Study of Low Cost Biofuel Production from Vegetable Waste

A DISSERTAION

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FOR THE MS DEGREE IN BIOTECHNOLOGY.**

SUBMITTED BY

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Declaration

This is to declare that the research work embodying the results reported in this thesis entitled "**Study of low cost biofuel production from vegetable waste**" submitted by Tribeni Ghosh, has been carried out under the supervision and able guidance of Professor Dr. M. Mahboob Hossain, Microbiology Program, BRAC University in partial fulfilment of MS in Biotechnology, at BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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DEDICATED
TO
MY BELOVED FAMILY

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Abstract

The necessity of an alternate clean energy source is increasing with the elevating energy demand of modern age. Microbial production of bioethanol can be the requirement of a substitute clean energy source is accumulative with the elevating energy demand of modern age. Microbial production of bioethanol can exchange the conventional fossil fuel with green energy. In this study, local yeast isolates were used for the manufacture of bioethanol using cellulosic vegetable wastes as substrate. This project was aimed for the resourceful bioconversion of lignocellulosic biomass into ethanol by microbial action. Wild-type yeasts isolated from sugarcane juice (SC.t), date juice (DJ.t) and grape juice (GRP.t) were used as the ethanol producing organism. Very low-priced and easily available raw materials (vegetable peel, cellulosic wastes) were used as fermentation media. The overall objective of this project is to meet the demand for a low-priced and extremely efficient integrated anaerobic *Saccharomyces spp*. fermentation process to produce ethanol as an energy source directly from the insoluble lignocellulosic substrate (kitchen-waste). Fermentation was enhanced with respect to temperature, reducing sugar concentration and pH. Analysis of fermentation characteristics was performed under the different substrate and environmental conditions; it was observed that temperature of 30°C and pH 6.0 were optimum for fermentation with maximum yield of ethanol. The maximum ethanol production by yeast was 11.49% using vegetable peels (previously treated with cellulolytic bacteria) as substrate at 48 hours under shaking condition may replace the conventional fossil fuel with green energy.

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pH- Negative logarithm of hydrogen ion concentration

^{0}C- Degree Centigrade

Temp- Temperature

Conc. - Concentration

DNS- 3, 5Dinitro salicylic acid

g or gm- Gram

hrs- Hours

fig. Figure

O.D- Optical density

µl- Micro liter

et.al.- And other people

Kg- Kilogram

L- Liter

mg- Milligram

ml- Milliliter

µm- Micrometer

Ppm- Parts per million

No. /no.-Number

V/v- Volume Per volume

W/v- Weight per volume

% - Percentage

/ - Per

α- Alpha

µ mol- Micro mole

ATP - Adenosine triphosphate

NADH - Nicotinamide adenine dinucleotide

Psi - Pound per square inch

Rpm - Round per minute

YPD - Yeast extract peptone dextrose

YMM- Yeast maintenance media

$CuSO_4$- Copper sulphate

$K_2Cr_2O_7$- Potassium dichromate

$MgCl_2$- Magnesium chloride

$CaCl_2$- Calcium chloride

CHAPTER 1.

INTRODUCTION

1.1 Introduction

The indispensable necessity of environmentally friendly energy source is increasing with time. After the utilization, conventional fossil fuels from nonrenewable sources cannot be used further and their exhaustion causes severe damage to the nature. Fossil fuels, as the name suggests, are very old. North Sea oil deposits are around 150 million years old, whilst much of Britain's coal began to form over 300 million years ago. Although humans probably used fossil fuels in ancient times, as far back as the Iron Age, it was the Industrial Revolution that led to their wide-scale extraction. (Grant, 2004).

And in the very short period of time since then – just over 200 years – the world consumed an incredible amount of them, leaving fossil fuels all but gone and the climate seriously impacted.

Fossil fuels are an incredibly dense form of energy, and they took millions of years to become so. And when they're gone, they're gone pretty much forever.

Clearly fossil fuel reserves are finite - it's only a matter of when they run out - not if. Globally - every year we currently consume the equivalent of over 11 billion tons of oil in fossil fuels. Crude oil reserves are vanishing at the rate of 4 billion tons a year – if we carry on at this rate without any increase for our growing population or aspirations, our known oil deposits will be gone by 2052.

We'll still have gas left, and coal too. But if we increase gas production to fill the energy gap left by oil, then those reserves will only give us an additional eight years, taking us to 2060. But the rate at which the world consumes fossil fuels is not standing still, it is increasing as the world's population increases and as living standards rise in parts of the world that until recently had consumed very little energy. Fossil Fuels will therefore run out earlier (Ecotricity.co.uk, 2016).

It's often claimed that we have enough coal to last hundreds of years. But if we step up production to fill the gap left through depleting our oil and gas reserves, the coal deposits we

know about will only give us enough energy to take us as far as 2088. This energy production from burning coal will emit more carbon dioxide.

Renewable energy offer us another way, a way to avoid this (fossil fuelled) energy time bomb. As the Saudi Oil Minister said in the 1970s, “The Stone Age didn’t end for lack of stone, and the oil age will end long before the world runs out of oil” (Ecotricity.co.uk, 2016).

One most important source of renewable energy is biofuel. Biofuels are produced from living organisms or from metabolic by-products (organic or food waste products). In order to be considered a biofuel the fuel must contain over 80 percent renewable materials. It is originally derived from the photosynthesis process and can therefore often be referred to as a solar energy source. There are many pros and cons to using biofuels as an energy source (AENews, 2016).

Currently biofuel is produced from plants as well as microbes. The oils, carbohydrates or fats generated by the microbes or plants are refined to produce biofuel. This is a green and renewable energy that helps in conserving fossil-fuel usage. But a new research has led to a new discovery of getting the microbes to produce fuel from the proteins instead of utilizing the protein for its own growth.

1.2 Ethanol as a biofuel

Kim and Dale, 2003 conducted a study to give some perspective on the size of the bioethanol feedstock resource, globally and by region, and to summarize relevant data that they believed that others will find useful, for example, those who are interested in producing biobased products such as lactic acid, rather than ethanol, from crops and wastes. The Authors has given a wide calculation of amount of Bioethanol that can be produced and how much of it will be replaced in the current energy use system. They have calculated about $73.9\ \mathrm{Tg}$ of dry wasted crops in the world that could potentially produce $49.1\ \mathrm{GL\ year^{-1}}$ of bioethanol. About $1.5\ \mathrm{Pg\ year^{-1}}$ of dry lignocellulosic biomass from these seven crops is also available for conversion to bioethanol. Lignocellulosic biomass could produce up to $442\ \mathrm{GL\ year^{-1}}$ of bioethanol. Thus, the total potential bioethanol production from crop residues and wasted crops is $491\ \mathrm{GL\ year^{-1}}$, about 16 times higher than the current world ethanol production. The potential bioethanol production could replace $353\ \mathrm{GL}$ of gasoline (32% of the global gasoline

consumption) when bioethanol is used in E85 fuel for a midsize passenger vehicle. Furthermore, lignin-rich fermentation residue, which is the co product of bioethanol made from crop residues and sugar cane bagasse, can potentially generate both $458\ \mathrm{TWh}$ of electricity (about 3.6% of world electricity production) and $2.6\ \mathrm{EJ}$ of steam.

