currently, Bangladesh is challenged by a huge energy demand from every sector. Albeit, it seems the country is lacking electricity, actually the production of gas is facing the real crisis, where not only the nation’s electricity production is depending upon it but also the transport sector, too, in the form of CNG. If we can ensure a cheap production of an energy which will mitigate the fuel need (as Biofuel), then the production of electricity can be increased. And we can see less carbon emitting National Highways and urban areas, as well. If this scenario is possible in a developing country like Brazil, then it is possible in Bangladesh.

2.3.6 Raw materials for ethanol production

a) Molasses or sugar

The name molasses is derived from Latin word *mel*, meaning honey Paturau (1989) and can be defined as the final effluent obtained in the manufacture of sugar in the repeated crystallization from various raw materials. It is a dark colored syrupy residual substance from which no crystalline sucrose can be obtained by simple means. Cane molasses is a major byproduct of the sugar industry (Yansong *et al*., 2000).There are various types of molasses which depends on the source from which they are obtained Rao (1997); beet molasses, cane molasses, black strap molasses, refinery molasses and high test

molasses are among the common once. Commercial cane-molasses is frequently called “black strap-molasses”.

Molasses can be converted into many value-added products by application of modern technologies. Many products can be made theoretically Rao (1997) and Paturau (1989), but in actual practice, the production of only a few products is commercially viable and hence, commercial scale plants are working in different countries to produce; ethyl alcohol, baker’s yeast, torula yeast protein molasses, L-lysine, acetone-butanol, citric acid, lactic acid, glutamic acid and mono sodium glutamate.

The industrial use of molasses arises from its sugar constituents. When compared to others, there was less molasses utilization in Bangladesh 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 Bangladesh also. This study is focused to ethanol production from sugar cane intermediate molasses obtained by redesigning sugar crystallization process by yeast (*Saccharomyces Cerevisiae*). The molasses from conventional sugar crystallization process was used as control and finally economic significance of sugar- ethanol product mix from redesigned sugar process was evaluated.

2.3.6.1 Composition and properties of cane molasses

The composition of molasses from sugar cane varies with the locality, variety of cane, character of soil, climate and method of processing. It is slightly acidic, having a pH of 5.5 – 6.5. The lower pH of cane molasses can be attributed to the presence of free aliphatic acids and is helpful in clarification. It contains 14 – 25% of carbohydrates as invert sugars (glucose and fructose) but no raffinose; while the total sugar is about 50 to 66% (mostly sucrose). The chemical composition of cane blackstrap molasses is shown in Table 2.4.

Constituent	**Percent**
Water	20.00
Total Solids	80.00

Sugars	62.00
Sucrose	32.00
Dextrose	14.00
Levulose	16.00
Major Nitrogenous Substances	2.77
Other Nitrogenous Substances	0.23
Soluble gums	2.00
Free acids	2.00
Combined acids	3.00
Ash	8.00

Table 2.4: Blackstrap molasses composition

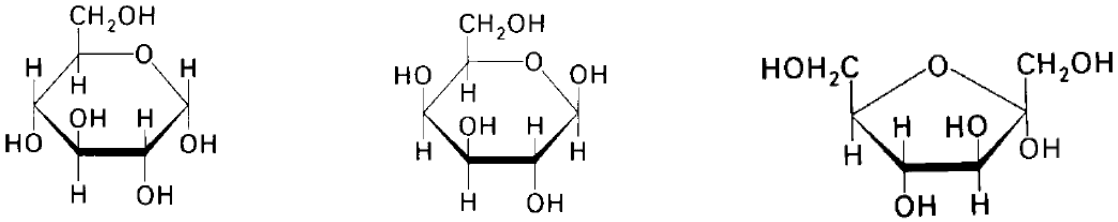


Figure 2.7: a) Structure of glucose, b) Structure of galactose, c) Structure of fructose

The simplest form of sugar is glucose, which is fermented by normal distillery and brewery yeasts. It has the formula $C_6H_{12}O_6$ and is made up of molecules with a single ring structure (Figure 2.7a). Very slight rearrangements of the atoms in the molecule can give other sugars with distinctly different properties. For instance, if the end groups are rotated it becomes galactose, which is not readily fermentable by normal yeasts (Figure 2.7b). Alternatively, by rearranging the ring structure, it becomes the fermentable fructose (Figure 2.7c). There are many other simple sugars, but only glucose and fructose will be considered here. Glucose does not exist extensively in a free state in nature. It is mostly polymerized as starch or cellulose, in which long chains of glucose units are formed. Glucose also exists in combination with fructose to form the disaccharide (two sugar molecules) or common table sugar (Figure 2.8).

Sucrose is the principal sugar contained in molasses and is readily fermentable either directly, or as its glucose and fructose components. It should be noted that the formula of sucrose is not exactly double that of glucose (or fructose), in that a molecule of water has been displaced in the synthetic process. This means that water is added when sucrose is broken down:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 2C_6H_{12}O_6$$

Sucrose Water Glucose

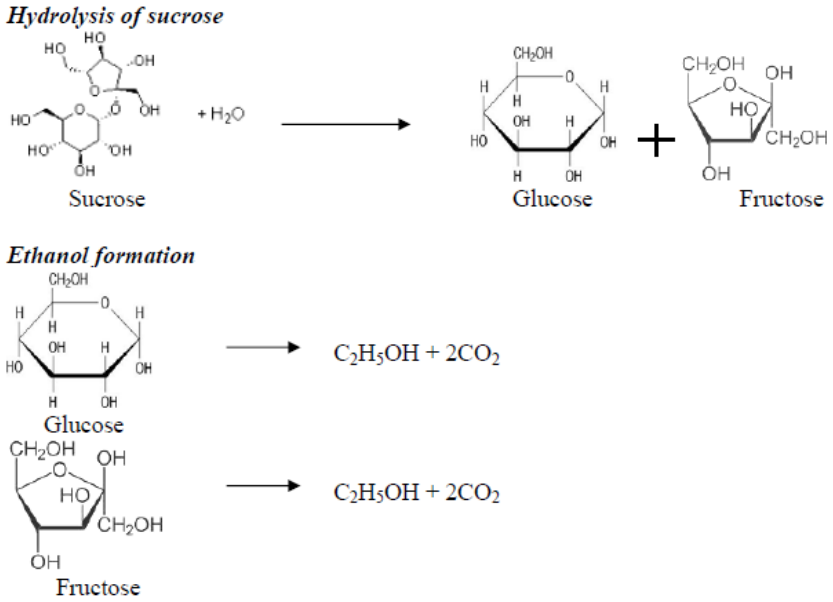


Figure 2.8: Sucrose hydrolysis and ethanol formation

Degradation with added water is referred to as hydrolysis. (This word comes from the Greek roots –hydro-, meaning water, and –lysis-, meaning cutting or breakage.) This addition of water should be taken into consideration when making any calculations of the amount of simple sugars (monosaccharides) produced from disaccharides when determining potential alcohol production by fermentation.

b) Kitchen waste or Lignocelluloses

Lignocellulose is the most abundant renewable source and is recognized as having great potential as a substrate for fermentation. Its hemicelluloses and cellulose components can be degraded and utilized efficiency (Timell, 1967; Yu & Saddler, 1984).

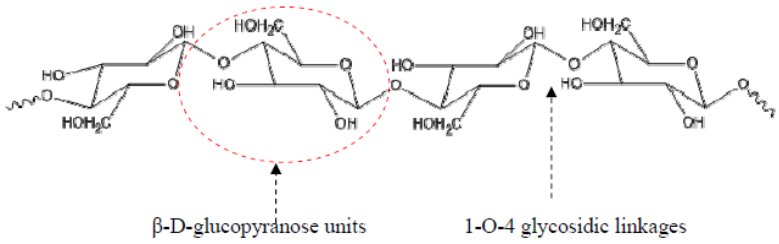


Figure 2.9: Cellulose molecular structure

Plant cell walls mostly consist of *lignocellulosic biomass.* Among its main components (cellulose, hemicelluloses and lignin), cellulose, a linear high molecular weight polymer of cellobiose (Figure 2.9), consist of two D-glucose, linked by β-1,4 bonds. In fact, there are several forms of cellulose with different degrees polymerization and molecular weight (Lynd *et al*., 2002; Fengel & Wegener, 1989). More ordered or crystalline cellulose is less soluble and less degradable (Zhang & Lynd, 2004).Cellulose in plant materials consists of both, crystalline and amorphous areas. Because of the high energy of a large amount of hydrogen bonds, cellulose crystalline areas are hard to degrade with enzymes or chemicals. Under normal conditions, due to the existence of hydrogen bonds, the cellulose is relatively insoluble. An efficient cellulose degradation technique, including effective enzymes, high temperatures, concentrated acid or alkaline, is necessary for both amorphous and crystalline cellulose in the conversion process. Cellulose provides structural support and chemical resistance for the plant. Considering the uniform hydrolysable glucose building blocks (Figure 2.10), the cellulose molecule would be the best carbohydrate source for the fermentation process.

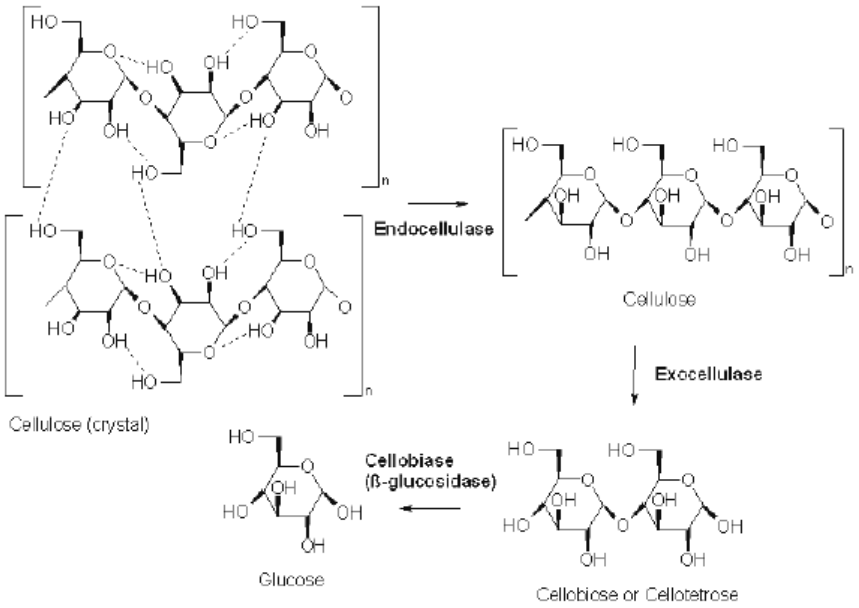


Figure 2.10: Reaction Route of Cellulose to Glucose (Wyman *et al*., 2009)

Hemicellulose, another important polysaccharide type in plant cell walls, is a branched hetero-polysaccharide consisting of various pentose and hexose units including D-xylose, L-arabinose, D-mannose, D-glucose / rhamnose and D-galactose. Besides these units, they may also contain acetic, D-uronic acid as well as 4-O-methylether. They are shorter chain molecules (around 200 DP) with covalent bonds to lignin to form cross-linked structures. Hemicellulose differs from cellulose by composition of several sugar units, by presence of shorter chains and by a branching of main chain molecules (Fengel & Wegener, 1989). Due to the linkages among hemicellulose, cellulose and lignin, removal of hemicellulose which is easier than removal of lignin and cellulose, is considered to be an efficient way to increase cellulose utilization. Due to the structural diversity, a wide range of enzymes including endoxylanase, exoxylanase, mannanase, arabinosidase, acetylesterase, and glucoronisidase are needed (Thygesen *et al*., 2003). Pretreatments, especially alkaline pretreatment, also were found to be effective in separating hemicellulose from lignin and cellulose.

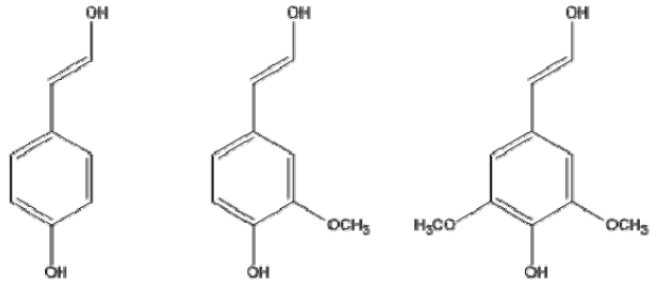


Figure 2.11: The structures of lignin building blocks

Lignin is linked to hemicelluloses and cellulose forming an impermeable barrier preventing enzymatic activity, provides additional strength and protection against fungi and insect attack (Swain, 1979;Howard, *et al*., 2003).The high molecular weight (in excess of 10,000 Da) phenylpropan structure of lignin leads tohigh insolubility in most solvents. Lignin significantly adds to the rigidity and moisture resistance of the biomass (Chandra *et al*., 2007). Furthermore, the existence of the lignin has been considered as an inhibitor for cellulase, due to the non-productive binding of lignin and cellulase depending on the lignin types (Mansfield *et al*., 1999). As a result, chemicals like surfactants (Börjesson *et al*., 2007), $MgSO_4$, and $CaCl_2$ (Liu *et al*., 2010), and exogenous proteins like bovine serum albumin (BSA) (Zheng *et al*., 2008) are added prior to enzyme or microbe loading to reduce the reaction between lignin and cellulase. In addition, many pretreatment methods have been developed to diminish the obstacle of lignin. Minimizing the effect of lignin inhibition depends on the source of lignocellulosic biomass, since lignin within the lignocellulosic materials is not uniform and varies in different plants andeven in different locations of the plant. For example, in the middle lamella and primary cell wall, lignin content is higher than in the secondary cell wall (Wiselogel *et al*., 1996).