They have identified Asia as the largest potential producer of bioethanol from crop residues and wasted crops, and could produce up to $291\ \mathrm{GL\ year^{-1}}$ of bioethanol. Rice straw, wheat straw, and corn stover are the most favorable bioethanol feedstocks in Asia. The next highest potential region is Europe ($69.2\ \mathrm{GL}$ of bioethanol), in which most bioethanol comes from wheat straw. Corn stover is the main feedstock in North America, from which about $38.4\ \mathrm{GL\ year^{-1}}$ of bioethanol can potentially be produced. Globally rice straw can produce $205\ \mathrm{GL}$ of bioethanol, which is the largest amount from single biomass feedstock. The next highest potential feedstock is wheat straw, which can produce $104\ \mathrm{GL}$ of bioethanol.

Increasing amounts of bioethanol are being produced from fermentation of biomass, mainly to counteract the continuing depletion of fossil resources and the consequential escalation of oil prices. Today, bioethanol is mainly utilized as a fuel or fuel additive in motor vehicles, but it could also be used as a versatile feedstock in the chemical industry. Currently the production of carbon-containing commodity chemicals is dependent on fossil resources, and more than 95% of these chemicals are produced from non-renewable carbon resources (Rass-Hansen et al., 2007).

1.3. Objectives

The main objective of this study is to produce inexpensive and highly efficient ethanol as an energy source directly from lignocellulosic substrate (vegetable waste) through integration of anaerobic *Saccharomyces* sp. by fermentation process. Stepwise objectives are:

- Isolation and Characterization of wild yeast isolates capable of ethanol production.
- Study of Thermo-tolerance, pH-tolerance, osmo-tolerance and ethanol-tolerance of the yeast isolates.
- Isolation of starch hydrolyzing bacteria from soil.
- Study fermentation kinetics of ethanol production at laboratory level.
- To economically produce ethanol by using available and cheap raw materials.

1.4 Hypothesis

Potential microorganism for industrial ethanol production is wild type isolates of yeast. After proper isolation, identification and characterization of stress tolerance (thermo-, ethanol-, pH-, osmo- & sugar tolerance) and then detailed characterization and optimization of physiochemical parameters for ethanol production the strain can be dubbed as an industrial strain.

Kitchen-waste can be served as cheap and available medium or source of Ethanol.

1.5 Scope and limitation of the study

Selected wild-type yeast isolates were screened for thermo-tolerance, pH-tolerance and ethanol tolerance. The yeast isolates was characterized using morphological, physiological and biochemical characteristics. The starch hydrolyzing bacteria were also isolated from bacteria for increasing ethanol production rate, but they have to constantly reisolated to preserve their starch hydrolyzing ability. The optimization of some ethanol production conditions was also investigated.

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

1.6 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 ethanol industry.

CHAPTER 2.

REVIEW OF LITERATURE

2.1. Overview

As a clean liquid fuel product of bioethanol fermentation is considered as an alternative to non-renewable energy. Processes those are involved in the conversion of biomass into fuel ethanol are getting attention in recent years. Ethanol fermentation technology has accomplished critical progression with its developing interest. Upgraded use of biomass resources and microbial activity on fermentation is the center of this ethanol creation technology. One promising strategy is the fermentation of lignocellulosic biomass where hydrolysis activity by particular microbial cellulase enzymes is included. Sugar-containing materials can be served as crude materials for fermentation prepare for ethanol subsidiaries generation. Significant raw materials those can be utilized as a part of ethanol generation must be changed over into basic sugars to be aged by the enzyme of particular microorganism, for example, yeast (Lin and Tanaka, 2006).

2.2. Bioethanol

Bioethanol (also known as ethyl or green alcohol) is a clear, colourless liquid that can be produced by the fermentation of virtually any source of sugar or starch, the most common sources being sugar cane, corn, wheat and sugar beet. Cellulosic biomass (e.g. grasses, woody crops, and organic wastes) can also be used to produce bioethanol through advanced processing techniques.

Currently, the largest producers in the global biofuel are the United States and Brazil, where millions of tons of sugar are processed (Mobile Emissions Today, 2006). Although at the moment bioethanol is mainly used in blends with gasoline as E10 and E20 (10 and 20% of ethanol mixed with 90 and 80% gasoline respectively), the demand has soared. For instance, consumption of bioethanol in most countries of the European Union is far greater than the quantity produced in those countries (Wikipedia, 2009a). According to a study by Hart's Global Biofuels Center (a division of Hart Energy Publishing LP, one of the world's largest energy industry publishers), the Global biofuel use may double by 2015 (Johnson, 2009).

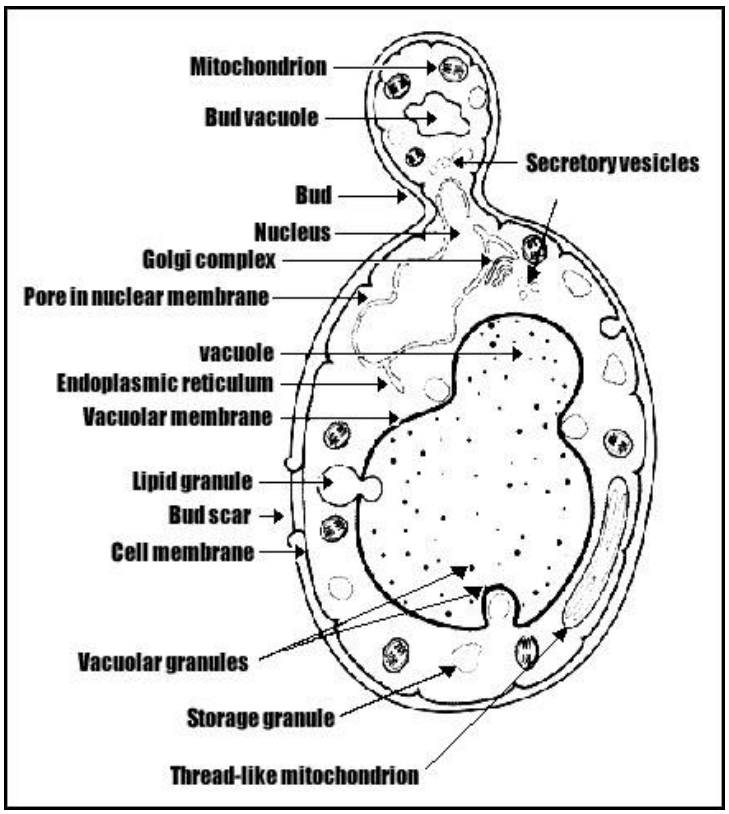
2.3. Microorganisms For Bioethanol Production

- Yeast
- Starch Hydrolyzing Bacteria

2.3.1. Yeast:

Bioethanol production from fermentable raw materials requires living organism that converts the sugars present in the substrates into ethanol. It is accounted for that microbial activity brings about a high return with a high rate and yeast is the most widely recognized organism for such activity.

Yeasts are unicellular fungi that can be classified into two phylogenetic groups i.e. teleomorphic and anamorphic ascomycetous or teleomorphic and anamorphic basidiomycetous yeasts that reproduce by budding or fission and that form their sexual states (i.e. asci), which are not enclosed in a fruiting body (Boekhout & Kurtzman, 1996; Kurtzman & Fell, 1998; Querol & Belloch, 2003). During sexual reproduction ascomycetous yeasts (i.e. yeasts of interest in this study) form asci, which contain varying numbers of ascosporeshapes. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or, as with certain yeasts, fuse with other ascospores (Van der Walt, 2000).



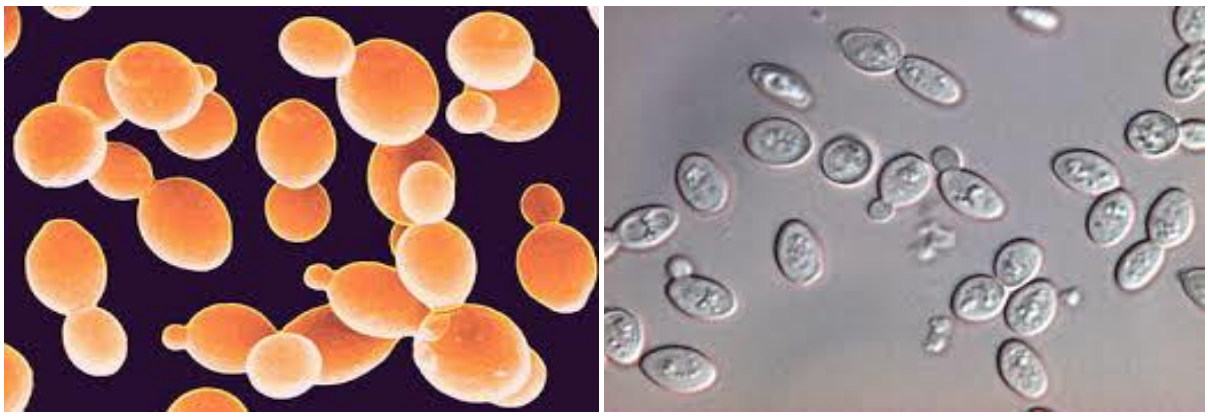


Fig 2.1: Yeast cell morphology

2.3.1.1 Yeast and fermentation

Throughout the history, ethanol production has been performing by "wild" yeast strains i.e. the yeast population naturally resident on the fermentation feedstock or other ingredients. By the mid-1900's, yeast strains were routinely chosen from "good" fermentations and utilized again and again. This practice, in spite of the rough yeast spread procedures being utilized, prompted to enhanced consistency and nature of definite items. At the point when business yeast makers

(driven principally by the always showing signs of change needs of the bread preparing industry) rose, they created enhanced yeast proliferation, quality control, and drying strategies that further enhanced consistency and nature of ethanol production. Around the world, almost all ethanol production is proficient utilizing a solitary sort and types of yeast, in particular, *Saccharomyces cerevisia*e Many several yeast species, have been distinguished in nature A boundless number of strains are conceivable; actually a huge number of yeast strains have been chosen for particular purposes. Many strength strains have been popularized for ethanol or CO_2 production, including particular strains for preparing, wine, brew, refined refreshments, and fuel ethanol. More than 90% of the glucose from starch is changed over to ethanol and carbon dioxide by the yeast cell in fermentation mode.

Table2. 1: Ethanol production by different *S. cerevisiae* strains

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

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

2.3.1.2 Yeast fermentation conditions

The yeast quantity also has an effect on performance. A higher quantity will result in a faster start of fermentation. It also helps to control contamination. Quantity must be optimized for cost effective performance. Nutrition is another critical parameter to think about. For optimal fermentation, yeast requires building block substances (C, N, P, S, O) in optimal ratios, minerals (e.g., K, Na, Mg, Ca, Zn, Fe, Mn, Cu, Co) and vitamins (B1, B5, B6, Biotin, etc.). Oxygen is typically present at low levels in business scale ethanol fermentations. Practically speaking, the process can't be totally anaerobic on the grounds that oxygen is required for the production of unsaturated fatty acids that are vital for yeast development and ethanol production. Most

substrates for business ethanol production have been observed by to be nitrogen constrained. It is in this way prescribed to add to grain-based ethanol fermentations yeast nutrients containing a nitrogen source usable by yeast. High ethanol concentration additionally stresses yeast. Avoidable yeast stress factors, for example, high temperatures, high osmotic weight, high sodium (and different ions) concentration, and high concentrations of organic acids.