Lignin building units are three aromatic alcohols: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 2.11), which are polymerized into an amorphous moncrystalline polymer (Higuchi, 1990). Functional groups in lignin including methoxyl groups, phenolic hydroxyl groups, benzyl hydroxyl group, carbonyl groups and small amount of terminal aldehyde groups are the other factors that will affect the degradation

of lignin. Also the hydroxyl and methoxyl groups in lignin precursors will interact with cellulose fibrils (Houtman & Atalla, 1995) (Figure 2.11). The lignin carbohydrate complexes (guaiacyl, synringyl and p-hydrophenil units) formed by the interactions are also considered to be restricting factor for biomass accessibility.

Although lignin is harmful for lignocellulosic biomass conversion, it is considered to be a valuable by-product in the bio-refining process. Products like filter material, expanded polyurethane foam and thermoplastic have been successfully produced from lignin extracted from plants (Glasser & Leitheiser, 1984; Kosikova *et al*., 2006). The overall efficiency of lignocellulosic biomass conversion would be maximized if the recovery of lignin would become a part of the overall biomass conversion process.

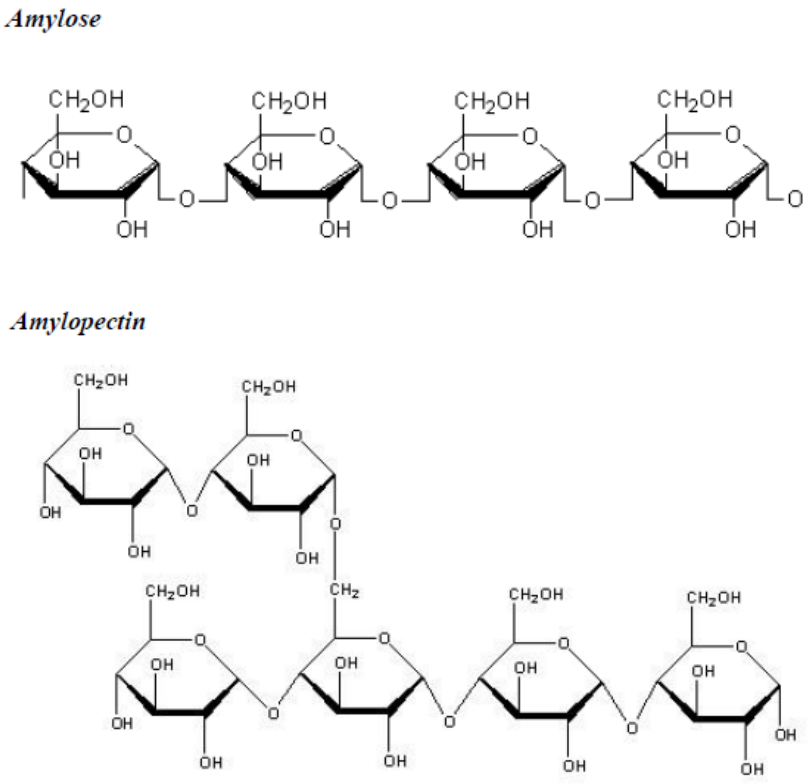


Figure 2.12: Starch molecular structures

Amylase hydrolysis

$$(C_6H_{10}O_5)_n + nH_2O \xrightarrow[\text{amylase}]{} n\ C_6H_{12}O_6$$

Diastase hydrolysis

$$2(\underset{\text{Starch}}{C_6H_{10}O_5)_n} + nH_2O \xrightarrow[\text{diastase}]{} n(\underset{\text{Maltose}}{C_{12}H_{22}O_{11}})$$

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow[\text{Maltase}]{} 2C_6H_{12}O_6$$

Ethanol formation

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

Figure 2.13: Starch hydrolysis and ethanol formation

Starch molecules are long chains of α-D-glucose monomers (Figure 2.12). Slightly more complex than the sugar fermentation process, starch needs to be broken down into glucose first through hydrolysis with amylase recovered from fungi, or diastase and maltase from sprouting grain (Figure 2.13). Then fermentation enzymes will ferment the fermentable sugars into ethanol and carbon dioxide.

2.4 Factors affecting fermentation

A number of factors like high temperature, low ethanol and sugar tolerance of the yeast limit the industrial production of ethanol at low production costs (Figure 2.14).

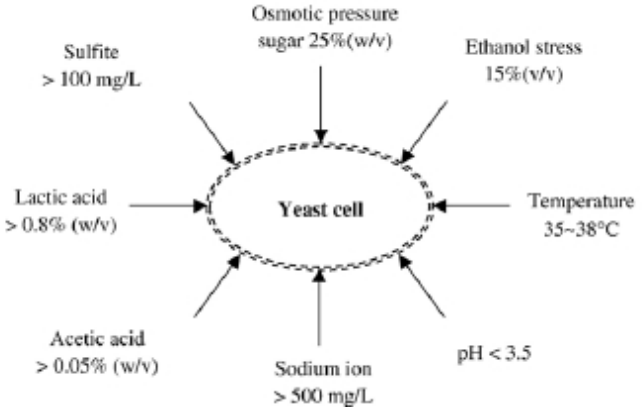


Figure 2.14: Potential environmental stresses on *Saccharomyces cerevisiae* during alcoholic fermentation

2.4.1 Effect of sugar concentration

Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. However, due to osmotic stress high substrate concentration is inhibitory to fermentation (Jones *et al.,* 1981). Borzani *et al* (1993) studied fermentation with various initial concentrations of sugar. They also demonstrated the logarithmic relationship between time of fermentation and initial concentrations of sugar. Bertolini *et al* (1991) isolated yeast strains from sample collected from Brazilian alcohol factories. These strains were capable of fermenting up to 30% of sucrose efficiently. The efficiency of selected strains varied from 89% to 92% depending upon the utilization of total sugar available in the medium. A maximum amount of 19.7% (v/v) ethanol accumulated from fermentation of 30% sugar as compared to 2 reference strains, which produced 18.0(v/v) and 15.6 (v/v). A repeated batch fermentation system was used to produce ethanol using an osmo-tolerant *S.cerevisiae* (US3) immobilized on calcium alginate (Sree *et al.,* 2000). The ethanol production in the fermentation broth was increased with the 5% (v/v) glucose but decreased beyond that. The final glucose utilization in the fermentation broth was found to be used up at the glucose concentration equal to or below 5% (v/v), but above the glucose concentration of 5% (v/v), the final glucose utilization became quite appreciable The maximum specific growth rate and maximum ethanol concentration were increasing with an increase of glucose concentration for 5% (v/v). A reduction of ethanol production and growth of yeast were decreased when glucose concentration was greater than 5% (v/v). (Apiradee Sripiromrak, 2006)

2.4.2 Effect of temperature

The fermentation process is always accompanied with evolution of heat that raises the temperature of the fermenter. As a result it becomes necessary to cool the large fermenters in the distilleries. This necessity often becomes a major operation and a cost factor in the production of ethanol. Temperature exerts a profound effect on growth, metabolism and survival o the fermenting organism.

Fermentation in industries is usually carried out at ambient temperature of 25- 35°C but temperature exceeds 40°C during fermentation especially in northern regions which

decreases the cell viability and productivity. Maintenance of high cell viability is a major characteristic of fermentation to get high ethanol yield. Fermentation at 35-40°C or above has advantages such as ethanol recovery and significant savings on operational costs of refrigeration control in distilleries for alcohol production. Therefore many studies have been carried out for development of yeast to ferment at high temperature of upto 40-45°C.

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

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

The fermentation activities of yeasts will decrease the temperature of 25°C and below due to catabolic activates of the enzymes decreases as temperature lower temperature Du Preez *et al*. (1987) and at 35°C the ethanol production was also reduced for all isolates. The evolution of heat at higher temperature during fermentation inhibited ethanol yield (Morimura *et al.,* 1997).

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

of 35°C, strain K211 gave stable ethanol concentration of 91 g/l and productivity of 2.7g/l-h.

2.4.3 Effect of ethanol on yeast fermentation

A limitation of ethanol fermentation is the capacity of yeast to tolerate ethanol concentration, because ethanol inhibits alcoholic fermentation, which limits the concentration of ethanol which can be produced by a given strain of yeast. The maximum concentration of ethanol which can be produced by yeast varies with species up to 20% by volume. The degree of inhibition is also related to other environmental factors, in particular high sugar concentration and high temperature which cause the inhibition of ethanol fermentation. Ethanol, which is produced during fermentation, is rather inhibitory to cell growth than that from an exogenous source (Wayman &Parekh, 1990).

Navarro (1980) studied the high intracellular ethanol concentrations were a consequence of resistance to diffusion through the membrane to the outside. At elevated temperature, the rate of ethanol production increased faster than the rate of excretion. Navarro and Durand (1978) also concluded that the effects of temperature on ethanol accumulation in *S. uvarum*. They found growth was arrested when a critical intracellular ethanol concentration had been reached, and this intracellular accumulation was greater at higher temperatures.

The toxic effect of ethanol has also been attributed to damaging the cell membrane or changing its properties. The extent of ethanol tolerance of certain yeasts is highly strain dependent and appears to be related to the unsaturated fatty acid and the fatty acyl composition of the plasma membrane (Wayman &Parekh, 1990).

2.4.4 Effect of Nutrients

Yeasts grow in simple media which contain fermentable carbohydrates to supply energy and carbon skeleton for biosynthesis, adequate nitrogen for protein synthesis, mineral

salts and one or more growth factors. Sources of carbon included monosaccharides, disaccharides and trisaccharides (Priest & Campbell, 1996).

The metabolic activities of yeasts are greatly affected by the temperature at which they grow. Temperatures above the optimum lower the growth rate, oxygen solubility and also change the cellular composition of yeasts. It is known that under oxygen-limited conditions, yeasts require nutritional supplements for growth (Slapack *et al*., 1987; Thomas *et al*., 2002). An increase in temperature does not inhibit substrate uptake nor does it significantly alter enzyme levels (Slapack *et al*., 1987). Helena da Cruz *et al*. (2003) concluded that nitrogen and carbon are the main nutrients in fermentation medium and this implies that the mutual interaction of these nutrients may play an important role in the metabolism of yeasts The supplementation of the growth media, containing maltose or glucose, with a more complex structural nitrogen source such as peptone induced higher biomass accumulation and ethanol production. In *S. diastaticus*, Amore *et al*., (2002) reported by doubling the nutrient components in the medium, resulting in the production of 9.1% (w/v) ethanol. Increasing the fermentation temperature from 40 to 45 °C Most yeast grows well on a variety of amino acids, purines, and pyrimidines as the sole source of nitrogen. They require trace amounts of biotin, thiamine, pyridoxine, calcium pantothenate and inositol for the maximum growth and fermentation rate (Wayman & Parekh, 1990). Amore *et al*., (2002) have also shown that role of magnesium in relieving the detrimental effect of high temperature may to some extent be related to the requirement of some of the glycolytic enzymes for this cation. In addition, increasing the cell density also resulted in an increase in ethanol production at the higher temperature resulted in a decrease in the rate and extent of glucose utilization and ethanol production.

2.4.5 Effect of pH

Hydrogen ion concentration has a significant influence on industrial fermentation due as much to its importance in controlling bacterial contamination as its effect on yeast growth, fermentation rates and by-product formation. The best ethanol yields are

generally obtained at pH 4.5-4.7. At higher pH, more glycerol and organic acids are formed at the expense of ethanol (Wayman & Parekh, 1990).

Under fermentation conditions, the intracellular pH of *S. cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 or between 5.9 and 6.75 when the external pH is varied between 6.0 and 10.0. The gap between the extracellular pH and the intracellular pH widens, greater stress is placed on the cells and more energy is expended to maintain the intracellular pH within the range that permits growth and survival of the yeast. A greater proportion of glucose is converted to ethanol if the pH is adjusted to 4.5. This increased conversion is independent of the presence of nutrient supplements in the medium (Thomas,*et al*., 2002). If the pH is adjusted to 7 or above, acetic acid is produced from acetaldehyde due to the increased activity of aldehyde dehydrogenase due to glycerol production which inhibits ethanol fermentation (Wang,*et al*., 2001).

2.5.6 Effect of salt concentration

The growth of Yeast cell & production capacity may inhibit due to higher salt concentration.

The physiological features, that distinguish different yeasts, include the range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can use as a source of carbon and energy under semi-anaerobic and aerobic condition, the relative ability to grow in the presence of 50-60% (w/v) D-glucose or 10% (w/v) sodium chloride plus 5% (w/v) glucose (a measure of osmo-tolerance) and the relative ability to hydrolyze and utilize lipids. These properties help investigators determine which yeast strains merit investigation for a particular application (Glazer & Nikido, 1995).