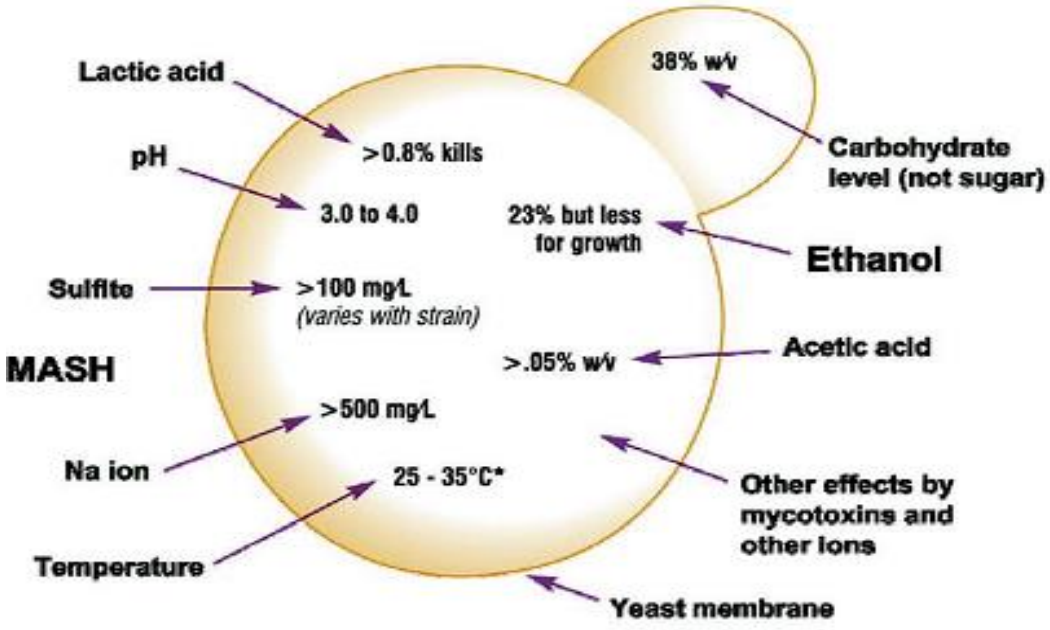


Fig 2.2: Stress factors that affect yeast metabolism

2.3.1.3 Yeast isolates selections

Some widely used, high alcohol productivity strains are *Saccharomyces cerevisiae*, S.uvarum (formerly *S.carlsbergensis*), and *candidautilis*e. *Saccharomyces anamensis* and *Schizosaccharomyces pombe* are also used in some instances. *Kluyveromyces* species, which ferment lactose, are good producers of ethanol from whey. Ethanol production by yeast is characterized by high selectivity, low accumulation of byproducts, high ethanol yield, high fermentation rate, good tolerance toward both increased ethanol and substrate concentrations, and lower pH value. Viability and genetic stability of yeast cells under process conditions and at high temperature are also desirable. Finding a strain that has all these characteristics is difficult. The most important ones used for making ethanol are members of the *Saccharomyces* genus, bred to give uniform, rapid fermentation and high ethanol yields, and be tolerant to wide ranges of, temperature, pH levels, and high ethanol

concentrations. Yeasts are facultative organisms which mean that they can live with or without oxygen. In a normal fermentation cycle, they use oxygen at the start, and then continue to thrive once it has all been used up. It is only during the anaerobic (without oxygen) period that they produce ethanol (Basappa, 1989).

Table 2.2: Commonly used yeast species in food, beverage and chemical industries (Jacobson & jolly, 1989)

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, K 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, P.tannophilus, Pichia stipis*

2. 3.1.4. Sugar degradation pathways of yeasts

There are three pathways yeast (more often than not *Saccharomyces cerevisiae*) can acquire energy through the oxidation of glucose

a) Alcoholic fermentation under anaerobic conditions

Yeast (e.g. *Saccharomyces cerevisiae*) produce ethanol by metabolize fermentable sugars in absence of oxygen and also Carbon dioxide is produced. The metabolic pathway leads to the production of ethanol as a metabolic product. Yeast uptakes different simple sugars as energy source. Enzymes of the metabolic pathway are responsible for the bioconversion. The anaerobic

process is an energy producing reaction. Optimized fermentation condition is needed for a higher production rate and for that stress tolerant yeast strains are required.

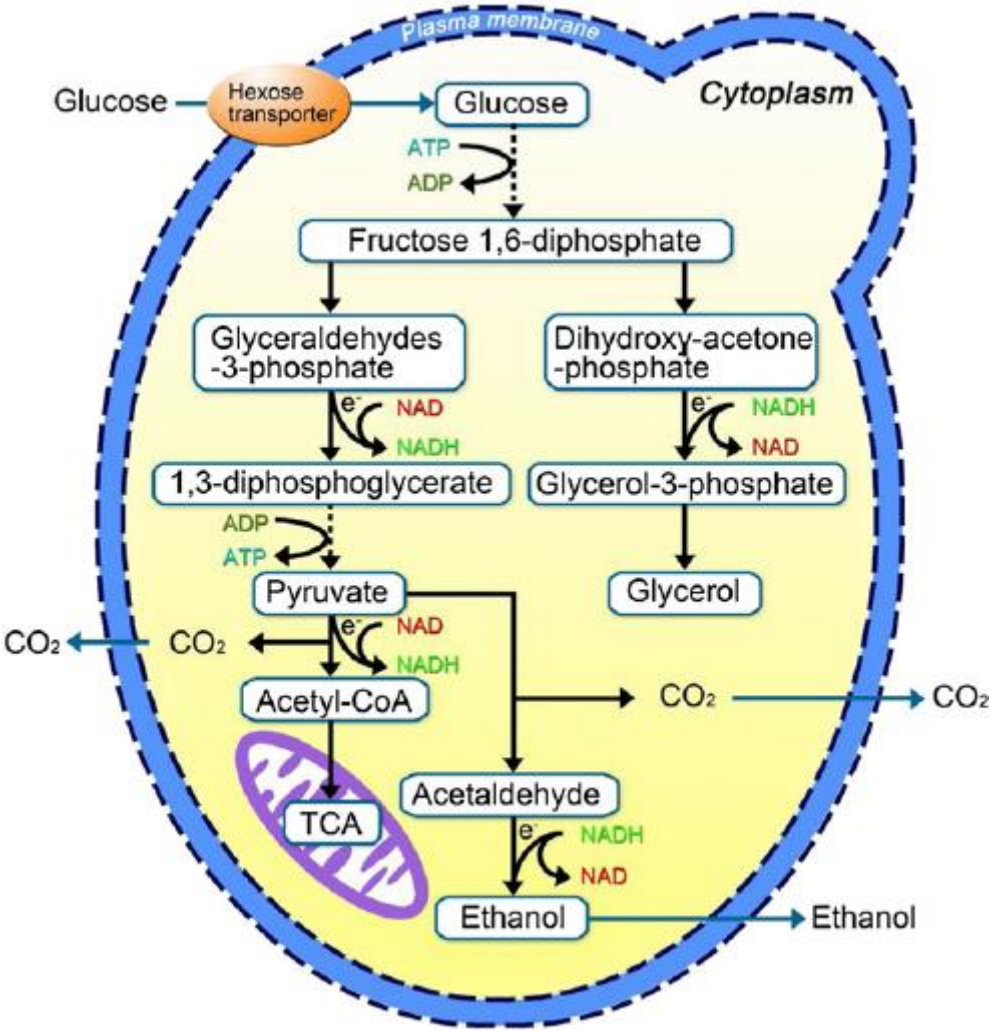


Figure 2.3: **A scheme of ethanol production pathway by yeast.**

Pyruvate can follow distinguished metabolic routes (Figure 2) depending on the environmental conditions, which in turn regulate the enzymes involved as well as their kinetics properties, but also of the yeast species [12]. Conversely, the carbon flux gets to a branching point in which may be divided among the respiratory and the fermentative pathways.