Microorganisms such as the yeast, *Saccharomyces cerevisiae* develop systems to counteract the effect of osmotic stress such as salt stress (NaCl).Specifically, salt-induced stress results in two different phenomena: ion toxicity and osmotic stress. Defense responses to salt stress are based on osmotic adjustments by osmolyte synthesis and cation transport systems for sodium exclusion. In osmo-stressed *S. cerevisiae*, polyols (glycerol in particular) are the major osmolytes produced

accumulated by cells. Other products synthesized by yeast during stress conditions are trehalose and glycogen that may collectively represent 25% of the dry cell mass depending on the environ-mental conditions. The disaccharide trehalose accumulates not only during salt adaptation, but also in response to a number of other stress conditions, and has been shown to protect cells against high temperature by stabilizing proteins and maintaining membrane integrity. Exposing yeast cells in a hyper osmotic environment leads to a rapid initial efflux of water into the medium, which, in other words, is what is meant as cell dehydration. Dehydration is a rapid process mediated solely by water efflux through the lipid bi-layer. Intracellular water is recruited from the vacuole into the cytoplasm thus partially compensating for the sudden increase in macromolecular concentration. Additionally, the cytoskeleton collapses leading to depolarization of actin patches. This cell dehydration leads to growth arrest. So the salt tolerant is capable of continuing their growth in high salt concentration.

2.4.7 Inhibition of growth and fermentation by substrate

The production of high concentration of ethanol is frequently limited by the inhibitory effect on productivity of the fermenting microorganism exerted by the substrate, the concentration of which affects osmotic pressure (Van Uden, 1989). Musts with lower sugar concentrations start to ferment sooner, and the sugar is completely fermented. High sugar concentration inhibits fermentation by their high osmotic pressure, which draws water from the yeast cells (Rehm & Reed, 1995).

Direct substrate inhibition of fermentative ability becomes significant somewhere between 15- 25% sugar concentrations (Van Uden, 1989). Values of specific ethanol production rate and specific uptake decrease almost linearly with the increase sugar concentration. When the substrates are introduced in several batches ethanol yields are higher (Casey & Ingledew, 1986; D'Amore & Stewart, 1987) and cell viability is close to 95% compared to 40% for single batch run (Casey & Ingledew, 1986).

2.4.8 Effect of Oxygen

The microorganisms involved in ethanol fermentation are facultative microbes since they are able to grow with or without the utilization of oxygen. Thus, two of different path ways of pyruvate metabolism are available (Abbott, 2005). In the presence of oxygen, more cell biomass is produced from initial substrate and the growth rate is increased (Alfenore, 2005). However for ethanol production, oxygen must be restricted from entering the fermenter. But, small concentration of oxygen must be proved to the fermenting yeast as it is a necessary component of in the biosynthesis of polyunsaturated fats and lipids (Cysewaski & Wilke, 1977). According to Kosaric and Vardar-Sukan (2001), typical amount of oxygen maintained in the broth is 0.05-0.10 mmHg. Any value higher than this will promote cell growth at the expense of ethanol productivity. The oxygen concentration which triggers aerobic or anaerobic growth processes, is however, varies from culture to culture depending on substrate concentration and cell density (Munnecke, 1981).

2.5.9 Effect of Immobilization

Immobilization is the restriction of cell mobility within a defined space. Immobilization provides high cell concentrations and cell reuse. It also eliminates washout problems at high dilution rates and the costly processes of cell recovery and cell recycle. High volumetric productivities can also be obtained with the combination of high cell concentrations and high flow rates. Immobilization may also improve genetic stability (Nicholas *et al*., 2005).

The most significant advantages of immobilized yeast cell systems are the ability to operate with high productivity at dilution rates exceeding the maximum specific growth rate, the increase of ethanol yield and cellular stability and the decrease of process expenses due to the cell recovery and reutilization (Lin & Tanaka, 2006).

Perspective techniques for yeasts immobilization can be divided into four categories: attachment or adsorption to solid surfaces (wood chips, de-lignified brewer's spent grains, DEAE cellulose, and porous glass), entrapment within a porous matrix (calcium alginate, k-carrageenan, polyvinyl alcohol, agar, gelatine, chitosan, and polyacrilamide), mechanical retention behind a barrier (microporous membrane filters, and microcapsules) and self-aggregation of the cells by flocculation. The application of these

different immobilization methodologies and carriers, their impact in microbial growth and physiology, internal and external mass transfer limitations, product quality and consistency, bioreactor design, bioprocess engineering and economics have been largely discussed(Verbelen *et al.*, 2006).

2.4.10 Effect of Metal

Limited availability of metal ions can also influence fermentation performance of yeasts. In addition, during fermentation, the concentrations of various nutrients change and yeasts must respond dynamically to such changes (Walker & Dijck, 2006). The common metals Copper, magnesium, Potassium, Zink and Iron have effect on ethanol production by Yeast.

2.4.11 Effect of UV Radiation

Hashimoto *et al.*, (2004) showed induced UV irradiation for a small period cause mutation and affect the fermenting ability in a positive manner. The cell survival rate decrease, if the exposure is prolonged.

Their experiment showed, after 30 seconds of UV exposure or 180 minutes of treatment with the chemical mutagen ethyl methanesulfonate (EMS), all the cells died. But mutants derived from three of their five strains showed improvement in alcohol production (12.3% from 10.4%, 12.8% from 9.5% and 11.2% from 9.7%).

2.5 Uses of Ethanol

Industry/ Sectors	Application	References
Chemical industry	Ethanol is a versatile product necessary for the establishment of a powerful chemical industry. It can be used to produce a long list of industrial chemical products and by-products. It also used as a highly performing solvent for agro industries preparations.	Chandel *et al.*, 2007
Medical	There is a market in Less Developing country's for ethanol which is actually imported from overseas for hospitals, clinics operational needs and for industrial maintenance purposes. For example, bioethanol had undergone bioconversion by yeast to produce probiotics and biotherapeutic agent.	Demirbas, 2007
Alcoholic beverages	Ethanol is used for the production of various types of liquor such as wine and beer.	Demirbas, 2007
Transportation	Substitute fossil fuel in transportation sectors and use as petrol substitute or additive. Besides using 100% bioethanol as a gasoline substitute, the mixing of ethanol and gasoline also can be done. It has several advantages such that it can increases octane number thus reduce toxicity and it is more efficient than gasoline in spark-ignition engines.	Palmarola *et al.*, 2005; Bon and Ferrara, 2007; Alam *et al.*, 2007

2.5.1 Other uses

It is easily soluble in water in all proportions. Absolute ethanol and 95% ethanol are themselves good solvents, somewhat less polar than water and used in perfumes, paints and tinctures. Alcoholic drinks have a large variety of tastes because various flavor compounds are dissolved during brewing. Ethanol is used in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62%. Ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi, and many viruses, but is ineffective against bacterial

spores. Wine with less than 16% ethanol cannot protect itself against bacteria. It is also used in preservation of biological specimens.

CHAPTER III

MATERIALS AND EXPERIMENTAL PROCEDURES

3.1 Materials

- Molasses
- Wild-type yeast strain collected from various fruits
- DinitroSalicylic acid (DNS), Sulphuric acid, sodium-potassium tartrate ($KNaC_4H_4O_6.H_2O$), Sodium hydroxide, YPD (yeast extract, peptone, dextrose) medium, Sodium-Metabisulfite, Phenol, Potassium-Sodium-Tartarate, NaOH, Potassium Iodide, Potassium dichromate, Soluble starch, sodium acetate, carbol-fuchsine,etc. were of analytical grades and purchased directly from Sigma (USA), E-Merck (Germany & India).
- Spectrophotometer (Model-TGOU, UK)
- Incubator (Model:DSI300D,Taiwan)
- Shaking Incubator (Model-WIS-20R, Daihan Scientific, Korea)
- Autoclave (Model:WAC-47, Daihan Scientific, Korea)
- pH Meter (pHep,HI-98107, Hanna instruments)
- Microscope (Model-CX-21, Olympus, Japan)
- Glasswares, Laboratory distillation apparatus, Neubauer counting chamber (Cell counter) / Haemacytometer, Magnetic Stirrer, Petri dishes, slants, micro-pipettes, Bunsen burner, hot plate, Desiccators, Balance, micro-burette, Laminer airflow cabinet (Model: SAARC) etc.

3.2 Collecting samples to isolate the wild yeast strains

The yeasts were isolated from rotten fruit samples of grapes and date-juice were collected from markets around Dhaka, Bangladesh.

3.3 Inoculum Development

The yeasts inoculums (10^6-10^7 cells/ml) were grown in a medium, containing (g/l): (0.3% yeast extract, 1% peptone, 2% glucose, 1.5% agar), adjusted to pH 5.0. The medium

was autoclaved at 121 °C and 15 psi and poured on petridish and cooled, then streaked by 48 hours old selected yeast strain from slant.

After preparation of inoculum broth (g/l) (0.3% yeast extract, 1% peptone, 2% glucose) the medium was autoclaved at 121 °C and 15 psi and poured on conical flask and cooled, then inoculated with 48 hours old selected yeast strain from petridish and incubated at 30 °C for twenty-four hours in vigorous shaking condition (180 rpm).

3.3.1 Cell count and viability

A hematocytometer was used to determine yeast cell counts in each conical flask. A 1 ml inoculum broth sample was serially diluted with a sterile saline solution (0.89% w/v NaCl) to a point where a reasonable number of cells could be counted (Alfenore *et al.*, 2007). Most of the time, the cell count recorded is 10^6 cells/ml or a fraction higher.

3.3.2 Maintenance of culture

The culture of yeast was maintained by sub-culturing on slants using YMM (Yeast maintenance Media) media, incubating for 48 hrs at 30ºC and thereafter storing in a refrigerator at 4ºC for future use.

3.4 Identification of the yeast

3.4.1 Morphological characterization

Yeast isolates were identified based on the morphological characters (Kreger-Van Rij, 1984; Mesa *et al*., 1999) and physiological characteristics.

3.4.2 Growth on solid medium

In our study, the morphology of cells of the isolate and its appearance on solid YEPD agar media was examined, The medium was autoclaved at 121 °C and 15 psi and poured on petridish and cooled , then inoculated with 48 hours old selected yeast strain and incubated at 30 °C for 48 hrs. The following features of the appearance of cultures were recorded; texture, color and surface of colonies.

3.4.3 Growth in liquid medium

In our study, Yeast isolate was cultured in YPD liquid medium. The medium was autoclaved at 121 °C and 15 psi and cooled. 15 ml portion of the medium was distributed into McCartney tubes, and then inoculated with 48 hours old selected yeast strain and incubated at 30 °C for 3 days. The culture was examined for the growth visually on the surface of YPD liquid medium and the shape of cells by compound microscope (Model-CX-21, Olympus, Japan).

According to Lorenz *et al.,* 2000, the morphological changes in the culture yeast (*Saccharomyces cerevisiae*) leads to filament formation under unfavorable fermentation conditions. This causes scum formation and foaming. The budding yeast *S. cerevisiae*, starved for nitrogen, differentiates into a filamentous growth form.

3.5 Physiological characterization

3.5.1 Fermentation of carbohydrates

Yeast fermentation broth base with Durham tube was used for testing of yeasts for carbohydrate fermentation. Yeast fermentation broth media were used for identification yeasts based on fermentation of specific carbohydrates of fermentation pattern. The carbohydrate used were; glucose (dextrose), galactose, maltose, sucrose, lactose trehalose, fructose and xylose. Yeast fermentation broth was modification of media developed by Wickerham for the determination of carbohydrate fermentation by yeasts for fermented carbohydrates by yeasts, the color of the medium changed from red to yellow due to the formation of acids and gas produced (Warren & Shadomy, 1991).

Testing of selected strain for carbohydrate fermentation, the ability to ferment seven different carbohydrates was examined anaerobically, was assessed by looking for the formation of gas (Co2) in Durham tube and color change of the fermentation media. In addition, medium prepared from Peptone 10 g, NaCl 5 g, Phenol red (Indicator / Chromogen), Carbohydrate 5 g and make the volume up to 1000 ml with distilled water. A volume of 15 ml aliquots dispensed in different McCartney tubes were autoclaved

121°c and 15 psi, after cooling the media then inoculated with 48 hrs old selected yeast strain and fermented for 72 hrs.

3.6 Stress tolerance characterization

3.6.1 Detection of thermo-tolerance

YEPD liquid medium was used for detecting thermo-tolerance and growth in liquid media of selected yeast strain. The medium was autoclaved at 121 °C and 15 psi and cooled. 10 ml portion of the medium was distributed into McCartney tubes, and then inoculated with 48 hours old selected yeast strain. The initial optical density of each tube was recorded on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 25°C, 30°C, 37°C, 40°C and 44°C for 2 days for observing thermo tolerance of yeast strain. The increase in optical density in a tube was recorded as evidence of growth. Without it, growth on YEPD agar media at 25°C, 30°C, 37°C and 40°C was also observed to ensure thermo-tolerance.

3.6.2 Detection of ethanol tolerance

The medium for the detection of ethanol tolerance of thermo-tolerant yeast was modified from Osho, 2005. YEPD liquid medium was used for detecting yeasts for ethanol tolerance. The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. One ml of various concentrations of absolute ethanol was varied from 5 to 25% (v/v), and then added to different flask of the same medium to constitute varying percentages of ethanol differing by 5% (v/v) from one flask to the others. Forty mililiter portion of the medium was distributed into 125 ml flask, and then inoculated with selected thermo-tolerant yeasts. The initial optical density of each flask was read off on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 40 °C for 5 days. The increase in optical density in a flask was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of yeasts.

In our study, YEPD broth was prepared containing 5%, 10%, 12%, 15%, 18% 20% and 25% of absolute ethanol. Each McCartney contained 15 ml of YEPD liquid media with appropriate concentration of ethanol and blank media was used as a control. Then each was inoculated by half loop full of Yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm.

3.6.3 Growth in different pH in Liquid Media

YEPD liquid medium was used for detecting the ability to grow in different pH of selected yeast strain. The medium was autoclaved at 121 °C and 15 psi and cooled. YEPD broth was prepared at different pH. Each test-tube contained 13 ml of YEPD media with different pH and blank media was used as a control. Then each was inoculated by half loop full of yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm for growth.

3.6.4 Osmo-tolerance of Yeast

Growth impairment under conditions of high osmotic strength is often associated with defects in the cell wall or components of the cytoskeleton (Novick & Botstein, 1985).
YEPD broth was prepared containing 6%, 9%, 12%, 15%, 18% and 20% of NaCl. Each test-tube contained 13 ml of YEPD media with appropriate concentration of salt and blank media was used as a control. Then each was inoculated by half loopful of Yeast cell and measured the initial optical density at 600 nm and incubated at 30°c for 48 h. After 48 h cell density was further recorded at 600 nm.

3.7 Ethanol fermentation characterization from Molasses

3.7.1 Pretreatment of Molasses

Sulfuric acid is used to convert calcium to calcium sulfate salts. Calcium acts as an inhibitor agent during fermentation. Sulfuric acid decreases the fermentation medium pH that control bacterial contamination. Sulfuric acid in diluted molasses can precipitate

calcium and reduce the scaling. Blackstrap molasses contains many nutrients for fermentation. Without it, it requires some nutrients elements. Urea is used as a nitrogen source in molasses fermentations for ethanol production, but it in alcoholic beverage it may produce carcinogenic ethylcarbamate, which is unacceptable. Diammonium phosphate may be added in Phosphorus deficient molasses. In our present study we used 0.30 ml concentrated Sulfuric acid and 0.10 gm urea in 250 gm molasses to treat and diluted it with tap water.

3.7.2 Fermentation media preparation

Sugarcane molasses was used as a fermentation media for our study. Molasses was collected from local market of Bangladesh at the district of Sirajgunj. It contains ~20% of reducing sugar. In our all experiments we estimated Reducing sugar concentration only.

3.7.3 Composition of Fermentation Media

Molasses	250 gm
Urea	0 .10 gm
Conc. (H_2SO_4)	0 .30 ml

Tap water was added up to 1000 ml and the media was boiled there after autoclave at 121°C and 15psi. The reducing sugar concentration was maintained by boiling the media.

3.7.4 Preparation of yeast cell suspension

Autoclaved molasses fermentation media broth (10 ml) was added to a 48 hours old slant culture of *S. cerevisiae* and the tube was placed in a rotary incubator at 30°C in vigorous shaking (180 rpm) to form a homogeneous suspension.

3.7.5 Fermentation of molasses

Fermentation was carried out in Erlenmeyer conical flasks. 250 ml fermentation media was taken into 500 ml Erlenmeyer flasks and then the homogenous suspension of yeast was inoculated into the media in an aseptic condition. The flask was cotton plugged and

incubated at different temperature to an incubator in both non-shaking and shaking condition.

3.8 Ethanol fermentation characterization from Kitchen wastes

3.8.1 Pretreatment of kitchen waste

Kitchen-wastes were collected from various households as discards. These solid wastes include peels of potatoes, papaya, pumpkin, cucumber, lady's finger, green banana, balsan apple, carrot and basil. 250 gm solid wastes were hewn, pulverized and placed in 1 liter tap water to get mingled by use of a mechanical blender. The complete breakage of solid substances can only ensure the proper nutrient availability for the microorganism in medium. Hydrochloric acid is used to convert calcium to calcium sulfate salts. Calcium acts as an inhibitor during fermentation. HCl decreases the fermentation medium pH that control bacterial contamination. The chemical hydrolysis of plant residues is induced by HCl in a boiling temperature and left plant carbohydrate units of Cellulose and Starch. Further degradation arise monomers of Amylose, Amylopectin and then Glucose. Kitchen-wastes contain many nutrients for fermentation. To supplement that, Urea is used as a nitrogen source. Downstream processing is needed before isolate the usable Ethanol.

3.8.2 Composition of Fermentation Media

Solid Kitchen waste	250 gm
Urea	0 .10 gm
Conc. (HCl)	2 .0 ml

Solid wastes contained different peels. Tap water added up to 1000 ml and boiled the media for one and half hours. After cooling the media, pH was adjusted to 6.0.

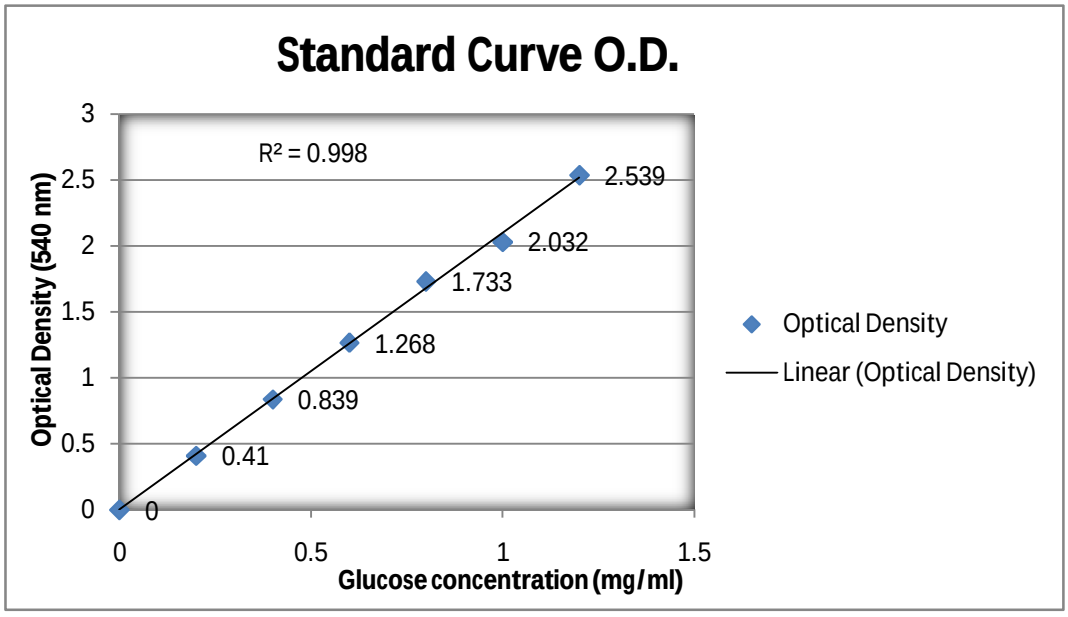
3.8.3 Fermentation of kitchen waste

Fermentation was carried out in Erlenmeyer conical flasks. 250 ml fermentation media was taken into 500ml Erlenmeyer flasks and then added the homogenous suspension of yeast was inoculated into the media in an aseptic condition. The flask was cotton

plugged and incubated in a rotary incubator at 30°c in shaking condition (120 rpm). Earlier experiments suggested more Ethanol is produced in shaking than non-shaking state.

3.9 Estimation of reducing sugars

The reducing substance (sugar) obtained due to the enzymatic reaction was estimated by dinitrosalycylic acid (DNS) method (Miller *et al*., 1959). A double beam UV/VIS – scanning spectrophotometer was used for measuring absorbance. Reducing Sugar contents before fermentation and after fermentation were determined by taking 1.0 ml diluted solution (1 ml sample in 100 ml distilled water) with 3.0 ml of DNS reagent in a test tube. Blank containing 1.0 ml of distilled water and 3.0 ml of DNS was run parallel. The tubes were heated in a boiling water bath for 15 min .After cooling the tubes at room temperature, 8 ml distilled water was added in each and absorbance was noted at 540 nm using spectrophotometer. Sugar concentration was determined from the standard curve of glucose and multiplies by dilution factor. A standard curve of glucose was



prepared as below:

Figure 3.1: Glucose standard curve for reducing sugar estimation.

3.10 Estimation of Ethanol: Conway Method

Ethanol was determined by Redox titration. In this method ethanol is oxidized to ethanoic acid when ethanol react with excess of potassium dichromate solution (0.05 N) and unreacted dichromate is then determined by adding potassium iodide (50% KI) solution which is oxidized by the potassium dichromate. Potassium iodide reacts with potassium dichromate and creates iodine. Then the iodine is titrated with a standard solution of sodium thiosulfate (0.1N). The titration reading is used to calculate the ethanol content after fermentation. One ml Fermented solution was diluted up to in 250 ml; 500 ml and 1000 ml distilled water and took each one ml diluted solution as a sample. A Conway unit is used for ethanol detection by this procedure. One ml potassium dichromate was placed into the Conway unit center and sample was placed around the center. The Conway unit was then covered by a glass plate for 24 hours for reaction. The water and ethanol slowly evaporate, come in contact with Potassium dichromate and then oxidized. More ethanol evaporates until eventually all the ethanol from the fermented dilute solution has left the sample and reacted with the dichromate. One Conway unit was used as a blank and in that unit 1 ml distilled was used as a sample.

Protocol

Add 1 ml 0.05 N potassium dichromate solution in Conway unit center

↓

Add 1 ml sample in Conway unit Round

↓

Reaction complete within 24 hours

↓

Add 50% KI solution 0.5 ml + 1-2 drop soluble starch in Conway unit center

↓

Take 0.1N sodium thiosulfate in Microburatte

↓

Titration

↓

Untill the center becomes Colourless

Calculation

$$\text{Percentage of Ethanol (\%)} = \frac{(\text{TR of blank sample} - \text{TR of FS}) \times 11.6 \times 0.1 \times DF \times 100}{0.793 \times 1000}$$

= Alcohol percentage (gm/100 ml)

Where,

Density of Ethanol 0.793 g/ml, DF-Dilution Factor, FS-Fermented solution ,TR-Titration Reading, **Volume of sodium thiosulfate used: 11.60 cm^3**

3.11 Optimization of fermentation process

Fermentation process carried out by yeast is known to vary with respect to substrate concentration, temperature, N-source and inoculums size. It is therefore imperative to optimize the fermentation conditions for yeast cells so that the production efficiency increases. Various physicochemical factors were investigated affecting ethanol production from molasses.

3.11.1 Effect of sugar concentration

To study the effect of sugar concentration on ethanol production by *S. cerevisiae*, the production media was prepared by diluting molasses to reducing sugar concentration 3.0, 4.0, 6.0, 6.5, 7.0 and 7.5 percent and fermentation was carried out in a volume of 250 ml media in a 500 ml conical flask. A twenty-four hour old inoculum of yeast was added at the medium. Samples were withdrawn at different time and estimated for residual sugars (Miller *et al*., 1959) as well as ethanol content in the media.

3.11.2 Effect of pH

To study the effect of pH on ethanol production by *S. cerevisiae*, fermentation media with the optimum reducing sugar concentration was used for the production of Ethanol. Fermentation was carried out at pH 5.0 and pH 6.0 in a volume of 250 ml media in a 500 ml conical flask. A twenty-four hour old inoculum of yeast was added to the medium. Samples were withdrawn at different time and estimated for residual sugars (Miller *et al*., 1959) as well as ethanol content in the media.

3.11.2 Effect of shaking

To study the effect of shaking on ethanol production by *S. cerevisiae*, fermentation media with the optimum reducing sugar concentration was used for the production of Ethanol. Fermentation was carried out at shaking (120 rpm) and non-shaking condition in a volume of 250 ml media in a 500 ml conical flask. A twenty-four hour old inoculum of yeast was added to the medium. Samples were withdrawn at different time and estimated for residual sugars (Miller *et al*., 1959) as well as ethanol content in the media.

CHAPTER IV

RESULTS

4.1 Identification of the selected yeast

4.1.1 Morphological characterization

The morphology of the vegetative cells of yeast was determined according to the method of Kreger-Van Rij (1984) and Kurtzman and Fell (1997) by growing in liquid and on solid media.

4.1.1.1 Growth on solid medium

Yeast isolates formed butyrous and smooth white raised colonies on YEPD medium. The budding stage of the yeast isolates was observed under (40X) microscope and confirmed them to be yeast.