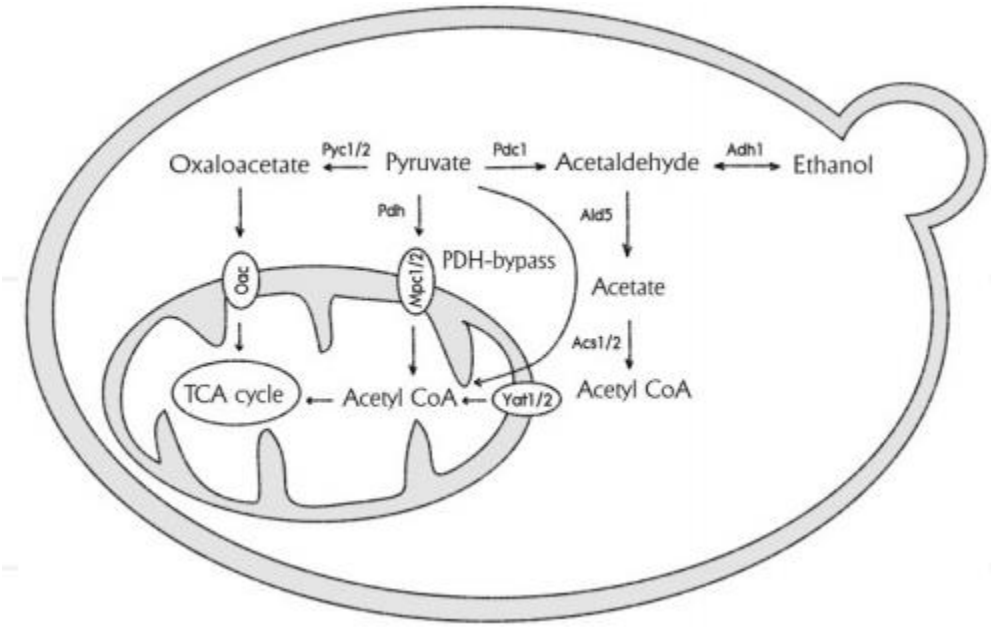


Fig 2.4: Pyruvate formed in glycolysis through alternative metabolic routes.

Pyruvate can be converted into 2 intermediates of TCA cycle: acetyl–CoA by the pyruvate dehydrogenase complex (Pdhp) and transported to the mitochondria by mitochondrial oxaloacetate carrier (Oacp); and/or oxaloacetate by pyruvate carboxylase (Pyc1p/2p) whose mitochondrial carrier is (Mpc1p/2p). Pyruvate can also be decarboxylated to give acetaldehyde by the pyruvate decarboxylase (Pdc1p). Adh1p- alcohol dehydrogenase; Ald5p - acetaldehyde dehydrogenase; Acs1p/2p - acetyl–CoA synthase; Yat1p/2p – carnitineacetyltransferase (adapted from [9]).

b) Respiration under aerobic conditions

Glycolysis of glucose yields pyruvate and two particles of ATP per molecule of glucose. Pyruvate is then oxidized to carbon dioxide and water by means of the citrus extract cycle and oxidative phosphorylation. This pathway yields a further 36-38 atoms of ATP per molecule of glucose and clearly the yeast would lean toward this course.

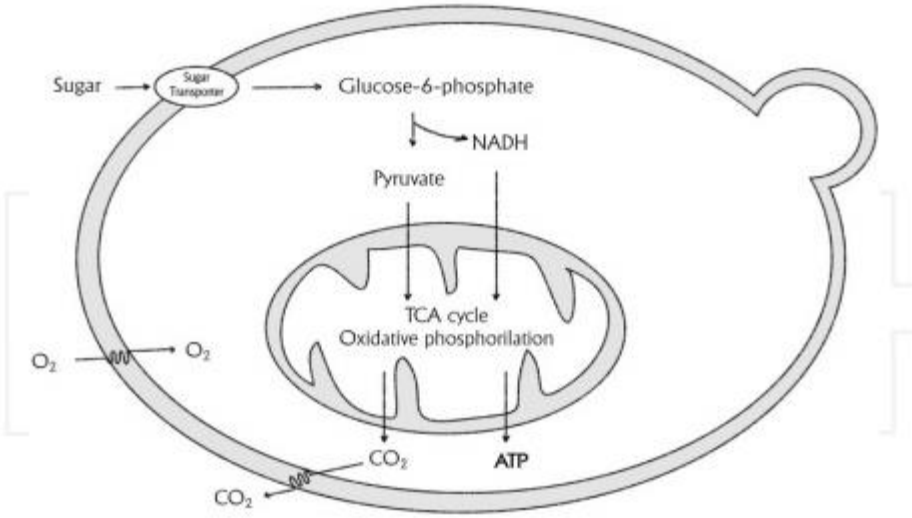


Fig 2.5: Metabolism of yeast under aerobic conditions

2.3.2. Starch hydrolyzing bacteria

Starch is a major component of agricultural crops. Enzymatic hydrolysis of polysaccharides such as starch and glycogen into oligosaccharides or simple sugar constituents is achieved by different amylolytic enzymes produced by a variety of microorganisms.

Amylases are isolated from a variety of microbial sources. α-amylase is produced by bacterial species of *Bacillus*(Muralikrishna and Nirmala, 2005; Asghar et al., 2007), *Pseudomonas*(Haq et al., 2002; Shiau and Hung, 2003) and *Clostridium*(Kılıc et al., 2005). Bacterial species such as *Bacillus subtilis* (Sumrinet al., 2011; Rajput and Li, 2012; Rajput et al., 2013), *B. licheniformis* and *Bacillus a*. are generally preferred for the production of α-amylase because they appear to be very productive (Nidhi et al., 2005; Kokab et al., 2007; Reda, 2007; Niazi et al., 2010). For the thermal stabilities of their α-amylase enzymes to be utilized in various fermentation processes, extreme thermophilic bacteria such as *Rhodothermusmarinus* and mesophilic bacteria such *as B. megaterium, B. maceransand B. Coagulans* are generally selected and utilized (Saroja et al., 2000; Gimbi and Kitabatake, 2002). Most thermostable α-amylase utilized in the industry is produced from *B. licheniformis* (Reda, 2007; Hmidet et al., 2010). Highly thermostable α-amylases are also obtained in hyperthermophilic and thermophilic Archaea such as *Pyrococcus furiosus, Thermococcus hydrothermalis, T. profundus, Sulfolobus acidocaldarius and S. solfataricus* (Goyal et al., 2005; Hernandez et al., 2006; Arikan, 2008).