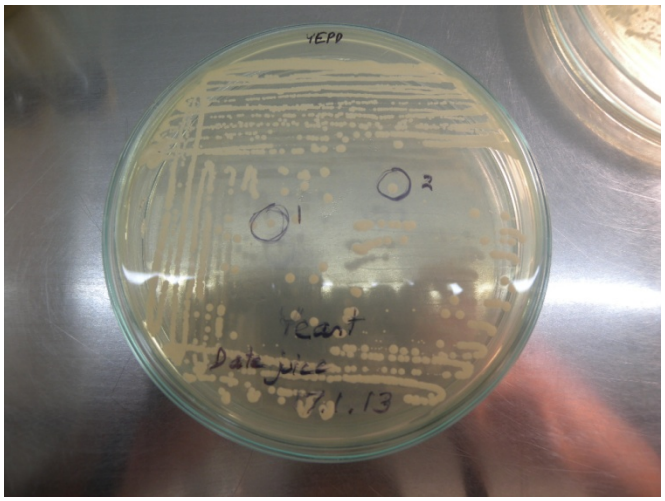


Figure 4.1: Colonies on YEPD agar medium after incubation of 48 hours at 30°c

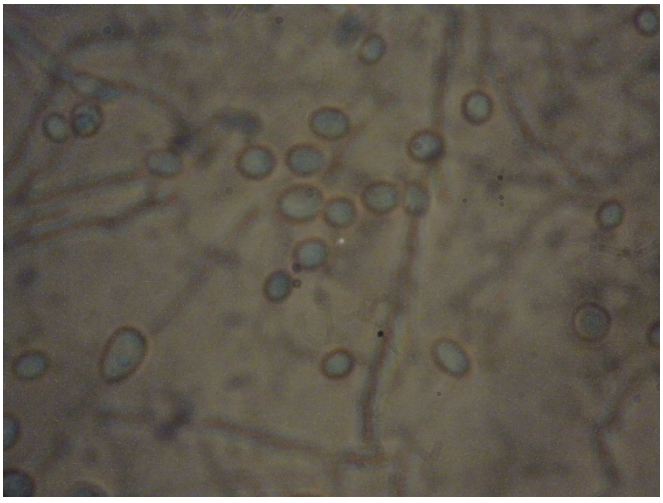
4.1.1.2 Growth in liquid medium

After 3 days of incubation at 30°C, heavy, dry climbing pellicles were formed on the surface of YEPD broth medium.

Figure 4.2: Growth in liquid medium

4.1.2 Microscopic observation

The cell morphology of the ethanol tolerant yeast isolated from Date-juice & Grapes strain here studied under compound microscope. An ovoidal to elongate have single, pairs, or triple budding cells were found. The isolate reproduces vegetatively by budding. The isolate obtained from Date-juice was larger than the isolate obtained from Grape.



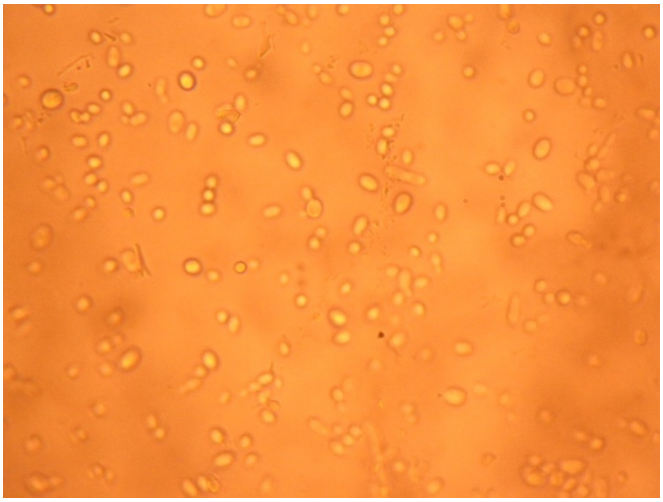


Figure 4.3: The cell morphology under compound microscope. a) Date-juice, b) Grapes

4.1.3 Physiological Characterization

4.1.3.1 Fermentation of carbohydrates

In this study, *Saccharomyces cerevisiae* showed variation of utilization of seven different sugars (Table 4.1). The Date-juice strain utilized Glucose, Sucrose,Fructose, Lactose, Maltose and Trehalose but failed to grow on Xylose. The strain obtained from Grapes utilized Glucose, Sucrose, Fructose, Lactose and Trehalose but failed to grow on Maltose and Xylose. After 48 hours the following results were shown:

Carbohydrate	Before Fermentation	After Fermentation
Glucose/ Dextrose	Pink	+ (yellow), gas formed
Sucrose	Pink	+ (yellow), gas formed
Maltose	Pink	+ (yellow), gas formed
Lactose	Pink	+ (yellow), gas formed
Fructose	Pink	+ (yellow), gas formed
Xylose	Pink	- (no color change)
Trehalose	Pink	+(yellow), gas formed

Table 4.1: Fermentation result of different carbohydrates for Date-juice strain of *S. cerevisiae*.

Carbohydrate	Before Fermentation	After Fermentation
Glucose/ Dextrose	Pink	+ (yellow), gas formed
Sucrose	Pink	+ (yellow), gas formed
Maltose	Pink	- (no color change)
Lactose	Pink	+ (yellow), gas formed
Fructose	Pink	+ (yellow), gas formed
Xylose	Pink	- (no color change)
Trehalose	Pink	+ (yellow), gas formed

Table 4.2: Fermentation result of different carbohydrates for Grapes strain of *S. cerevisiae*.

Fig 4.4: Media color change after Fermentation of Carbohydrate.

4.2 Stress tolerance results

4.2.1 Thermo-tolerance

Five YPD Agar containing plates were streaked by Yeast cell and incubated for 48 hours at 25°C, 30°C, 37°C, 40°C and 44°C. Date-juice strain of yeast was able to grow at 25°C-44°C, but the Grapes strain failed at 44°C. To confirm the results obtained from solid media, thermo-tolerance was repeated in liquid media. Growth at liquid media is shown in the given table.

Temperature	Strain	O.D. At Inoculation	O.D. after 24 hours	O.D. after 48 hours	O.D. Change in

					48 hours
25°	Date-juice	0.559	1.501	2.290	1.731
	Grapes	0.519	1.424	2.063	1.544
30°	Date-juice	0.515	1.848	2.311	1.796
	Grapes	0.441	1.790	2.162	1.721
37°	Date-juice	0.671	1.744	2.058	1.387
	Grapes	0.523	1.025	1.901	1.378
40°	Date-juice	0.465	1.456	1.918	1.453
	Grapes	0.350	0.301	1.261	0.911
44°	Date-juice	0.511	0.693	0.802	0.291
	Grapes	0.687	0.530	0.471	-0.216

Table 4.3: Growth in different temperature in liquid media.

From the table, it is evident that the Date-juice strain is slightly thermo tolerant as it has ability to grow up to 44°C. And the most suitable condition for growing of yeast proved to be 30°C.

4.2.2 Ethanol tolerance

The isolate was selected for screening of ethanol tolerant yeast (Table 4.4).The isolate can grow up to 20% ethanol containing liquid YEPD media. Maximum growth for the date-juice was seen in 5% ethanol containing media, but for the Grapes was 10%. Growth were recorded at 5%, 10%, 12%, 15%, 18%, 20%, and 25% of ethanol containing liquid media and O.D is given gradually:

Ethanol %	**Strain**	**O.D. At Inoculation**	**O.D. after 24 hours**	**O.D. after 48 hours**	**O.D. Change in 48 hours**
5	Date-juice	0.364	0.654	1.920	1.556
	Grapes	0.433	0.891	1.403	0.970
10	Date-juice	0.365	1.296	1.825	1.460
	Grapes	0.445	0.638	1.452	1.007
12	Date-juice	0.247	0.588	1.206	0.959

	Grapes	0.336	0.409	0.560	0.224
15	Date-juice	0.352	0.683	0.833	0.941
	Grapes	0.246	0.288	0.305	0.063
18	Date-juice	0.269	0.670	1.293	1.024
	Grapes	0.244	0.283	0.309	0.065
20	Date-juice	0.290	0.313	0.384	0.094
	Grapes	0.192	0.277	0.503	0.311
25	Date-juice	0.220	0.201	0.166	-0.054
	Grapes	0.259	0.237	0.109	-0.150

Table 4.4: Growth in different ethanol concentration containing media.

4.2.3 pH sensitivity

The ethanol producing strains have ability to grow at wide range of pH. At pH 2 the growth was not remarkable, but the strain somehow managed to survive the high acidic condition. They withstand the alkaline condition up to pH 10. Maximum growth was seen at pH 5. The Date-juice strain is more resistant than the Grapes strain in this experiment. After 48 hrs, cell density was recorded at 600 nm:

pH	**Strain**	**O.D. At Inoculation**	**O.D. after 24 hours**	**O.D. after 48 hours**	**O.D. Change in 48 hours**
2	Date-juice	0.335	0.548	0.773	0.438
	Grapes	0.363	0.462	0.496	0.133
3	Date-juice	0.361	1.044	1.407	1.046
	Grapes	0.404	0.755	1.466	1.062
4	Date-juice	0.390	1.502	1.859	1.469
	Grapes	0.369	0.795	1.468	1.099
5	Date-juice	0.407	1.396	1.887	1.480
	Grapes	0.355	0.849	1.572	1.237
6	Date-juice	0.459	0.573	1.847	1.388
	Grapes	0.376	0.871	1.571	1.195

7	Date-juice	0.473	1.113	1.822	1.349
	Grapes	0.439	0.723	1.319	0.880
8	Date-juice	0.328	0.832	1.614	1.286
	Grapes	0.254	0.569	1.035	0.781
9	Date-juice	0.336	0.638	1.517	1.181
	Grapes	0.296	0.833	1.287	0.991
10	Date-juice	0.473	0.773	1.377	0.904
	Grapes	0.365	0.702	1.283	0.918

Table 4.5: Growth in different pH containing liquid media

4.2.4 Osmo-tolerance of Yeast

YEPD broth was prepared containing 6%, 9%, 12%, 15%, 18%, and 20% of NaCl. Each McCartney contained 15 ml of YEPD media with appropriate concentration of salt and blank was used as a control. Then each was inoculated by half loopful of Yeast cell and measured the initial optical density at 600 nm and incubated at 30°c for 48 h. After 48 h cell density was further recorded at 600 nm. Growth were recorded at 6%, 9%, 12%, 15%, 18%, and 20% of salt containing media and O.D is given gradually :

NaCl %	**Strain**	**O.D. At Inoculation**	**O.D. after 24 hours**	**O.D. after 48 hours**	**O.D. Change in 48 hours**
6	Date-juice	0.206	0.569	1.325	1.119
	Grapes	0.221	0.343	0.411	0.190
9	Date-juice	0.209	0.379	0.661	0.452
	Grapes	0.226	0.267	0.315	0.089
12	Date-juice	0.218	0.258	0.329	0.111
	Grapes	0.222	0.249	0.301	0.079
15	Date-juice	0.242	0.267	0.302	0.060
	Grapes	0.260	0.254	0.294	0.034
18	Date-juice	0.252	0.281	0.314	0.062

	Grapes	0.224	0.286	0.351	0.127
20	Date-juice	0.324	0.332	0.356	0.032
	Grapes	0.227	0.222	0.341	0.114

Table 4.6: Growth in different NaCl containing liquid media

The results showed both strains are resistant against higher osmotic pressure. Both strains had their highest growth in 6% NaCl containing media and gradually lowest in 15% and 20% NaCl containing media.

4.3 Ethanol Fermentation

4.3.1 Ethanol production at room temperature using Molasses medium

In Non Shaking condition, a low amount of ethanol produced at 24 hours using initial reducing sugar concentration of the fermentation media at 3.0%, 30°C temperature and pH 5.0. Interestingly, the production is greatly enhanced in shaking condition (120 rpm). The results of 4 and 6% glucose concentration were mild. Maximum production was recorded in 6.5 and 7%. In 7.5% concentration production plummeted. The maximum production was 5.93% in both shaking and non-shaking condition by the Date-juice strain using molasses medium.

Figure 4.5: a) Sugar estimation by DNS method, b) After 24 hours fermentation in shaking condition, c) Conway unit used for Ethanol detection

Glucose conc.	Date-juice (Shaking)	Date-juice (Non-shaking)	Grapes (Shaking)	Grapes (Non-shaking)

3%	4.46	2.27	4.83	2.27
4%	3.37	5.53	5.53	4.1
6 %	5.19	4.46	5.93	3.37
6.5%	5.93	3.37	3	3
7%	5.93	3.73	4.10	3.37
7.5%	4.83	3	3	1.9

Table 4.7: Alcohol production comparison between Various Fruit Yeast strains in 24 hours fermentation

Glucose conc.	Date-juice (Shaking)	Date-juice (Non-shaking)	Grapes (Shaking)	Grapes (Non-shaking)
3%	→0.15→0.13	→0.81→0.35	→0.41→0.12	→0.82→0.77
4%	→0.40→0.40	→1.73→0.36	→2.09→1.18	→3.10→2.54
6 %	→0.86→0.73	→4.03→1.93	→5.96→5.80	→4.12→3.39
6.5%	→0.76→0.79	→5.40→3.28	→5.50→5.08	→6.36→6.11
7%	→0.94→0.93	→6.22→3.94	→5.07→3.66	→6.74→6.04
7.5%	→1.15→0.44	→5.30→2.63	→5.35→3.24	→5.98→5.56

Table 4.8: Glucose depletion comparison between Various Fruit Yeast strains in 24, 48 hours fermentation

Glucose conc.	Date-juice (Shaking)	Date-juice (Non-shaking)	Grapes (Shaking)	Grapes (Non-shaking)
3%	4.97 →4.95	4.84 →4.9	4.57 →4.76	4.79 →4.74
4%	5.11 →5.12	4.89 →5.08	4.58 →4.55	4.71 →4.74
6 %	5.22 →5.23	4.9 →4.96	4.86 →5.11	4.83 →4.87
6.5%	5.12 →5.12	4.75 →4.92	4.63 →4.63	4.76 →4.79
7%	5.17 →5.46	5 →5.03	4.81 →4.7	4.91 →4.87
7.5%	5.09 →5.34	4.95 →5.01	4.76 →4.69	4.82 →4.83

Table 4.9: pH comparison between Various Fruit Yeast strains in 48 hours fermentation (Initial 5.0)

Glucose% / pH	Alcohol
6.5% / 5	3 →6.67 →7.02
6.5% / 6	3.73 →7.75 →6.66
7% / 5	2.27 →6.15 →5.19
7% / 6	2.07 →6.83 →6.66

Table 4.10: Production of alcohol by Date-juice yeast strain at room temperature in 72 hours fermentation (Shaking at 120 rpm)

Initial glucose% / pH	Glucose % change
6.5% / 5	0.96 →0.90 →0.98
6.5% / 6	3.71 →0.98 →1.08
7% / 5	1.60 →1.03 →1.17
7% / 6	2.92 →0.93 →1.13

Table 4.11: Glucose depletion by Date-juice yeast strain in 72 hours fermentation (Shaking at 120 rpm)

Initial glucose% / pH	pH change
6.5% / 5	5.20 →5.52 →5.38
6.5% / 6	5.33 →5.62 →5.675
7% / 5	5.17 →5.37 →5.54
7% / 6	5.30 →5.75 →5.71

Table 4.12: pH comparison of Date-juice Yeast strain in 72 hours fermentation (Shaking at 120 rpm)

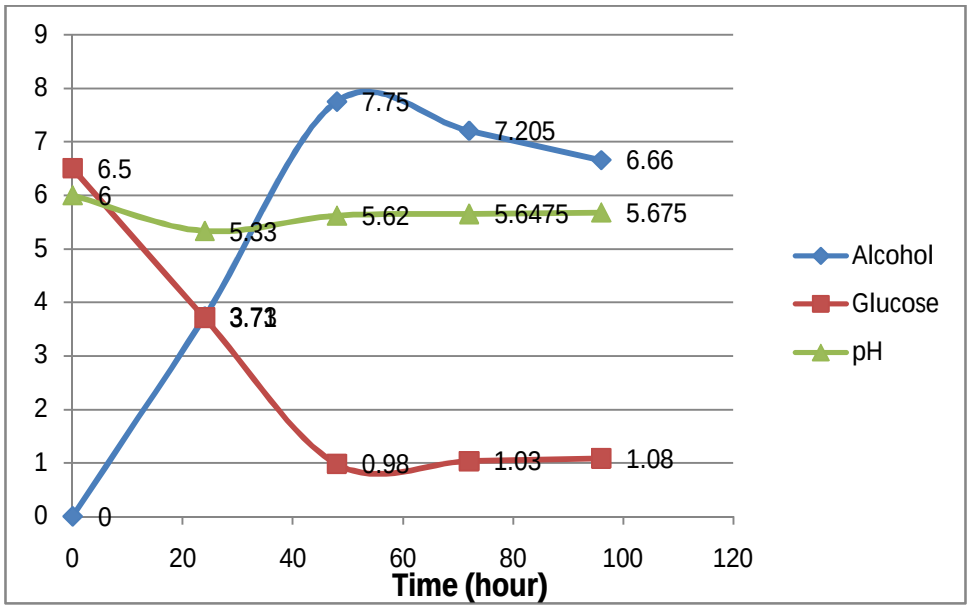


Figure 4.6: Fermentation Kinetics of Date-juice yeast strain (Shaking) in 6.5% Glucose conc. & pH 6.0

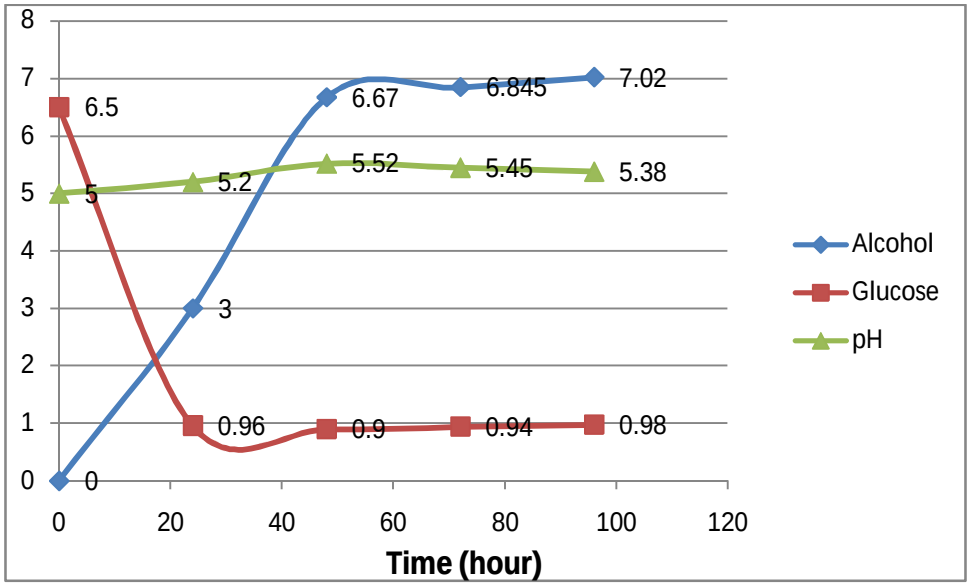


Figure 4.7: Fermentation Kinetics of Date-juice yeast strain (Shaking) in 6.5% glucose conc. & pH 5.0

4.3.2 Ethanol production at room temperature using Kitchen waste medium

Fermentation carried out in 500 ml Erlenmeyer conical flasks at 30°C in shaking condition. Earlier experiments suggested more Ethanol was produced in shaking than non-shaking state. After 48 hours of fermentation maximum 7.3% Ethanol production observed.

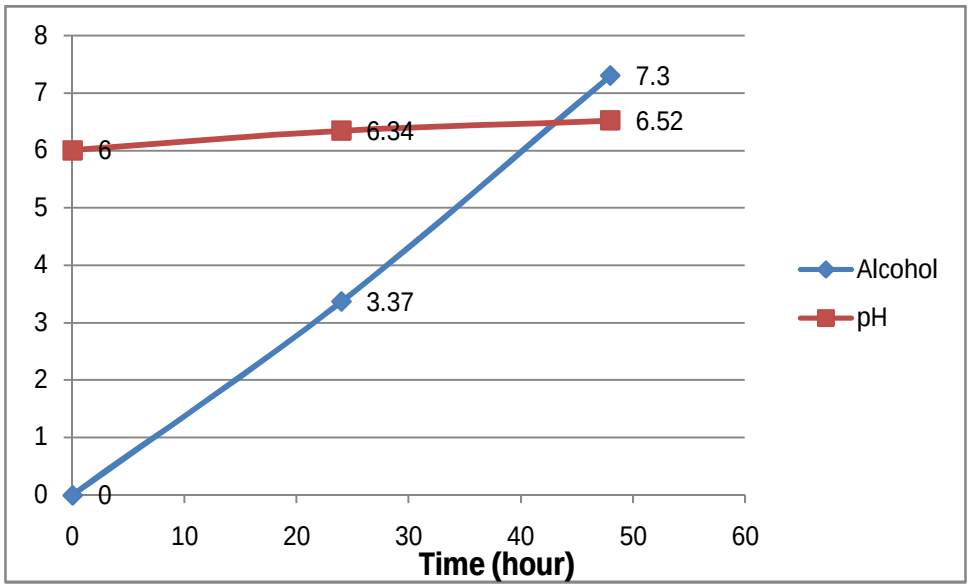


Figure 4.8: Fermentation kinetics of Date-juice yeast strain (Shaking) using Kitchen waste at pH 6.0

Fermentation Medium	Alcohol production	pH change	Glucose% change
Molasses	0→3.73→7.75	6→5.33→5.62	6.5 →3.71→0.98
	0→2.07→6.83	6→5.30→5.75	7 →2.92→0.93
Kitchen waste	0→3.37→7.3	6→6.34→6.52	

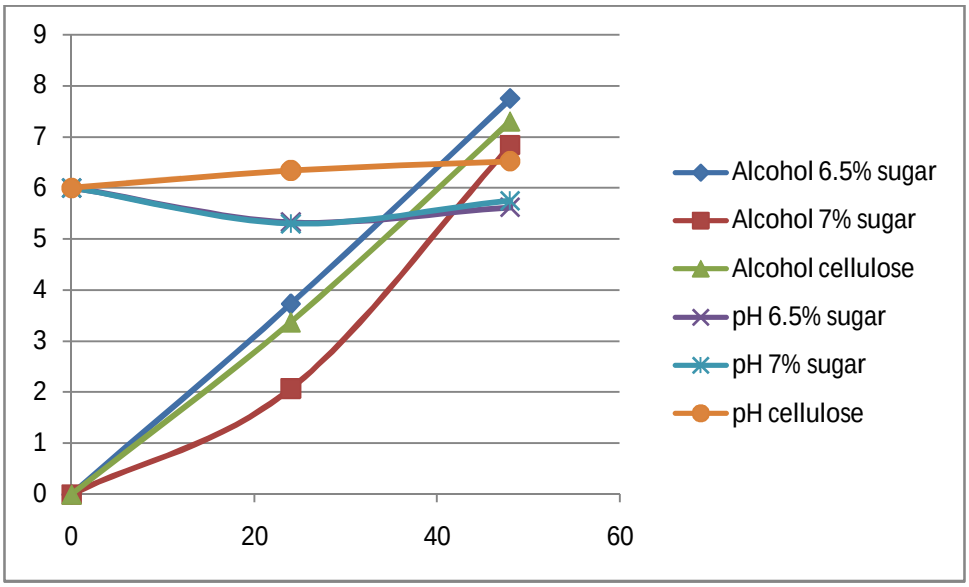


Table 4.13 & Figure 4.9: Date-juice strain fermentation in 48 hours (Molasses versus Kitchen waste) at pH 6.0

4.4 Optimization of fermentation process

4.4.1 Effect of sugar concentration

The effect of different sugar concentration was studied through initial twenty-four hour fermentation in a volume of 250 ml media in a 500 ml conical flask in shaking and non-shaking condition. 6.5% and 7% glucose concentration proved to be the optimum sugar concentration for Ethanol production. Date-juice strain seems to be the most productive strain than the Grapes strain in this experiment. Another round of seventy-two hours fermentation was carried out to identify the maximum production level of the microorganism. Maximum 7.75% of alcohol production is observed in the experiment.

4.4.2 Effect of pH

Initial round of fermentation were carried out at pH 5.0, because that is the optimum growth condition for the microorganism. To compare the effect of pH, another round of fermentation was performed at pH 6.0, as well as 5.0. Interestingly, pH 6.0 proved to be more suitable condition for the production of Ethanol.

In molasses media fermentation generally the pH descends, while fermenting the kitchen wastes pH rises.

4.4.3 Effect of shaking

Fermentation carried out in both shaking (120 rpm) and non-shaking condition. And shaking condition proved to be better than the stagnant form.

CHAPTER V
DISCUSSION

Based on the colony characteristics (white and creamy texture) ovoid microscope shape, the presence of ascospore, and budding pattern (multipolar), the selected isolate (Date-juice and grapes) were found to belong *sacharomyces* type unicellular ascomycete according to (Lodder, 1971) and (Boekhout & Kurtzman ,1996) (Fig 4.1; 4.3).

The isolates were tested for fermentation of carbohydrates and Date-juice strain was capable to ferment six sugars out of the seven sugars tested (Table 4.1). Glucose, Sucrose, Fructose, Lactose, Maltose and Trehalose were successfully fermented by this strain but it can't ferment Xylose. The Grapes failed to ferment Maltose and Xylose, but utilized five other carbohydrates (Table 4.2), which proved the identity both of the microorganisms are *Saccharomyces cerevisiae*.

Sacchromyces yeasts are the most ethanol tolerant of the eukaryotic organisms, and able to tolerate over 20% ethanol. In a previous study by (Casey & Ingledew, 1986), yeast strain *TGY2* could tolerate up to 16% (v/v) ethanol. Almost Similar ethanol-tolerance of 16.5% (v/v) has been observed for *saccharomyces cerevisiae* by (Teramoto, *et al.*, 2005).

Both strains (Date-juice, grapes) were screened for ethanol tolerance and showed up to 25% ethanol tolerance in YEPD liquid growth media. A slow growth rate was observed at 10-20% ethanol containing media (Table 4.4).

The optimal pH range for growth of yeast can vary from pH 4.0 to 6.0, depending on temperature, the presence of oxygen, and the strain of yeast. This likely is due to the optimum pH value for the activity of plasma membrane-bound proteins, including enzymes and transport proteins (Narendranath & Power, 2005). In our study the strain Date-juice can grow in a wide pH range from 2 to 10, but ph 5.0 showed to be the

optimum pH for it (Table 4.5). The Grapes strain showed growth in pH 3 to 10, but in pH 2, it grows reluctantly. One of most important findings of this experiment is the ability of growing in high acidic condition by the Date-juice strain.

The results proved both strains have resistance against higher osmotic pressure. Both strains showed their highest growth in 6% NaCl containing media and gradually lowest in 15% and 20% NaCl containing media (Table 4.6).