Table 2.3: Amylase-producing bacteria isolated from different sources

Bacteria	Isolated from
B. thermooleovorans	Hot spring
B. pseudofirmus, B. cohnii, B. vedderi, B. agaradhaerens, N. halobia	Soil and water of soda lakes
L. plantarum, L. fermentum	Traditional fermented foods
Halobacillus sp.	Saline soil
B. subtilis	Traditional fermented food
C. taiwanensis	Hot spring
A. amylolyticus	Geothermal soil of active fumaroles
B. sphaericus	Hot spring
S. marcescens	Seas and lake
Chromohalobacter sp.	Solar evaporated saltern pond
B. licheniformis, B. subtilis	Digestive tract of fish
B. megaterium	Soil
B. agaradhaerens	Salt-enriched soil
B. licheniformis, Gracilibacillus sp.	Salt lake
P. luteola	Olive washing wastewater contaminated soil
B. amyloliquefaciens	Rhizosphere of plant
C. alkanolyticum	Intestine of freshwater fish

B. thermooleovorans: Bacillus thermooleovorans, B. pseudofirmus: Bacillus pseudofirmus, B. cohnii: Bacillus cohnii, B. vedderi: Bacillus vedderi, B. agaradhaerens: Bacillus agaradhaerens, N. halobia: Nesterenkonia halobia, L. plantarum: Lactobacillus plantarum, L. fermentum: Lactobacillus fermentum, B. subtilis: Bacillus subtilis, C. taiwanensis: Caldimonas taiwanensis, A. amylolyticus: Anoxybacillus amylolyticus, B. sphaericus: Bacillus sphaericus, S. marcescens: Serratia marcescens, B. licheniformis: Bacillus licheniformis, B. megaterium: Bacillus megaterium, P. luteola: Pseudomonas luteola, B. amyloliquefaciens: Bacillus amyloliquefaciens, C. alkanolyticum: Corynebacterium alkanolyticum.

2.3.2.1 Enzymatic action

Amylase enzymes catalyze the breakdown of starch into sugars. Starch is a mixture of amylose and amylopectin polysaccharide polymers. Amylose consists of long strands of glucose sugars linked by α-(1→4) glycosidic bonds, while amylopectin has not only α-(1→4) glycosidic bonds, but also α-(1→6) glycosidic branches (Figure 2.6). Depending on the source, starch is usually 20-25% amylase and 75- 80% amylopectin. α-Amylase acts randomly along the starch chain hydrolyzing the α-(1→4) glycosidic bonds to produce a combination of maltotriose, maltose and limit dextrins. The typical optimal pH for α-amylase is 6.7-7.0. β-Amylase, which works from the non-reducing end of the polymer, hydrolyzes the second α-(1→4) glycosidic bond to produce the two-glucose sugar maltose. γ-Amylase also works from the non reducing end of amylase to

cleave α-(1→4) glycosidic bonds to produce glucose, it will also cleave α-(1→6) linkages found in pectin.

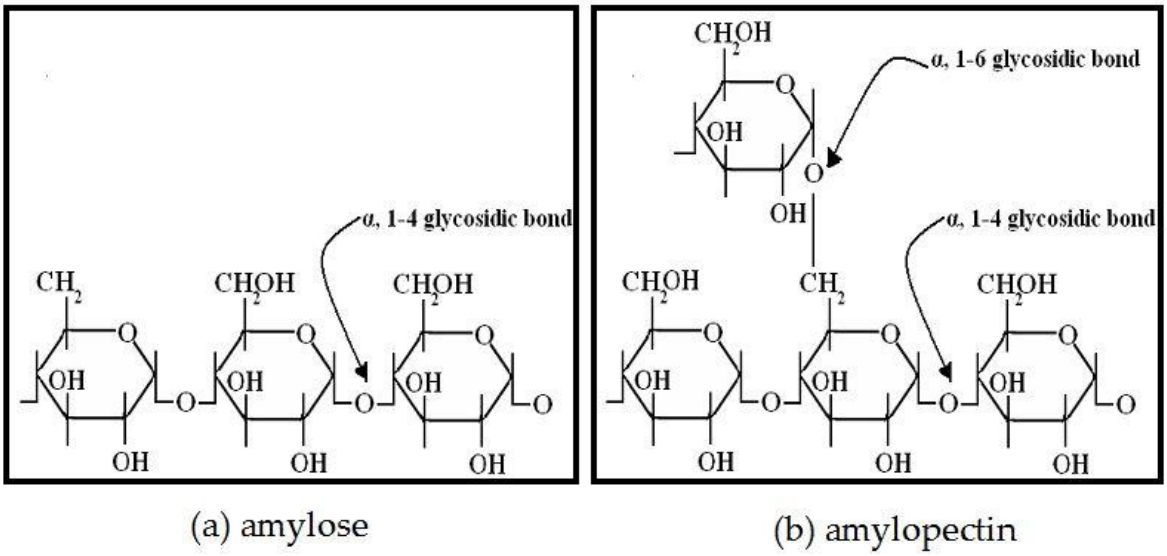


Fig 2.6: starch subunit: (a) amylose & (b) amylopectin

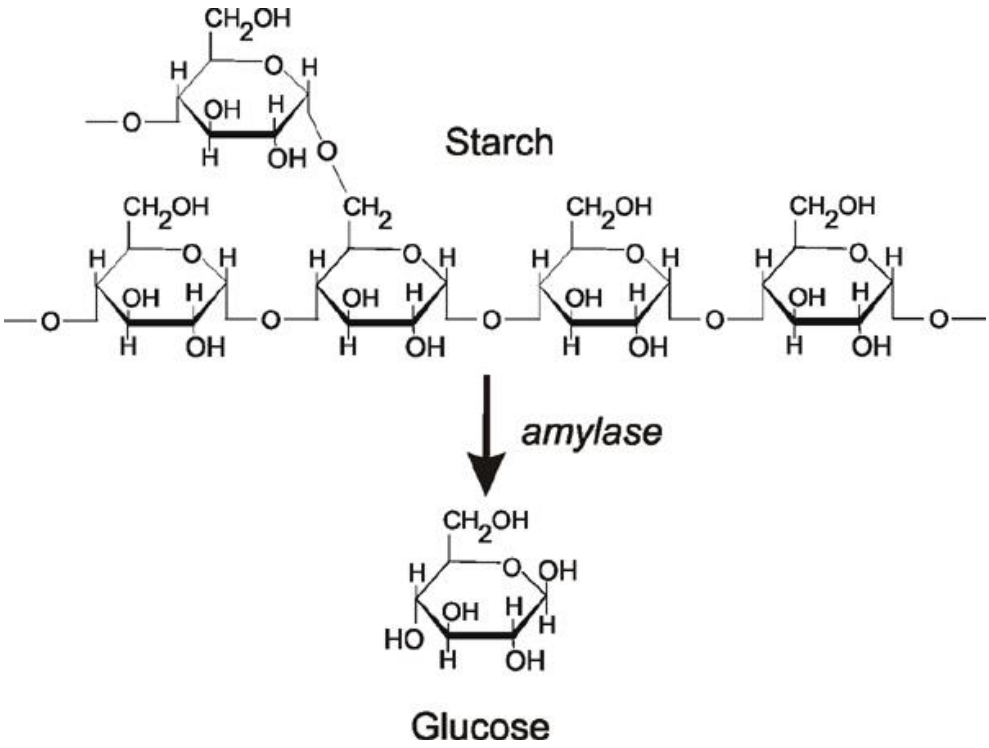


Fig 2.7: Starch hydrolysis by amylase.