A series of experiments had been conducted at different glucose concentration to determine the optimum condition for twenty-four hours alcoholic fermentation. In shaking condition, the Date-juice strain showed highest 5.93% production in 6.5% and 7% glucose level. In the same parameters the Grapes strain produced 5.93% in 6% glucose concentration. In the non-shaking condition, the Date-juice strain showed 5.53% production in 4% glucose level and the Grapes resulted maximum 4.1% in the same glucose concentration (Table 4.7).

The seventy-two hours fermentation results showed the detailed characterization of the Date-juice strain, which proves to be the better strain in every aspect. Maximum 7.75% alcohol production was recorded after forty-eight hours in 6.5% glucose and pH 6 (Table 4.10). This proved to be the highest production achieved in molasses media in this experiment.

In a similar study in Bangladesh, five isolates produced alcohol by fermenting molasses at wide range of temperature (25-37°c).The production was maximal at 30°c after 48 hours of incubation. The isolates TY,BY,GY-1,RY and SY produced alcohol 12.0%, 5.90%, 5.80%, 6.70% and 5.80%, respectively at 30°c after 48h ours of incubation. Using glucose as substrate in the fermented media varied between 2.3%-5.90%, the alcohol production rate were maximal up to 36 hours and stopped at 48 hours (Khan, *et al.*, 1989*).*

Another round of fermentation carried out in media prepared from kitchen-wastes. This particular media contain plant organelles and a rich source of cellulose and starch. The Date-juice strain was employed to ensure a cheap medium for the production of biofuel in near future. Using the same optimum condition, which is achieved by the molasses medium, 7.3% of alcohol production was recorded (Table 4.13).

CHAPTER VI
CONCLUSION

The cell morphology of the yeast cells under microscope is ovoidal to elongate, single or in pairs and budding cells are also recognized. All the physiological and biochemical characters observed suggested that the strain is *Saccharomyces cerevisiae.* The comparison of two wild-type isolate showed the Date-juice strain is more potent microorganism in ethanol fermentation.

The fermentation of molasses using *S. cerevisiae* (Date-juice strain) under optimized conditions, such as reducing sugar concentration 6.5%-7%, temperature 30°C and pH 6.0 are suitable in fermentation efficiency for ethanol production by free cells in 48 hrs shaking condition. After that, ethanol production decrease due to substrate limitation or by product (feedback) inhibition.

Pilot plant study of this strain with optimized conditions need to be done to make it an industrially suitable strain. Productivity can also be improved by mutation through radiation or genetic manipulation. Metabolic pathway engineering to direct ethanol production may another way for improvement. The most auspicious finding is that ethanol has produced from discards, which declares in future we can use this cheap product for a valuable necessity (biofuel).

CHAPTER VII
REFERENCES

Abbott DA (2005). Growth rates of *Brettanomyceyeasts*hinder their ability to compute with *Sacchromycescerevisiae*inbatchcornMashfermentations. Appl.Microbiol. Biotechnol,66(6): 22-25.

ABRACICLO."ProduçãoMotocicletas 2010" (in Portuguese).

Alam MZ, Kabbashi NA &Razak AA (2007).Liquid State Bioconversion of DomesticWastewater Sludge for Bioethanol Production. Bioenvironmental Engineering Research Unit (BERU), International Islamic University Malaysia (IIUM) Kuala Lumpur, Malaysia.

Alexopoulos CJ (1962). Sub-class hemiascomycetidae the yeast and leaf-curl fungi. In:Introductory Mycology. Second Edition. Toppan Printing Company, Japan. pp. 241-258.

Alexopoulos CJ, Mims CW & Blackwell M (1996).Introductory Mycology.Forth Edition. United States of America: John Wiley & Sons. pp. 280.

Alfenore S, Molina-Jouve C, Guillouet SE, Uribelarrea JL, Goma G &Benbadis L (2002). Improving ethanol production and viability of *Saccharomyces cerevisiae*by a vitamin feeding strategy during fed-batch process.App. Microbiol. Biotechnol,60: 67-72.

Alfenore S, Molina-Jouve C, Guillouet SE, Uribelarrea JL, Goma G&Benbadis L (2007). Improving ethanol production and viability of*Saccharomyces cerevisiae* by a vitamin feeding strategy during fedbatchprocess. Appl. Microbiol. Biotechnol., 60: 67-72.

Amore TD, Celotto G, Russell I & Stewart GG (2002).Selection and optimization of yeast suitable for ethanol production at 40 °C.Enzyme andMicrobial Technology, 11(7): 411-416.

ANFAVEA - AssociaçãoNacional dos Fabricantes de VeículosAutomotores (Brasil). January 2011."Produção de AutomóveisporTipo e Combustível - 2010(Tabela 10)" (in Portuguese) (PDF).
"Anúario da IndustriaAutomobilisticaBrasileira 2010: Tabelas 2.1-2.2-2.3 Produçãoporcombustível - 1957/2009" (in Portuguese).

ApiradeeSripiromrak (2006).ISOLATION AND CHARACTERIZATION OF THERMOTOLERANT YEAST FOR ETHANOL PRODUCTION,Suranaree University of Technology.

Bansal R & Singh RS (2003).A comparative study on ethanol production from molasses using*Saccharomyces cerevisiae* and *Zymomonasmobilis*. Indian J Microbiol, 43:261-64.

Bass D, Howe A, Brown N, Barton H, Demidova M, Michelle H,Li L, *et al.*(2007). Yeast forms dominate fungal diversity in the deepoceans. Proceedings of the Royal Soceity of London, B, BiologicalSciences, 274: 3069-3077.

Bassapa SC (1989).Recent development in ethanol fermentation process. Central Food Technology Research Institute.

Bertolini MC, Ernandes JR &Laluce C (1991). New yeast strains for alcoholic fermentation at higher sugar concentration. BiotechnolLett,13: 197-202.

Bettiga M, Bengtsson O, Hahn-Hägerdal B &Gorwa-Grauslund MF (2009). Arabinose and xylose fermentation by recombinant *saccharomyces cerevisiae*expressing a fungal pentose utilization pathway.Applied and Environmental Microbiology, 8(40).

Boekhout T &Kurtzman CP (1996). Principles and methods used in yeast classification, and an overview of currently accepted yeast genera. In:Nonconventional Yeasts in Biotechnology: A Handbook (Wolf, K.). Springer-Velag, Berlin, Heidelberg. pp. 1-99.

Bon EPS & Ferrara MA (2007).Bioethanol Production via Enzymatic Hydrolysis of Cellulosic Biomas.The Role of Agricultural Biotechnologies for Production of Bioenergy in Developing Countries.12 October 2007, Rome.

Börjesson J, Peterson R &Tjerneld F (2007). Enhanced enzymatic conversion of softwood lignocellulose by poly(ethylene glycol) addition. Enzyme MicrobTechnol, 3/5;40(4):754-62.

Borzani W, Garab A, Pires MH, Piplovic R & De laHiguera GA (1993). Batch ethanol fermentation of molasses: a correlation between the time necessary to complete the fermentation and the initial concentrations of sugar and yeast cells. World J MicrobiolBiotechnol,9: 265-68.

Casey GP &Ingledew WM (1986).Ethanol tolerance in yeasts. CRC .Crit. Rev. Microbiol,13: 219-280.

Chandel AK, Chan ES, Rudravaram R, Narasu ML, Rao LV &Ravindra P (2007). Economics and Enviromental Impact of Bioethanol Production Technologies: an Appraisal. Biotechnology and Molecular Biology Review, 2(1): 014-032.

Chandra RP, Bura R, Mabee WE, Berlin A, Pan X & Saddler JN (2007). Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics. Advances in Biochemical Engineering/Biotechnology, 108:67-93.

Chatanta DK, Attri C, Gopal K, Devi M, Gupta G &Bhalla TC (2008). Bioethanol production from apple pomace left after juice extraction. Internet J.Microbiol.,Vol. 5.

Claassen PAM, Van Lier JB, Lopez Conteras AM, Van Niel EWJ, Sijtsma L &Stams AJM (1999). Utilisation of biomass for the supply of energy carriers. Applied Microbiology and Biotechnology, 52(6):741-55.

Conti SF & Naylor HB (1959).Electron microscopy of ultrathin section of *Schizosaccharomycesoctosporus*.I. cell division.Journal of Bacteriology, 78: 868-877.

Cysewaski GR &Wilke C (1977). Rapid ethanol fermentation using vaccum and and cell Cycle .J. Biotechnol. Bioeng, 19: 1125-1143.

D'Amore T & Stewart GG (1987).Ethanol tolerance of Yeast.Enz.Microbiol.Technol,**9**: 321- 330.

Demirbas A (2007).Progress and Recent Trends in Biofuels, Progress in Energy and Combustion Science, 33:1-18.

Detroy RW, Cunningham RL, Bothast RJ, Bagby M & Herman A (2004).Biuconversion of wheat straw cellulose/hemicellulose to ethanol by *saccharomyces uvarum*and *pachysolentannophilu*. Biotechnology and Bioengineering, 24(5):1-9.

Du Preez JC, Bosch MN & Prior BA (1987). Temperature profiles of growth and ethanol tolerance of the xylose fermenting yeasts *Candida shematae* and *Pichiastipitis* . Appl. Microbiol. Biotechnol, 25: 521-552

Fengel D & Wegener G (1989).*Wood, Chemistry, Ultrastructure and Reactions* (Walter de Gruyter, Berlin-New York), 66-100

Glasser WG &Leitheiser RH (1984).Engineering plastics from lignin. 11. Hydroxypropyllignins as components of fire resistant foams. Polymer Bulletin, 12(1):1-5.

Glazer AN &Nikido H (1995). Microbial diversity. In:Microbial Biotechnology: Fundamental of Applied Microbiology. New York: Freeman and company. pp. 76-87.

Goettemoeller J &Goettemoeller A (2007). Sustainable Ethanol: Biofuels, Biorefineries, Cellulosic Biomass, Flex-Fuel Vehicles, and Sustainable Farming for Energy Independence. Praire Oak Publishing, Maryville, Missouri, ISBN: 9780978629304, pp: 42.

Hánh-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G &Zacchi G (2006).Bio-ethanol – the Fuel of Tomorrow from the Residues of Today. Trends in Biotechnology, 24(12): 549-556.

Hashimoto S, Ogura M, Aritomi K, Hoshida H, Nishizawa Y &Akada R (2005).Isolation of Auxotrophic Mutants of Diploid Industrial Yeast Strains after UV Mutagenesis. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2005, Vol. 71, No. 1: 312–319

Helena da Cruz S,Batistote M &Ernandes JR (2003).Effect of sugar catabolite repression in correlation with the structural complexity of nitrogen source on yeast growth and fermentation.Journal of Industrial and Brewing,109(4): 349-355.

Higuchi T (1990). Lignin Biochemistry: Biosynthesis and Biodegradatio, *Wood SciTechnol,* 24: 23-63.

Himmel ME, et al., (2007). Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Science, 315: 804-807.

Houtman CJ &Atalla RH (1995).Cellulose-lignin interactions (A computational study). Plant Physiology, 107(3):977-84.

Howard RL, Abotsi E, Van Rensburg ELJ & Howard S (2003). Lignocellulose biotechnology: Issues of bioconversion and enzyme production. *Afr J Biotechnol,* 2: 602-619

Ibeto CN, Ofoefule AU &Agbo KE (2011). A Global Overview of Biomass Potentials for Bioethanol Production: A Renewable Alternative Fuel. Trends in Applied SciencesResearch, 6:410-425.

Isaias M, Leal V, Ramos LM & Da-Silva JA (2004).Assessment of greenhouse gas emissions in the production and use of fuel ethanol in Brazil.Secretariat of the Environment, Government of the State of Sao Paulo.

Jacobson GK & Jolly SO (1989).Yeasts, molds and algae.Biotechnology, 7: 279-314.

JonesRP, PammentN & GreenfieldPF (1981).Alcoholic fermentation by yeasts- the effect of environmental and other variables.Proc.Biochem, 16: 42-49.

Jong-Gubbels P, Van Dijken JP &Pronk JT (1996). Metabolic fluxes in chernostat cultures of *schizosaccharomycespombe*grown on mixtures of glucose and ethanol. Microbiology, 142:1399-407.

Karakashev D, Thomsen AB &Angelidaki I (2007). Anaerobic biotechnological approaches for production of liquid energy carriers from biomass. Biotechnology Letters, 29(7):1005-12.

Khan AR, Malek MA, Choudhury N & Khan SI (1989).Alcohol production from molasses and liquid sugar using some indigenous yeast isolates. Bangladesh journal of Microbiology,6(1):37-42.

Kosaric N & Vardar-Sukan F (2001). Potential source of energy and chemical products .In: The Biotechnology of Ethanol: Classica land Future Application.(Roehr,M.ed.),Wileyvch, Weinheim, pp. 45-49.

Kosikova B, Labaj J, Slamenova D, Slavikova E &Gregorova A (2006). Novel environmentally friendly use of lignin biomass component. In: Brenes MD, editor. Biomass and Bioenergy.Nova Science Publisher Inc. p. 169-200.

Kreger-Van Rij NJW (1984).The Yeast a Taxonomic Study. New York: Elsevier Science Publishing Company. 1082 pp.