2.4. Fermentation of lignocellulosic biomass

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. The microbial production of ethanol was first reported by Pasteur in 1861 (Moreira, 1983). 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). Microbial enzymes will convert sugars into bioethanol where different organic raw materials can be used as substrate. Sugarcane and corn are being used as fermentation raw materials on Brazil and USA for decades (Chatanta et al., 2008).

Various raw materials like sugarcane juice and molasses (Morimura et al., 1997 and Agrawal et al., 1998), sugar beet, beet molasses (EI Diwany et al., 1992 and Agrawal et al., 1998), sweet sorghum (Bulawayo et al 1996) and starchy materials like sweet potato (Sree et al 1999), corn cobs and hulls (Beall et al., 1992 and Arni et al., 1999), cellulosic materials like cocoa, pineapples and sugarcane waste (Othman et al., 1992) and milk/cheese/whey using lactose hydrolyzing fermenting strains (Silva et al., 1995, Ghaly and Ben Hassan 1995) have been reported. The crucial aspects of microbial selection and adaptation include: substrate selection and preparation, suitable microbial strain selection and adaptation optimization of fermentation conditions and improvement of fermentation technology. Of these, simple sugar bearing materials such as molasses of sugar cane and molasses of sugar beet are the easiest to process because they are ready for conversion with limited pre-treatments as compared with starchy or cellulosic materials (Yadav et al., 1997). Therefore, effective bioconversion of cellulosic compounds into fermentable sugars is important. Sugar containing raw materials are used for ethanol fermentation. These organic raw materials are commonly classified into three groups: sugars, starch and cellulose (Jackman, 1987).

 Sugars from molasses, fruits, sugarcane can be converted directly into ethanol. Though molasses is used mostly for ethanol fermentation that contains 50%, 50% of organic and inorganic compounds with water.

 Starches from corn, potato and root crops are needed to be hydrolyzed into fermentable sugars so that microbial enzymes can be functional efficiently for fermentation.

 Cellulose from wood, agricultural residues, vegetable peels, algae must be converted into simple sugars likewise for enzymatic action to be fermented.

Table 2.4: Different substrates for ethanol production and their comparative production potential

Crop	Ethanol production potential (L/ton)
Sugar cane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse and other cellulose biomass	280

Source: Nigam and Agarwal, 2004.

2.5. Bioconversion of different sugars

2.5.1. Glucose

Glucose is the simplest form of sugars which is readily fermentable by normal yeasts. Glucose can also be converted into fructose by rearranging the ring structure (Huang et. al., 2011)**.** Naturally, glucose is not commonly found in a free state. It is mostly polymerized as starch or cellulose. Sucrose is another sugar composed of fructose and glucose. Hydrolysis involved in the fermentation will be resulted in the alcohol production.

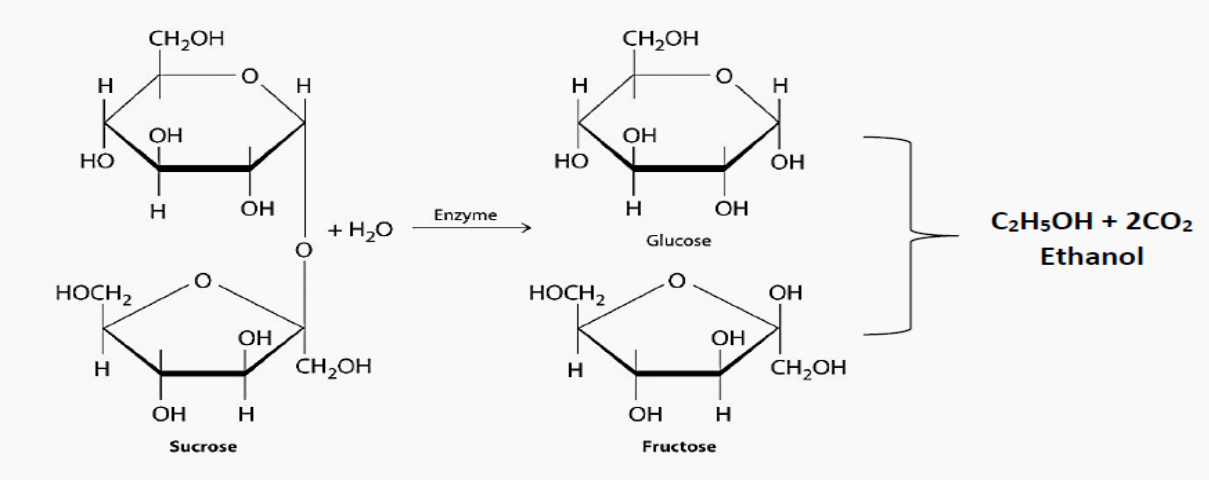


Figure 2.8: Sucrose hydrolysis and ethanol formation

2.5.2. Starch

Starch molecules are long chains of α-D-glucose monomer which is the principle food reserves of plants and can be derived from grains or tubers. Starch is a mixture of two polysaccharides, amylose and amylopectin. Amylose is a linear polymer composed of α-d-glucopyranosyl units, (1-4) linkage. The other polymer is amylopectin which contains α(1-4) linkages and α(1-6) branch points (Roy et al., 2000). Starch needs to be broken down into glucose to be fermented by yeast. The hydrolysis of starch can be processed by enzymatic action of microorganisms or pure enzymes. Treatment of different enzymes or acids may be resulted in Starch hydrolysis leading to molecular fragments varying from large molecular weights down to small oligosaccharides and D-glucose (Haissiget al., 2006). Enzymes such as diastase and maltase have important role in starch hydrolysis.