Kurtzman CP & Fell JW (2006). Yeast systematics and phylogeny: implications of molecular identification methods for studies in ecology. In C. Rosa, & G. Péter (Eds.), Biodiversity and Ecophysiology of Yeasts: The Yeast Handbook (pp. 11-30). New York: Springer.

Kurtzman CP & Fell JW (1997).The Yeasts:A Taxonomic Study. Fourth Edition. Amsterdam: Elsevier Science Publishing Company. 1055 pp.

Lachke A (2002). Biofuel from D-xylose – the second most abundant sugar. Resource, 5:50-8.

Laluce C, Abud CJ, Greenhalf W &Sanches Peres MF (1993).Thermotolerancebehaviour in sugarcane syrup fermentations of wild type yeast strains selected under pressures of temperature, high sugar and added ethanol. BiotechnolLett,15: 609-14.

Laplace JM, Delgenes JP,Molleta R& Navarro JM (1992). Fermentation of lignocellulosic sugars to ethanol -selection of mutants of Pichiastipitis affected for D-glucose utilization. Enzyme Microb.Tcchnol, 14: 644-648.

Laplace JM, Delgenes JP, Molleta R & Navarro JM (1993). Ethanolproduction from glucose and xylose by separated and co-culture processes using high cell density systems. Process Biochem, 28: 519-525.

Licht FO (2006). World Ethanol Market: The Outlook to 2015, Tunbridge Wells, Agra Europe Special Report, UK.

Licht FO (2010). Renewable Fuels Association, Ethanol Industry Overlook 2010, pp. 2 and 22. 2010.

Lin Y & Tanaka S (2006). Ethanol fermentation from biomass resources: current state and prospects, Appl. Microbiol. Biotechnol, 69:627–642.

Liu H, Zhu J & Fu SY (2010).Effects of lignin-metal complexation on enzymatic hydrolysis of cellulose. Journal of agricultural and food chemistry, 58(12):7233-8.

Lodder J (1971). *The yeasts: A Taxonomic study.* NorthHoll and Publishing, Amsterdam

Lorenz MC, Cutler NS &Heitman J (2000).Characterization of Alcohol-induced Filamentous Growth in *Saccharomyces cerevisiae*. Mol. Biol. Cell, 11(1): 183-199.

Lynd LR, Weimer PJ, Van Zyl WH & Pretorius IS (2002). Microbial cellulose utilization: Fundamentals and biotechnology, *Microbial MolBiol R,* 66: 506-577

Mansfield SD, Mooney C & Saddler JN (1999).Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnology Progress, 15(5):804-16.

Matsushika A, Watanabe S, Kodaki T, Makino K &Sawayama S (2008). Bioethanol production from xylose by recombinant *saccharomyces cerevisiae*expressing xylose reductase, NADP+-dependent xylitol dehydrogenase, and xylulokinase. Journal of Bioscience and Bioengineering, 3;105(3):296-9.

Mesa JJ,Infante JJ,Rebordinos L &Cantoral JM (1999). Characterization of yeasts involved in the biological ageing of sherry wines. Lebensm.Wiss.Technol., 32: 114-120.

Mielenz JR (2001). Ethanol production from biomass: Technology and commercialization status. CurrOpinMicrobiol, 6/1;4(3):324-9.

Miller GL (1959). Use of dinitrosalicyclic acid reagent for determination of reducing sugar.Analytical chemistry,31: 426-28.

Miller JJ (1989). Sporulation in *Saccharomyces cerevisiae*. In:(Rose, A.H. and Harrison, J.S.)The Yeast (Vol. 3). London: Academic Press. pp. 491-550.

Ministério da Agricultura, Pecuária e Abastecimento."Portaria Nº 143, de 27 de Junho de 2007" (in Portuguese).

Morais PB, Rosa CA, Linardi VR, Carazza F &Nonato EA (1996). Production of fuel alcohol by *saccharomyces* strains from tropical habitats. Biotechnology Letters, 18(11):1351-6.

Moreira AR (1983). Biotechnol. Ser. 4: 485-400.

Morikawa Y, Takasawa S, Masunaga I &Takayama K (2004).Ethanol productions from D-xylose and cellobiose by *kluyveromycescellobiovorus*. Biotechnology and Bioengineering, 27(4):1-5.

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

Munnecke DM (1981). Basic principles of ethanol fermentation.In: Biomass Conversion Process of Energy and Fuels. (Sofer, S. Sand Zaborsky,O.R. eds). Plenum press,New York, Pp.567-568.

Narendranath NV & Power R (2005). Relationship between pH and medium dissolved solids in terms of growth and metabolism of *Lactobacilli* and *Saccharomyces cerevisiae* during ethanol production. Appl. Env. Microbiol, 71(5): 2239-2243.

Navarro JM (1980). Alcoholic fermentation: influence of the culture conditions on inhibition by ethanol. Cellular and Molecular Biology, 26(2): 241-246.

Navarro JM & Durand G (1978). Alcohol fermentation: effect of temperature on ethanol accumulation within yeast cells. Ann. Microbiol, 129(2): 215-224.

Nicholas NN, Dien BS, Wu Y & Cotta MA (2005).Ethanol fermentation of starch from field peas. J. Cereal Chem, 82: 554-558.

Norr AA, Hameed A, Bhatti KP &Tunio SA (2003). Bio-ethanol fermentation by the bioconversion of sugar from dates by *Saccharomyces cerevisiae*strain ASN-3 and HA-4. Biotechnol,2(1):8-17.

Novick P & Botstein D (1985). Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell*, 40: 405-416.

Ok T &Hashinaga F (1997). Identification of sugar tolerant yeasts isolated from high sugar fermented vegetables extracts. J Gen ApplMicrobiol, 43: 39-47.

oregon.gov, biomass forum

Osho A (2005). Ethanol and sugar tolerance of wine yeasts isolated from fermenting cashew apple juice. African Journal of Biotechnology, 4(7): 660-662.

Palmarola-Adrado B, Choteborská P, Galb M &Zacchi G (2005).Ethanol production from non-starch carbohydrates of wheat bran.Bioresource Technology, 96: 843-850.

Paturau MJ (1989).: By products of the cane sugar industry. An introduction to their industrial Utilization (137-190).Third completely revised edition.Elsevier publishing company.

Popa, B (29 Jan 2009). "Emissions: Gasoline vs. Diesel vs. Bioethanol". autoevolution.com

Priest FG & Campbell I (1996). Brewing Microbiology. Corn Wall: Chapman & Hall. 306 pp.

Ragauskas AJ, et al., (2006). The path forward for biofuels and biomaterials. Science, 311: 484-489.

Rao MJP (1997). Industrial Utilization of Sugar Cane and Its Co-Products.IAPCK publishers and distributors. New Delhi, India.

Recek M, Cadez N &Raspor P (2002). Identification and characterization of yeast isolates from pharmaceutical waste water. Food Technology and Biotechnology, 40(1): 79-84.

Rehm HG & Reed G (1995).Enzames, Biomass, Food and Feed,2nd ed. Biotechn. Vol. 9.Weinheim:VCHVerlagsgesellschaftmbh

Singha LK, Chaudharya G, Majumderb CB &Ghosha S (2011).Utilization of hemicellulosic fraction of lignocellulosic biomaterial for bioethanol production. Advances in Applied Science Research, 2(5):508-21.

Slapack GE, Russell I, & Stewart GG (1987).Thermophilic Microbes in Ethanol Production. Boca Raton: CRC Press. 186 pp.

Solomon BD, et al., (2007). Grain and cellulosic ethanol: History, economics, and energy policy. Biomass & Bioenergy, 31: 416-425.

Sree NK, Sridhar M, Suresh K, Bharat IM &Rao LV (2000). High alcohol production by repeated batch fermentation using immobilized osmotolerant*S.cerevisiae.* J IndustMicrobiolBiotechnol,24: 222-26.

Sun Y & Cheng J (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. BioresourTechnol, 5;83(1):1-11.

Swain T (1979). Tannins and lignins. In: Rosenthal GA, Janzen DH, editors. Herbivores: Their interaction with secondary plant metabolites. New York: Academic Press. p. 657–682.

Taherzadeh MJ &Karimi K (2007). Process for ethanol from lignocellulosic materials I: Acid-based hydrolysis processes, BioResources, 2(3): 472-499.

Teramoto Y, Sato R & Ueda S (2005). Characteristics of fermentation yeast isolated from traditional Ethiopian honey wine, ogol. Afr .J. Biotechnol. 4 (2): 160-163.

Thomas KC, Hynes SH &Ingledew WM (2002). Influence of medium buffering capacity on inhibition of *Saccharomyces cerevisiae*growth by acetic and lactic acids. Applied and Environmental Microbiology, 68: 1616-1623.

Thygesen A, Thomsen AB, Schmidt AS, Jørgensen H, Ahring BK & Olsson L (2003). Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. Enzyme MicrobTechnol, 4/8;32(5):606-15.

Timell TE (1967).Wood Sci. Technol, 1: 45-70.

Tortora GJ, Funke BR & Case CL (2010). Microbiology: An Introduction, Tenth Edition. Pearson Benjamin Cummings, Pearson Education, Inc. San Francisco, Pp. 330.

Uma V &Polasa H (1990).*S.cerevisiae* of palm wine enhanced ethanol production by using mutagens. J IndustMicrobiolBiotechnol, 5: 1-4.

UNICA.Abraciclo (2010-01-27). "Motos flex foram as maisvendidasem 2009 nacategoria 150cc" (in Portuguese).

Van Uden N (1989). Alcohol Toxicity in Yeasts and Bacteria. Boca, FL: CRC Press, Inc.

vanWyk JPH (2001). Biotechnology and the utilization of biowaste as a resource for bioproduct development. Trends in Biotechnology, 19: 172-177.

Vasconcelos DNJ, Lopes EC and De Francaet PF (2004). Continuous Ethanol production Using Yeast Immobilized on Sugar –Cane stalks. Brazilian Journal of chemical engineering. V21, No 03, pp357-365.

Verbelen PJ, De Schutter DP, Delvaux F, Verstrepen KJ &Delvaux FR (2006).Immobilized yeast cell systems for continuous fermentation applications, Biotechnol. Lett, 28:1515-1525.

Visser W, Scheffers WA, Batenburg-Van Der Vegte WH & Van Dijken JP (1990).Oxygen requirements of yeasts. Applied and Environmental Microbiology, 56(12):3785-92.

Walker GM & van Dijck P(2006). Physiological and molecular responses of yeasts to theenvironment. In: Querol, A., Fleet, G.H. (Eds.), Yeasts in Food and Beverages. TheYeast Handbook. Springer-Verlag, Berlin Heidelberg, pp. 111–152.

Wang M. "Energy and Greenhouse Gas Emissions Effects of Fuel Ethanol" (PDF).

Wang M, Saricks C &Santini D. "Effects of Fuel Ethanol Use on Fuel-Cycle Energy and Greenhouse Gas Emissions" (PDF). Argonne National Laboratory.

Wang M, Wu M &Huo H (2007). Life-cycle energy and greenhouse gas emission impacts of different corn ethanol plant types. Environmental Research Letter, 2:1-13.

Wang ZX, Zhuge J, Fang H & Prior BA (2001). Glycerol production by microbial fermentation: A review. Biotechnology Advances, 19: 201-223.

Warren P &Shadomy L (1991). Yeast fermentation broth base with carbohydrate and Durham tube. In**:** Manual of Clinical Microbiology.5th ed. Washington D.C

Wayman M & Parekh SR (1990).Microbiology of fermentation catalysts. In:Biotechnology of Biomass Conversion. Milton Keynes: Open university press. pp. 75-100.

Wiselogel A, Tyson S & Johnson D (1996).Biomass feedstock resources and composition. In: Wyman CE, editor. Handbook on Bioethanol: Production and Utilization. Washington DC: Taylor & Francis. p. 105-118.

Worldwatch Institute and Center for American Progress (2006). American energy: The renewable path to energy security.

Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY, et al. (2009). Comparative sugar recovery and fermentation data following pretreatment ofpoplar wood by leading technologies.Biotechnology Progress,25(2):333-9.

Xu Q, Song Q, Ai X, McDonald TJ, Long H & Ding S (2009). Engineered carbohydrate-binding module (CBM) protein-suspended single-walled carbon nanotubes in water. Chemical Communications (Cambridge, United Kingdom), 3:337-339.

Yadav A, Dilbaghi N & Sharma S (1997).Pretreatment of sugarcane molasses for ethanol production by yeast.Indian J of Microbiol,37: 37-40

YansongG, Min Q, Quan Z,Zhengmao Z&Guoqiang C (2000).hyperproduction of alcohol using yeast fermentation in highly concentrated molasses medium. Bioline International 2001, Vol. 6 No3 pp (225-2300).

Yu EKC & Saddler JN (1984).Butanol and buanediol Production from Pretreated biomass.Proceedings of Biomass Convension Technology. Pp 1-10.

Zhang HY & Lynd LR (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulose systems, *BiotechnolBioeng,* 88: 797-779.

Zheng Y, Pan ZL, Zhang RH, Wang DH & Jenkins B (2008). Non-ionic surfactants and non-catalytic protein treatment on enzymatic hydrolysis of pretreated creeping wild ryegrass. Applied biochemistry and biotechnology, 146(1-3):231-48.