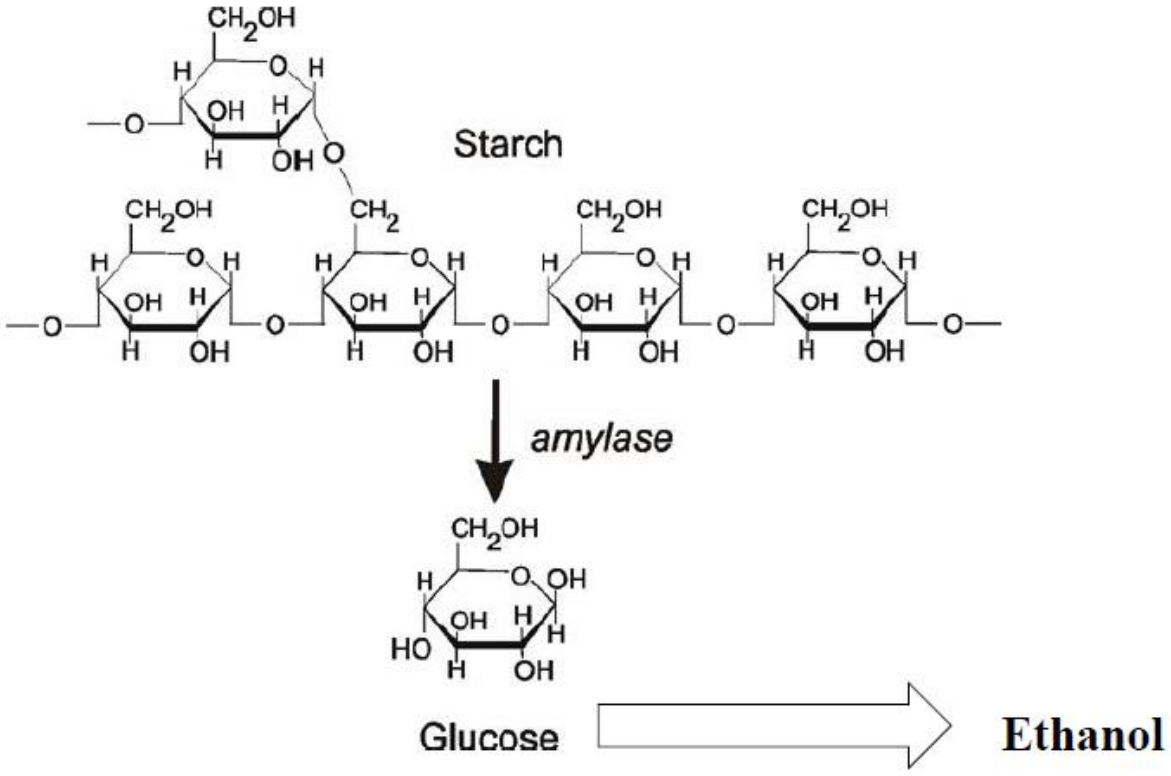


Figure 2.9: Starch hydrolysis and ethanol formation

2.5.3. Cellulose

Cellulose is the main structural sugar of lignocellulosic biomass (Xin-Qing et al., 2011). Cellulose is a homologous polymer where glucose units are linked by β-1, 4 glycodsidic bonds. To become fermentable sugar, cellulose has to be hydrolyzed into simpler form. This hydrolysis can be attained by a complex enzyme system called cellulase (exoglucanase, endoglucanase and β glucosidase etc.); lesser hemicellulase (pentose, D- Xylose, D-arabinose, D-glucose, and D-galactose etc.) and least of all lignin (Sadhu and Maiti, 2013).

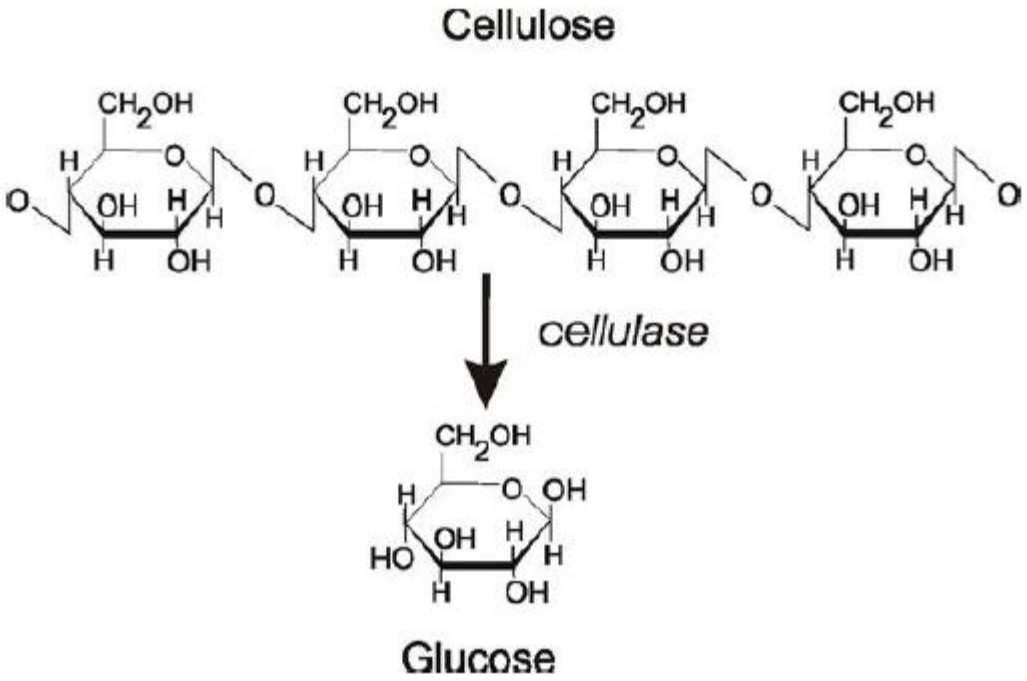


Figure 2.10: Cellulose hydrolysis

2.6. Factors affecting fermentation

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

2.6.1 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 osmotolerant *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)

2.6.2 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 up to 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 at35°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 thermotolerant flocculating yeast *Saccharomyces cerevisiae*strain KF-7 by maintaining a high concentration of KCl in the molasses medium. Among selected strains, K211 had the highest cell viability and ethanol productivity in a molasses medium containing 25% (w/v) total sugar at 35°C. As a result of repeated batch fermentation tests with K211, stable ethanol production was achieved with an ethanol concentration of 92g/l and a productivity of 3.5 g/l-h at 33°C in 22% molasses medium. Even at higher temperature of 35°C, strain K211 gave stable ethanol concentration of 91 g/l and productivity of 2.7g/l-h.

2.6.3 Effect of ethanol on yeast fermentation

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 and Rarekh, 1990).

2.6.4 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 and 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 and 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 and 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.

2.6.5 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 and 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).

2.6.6 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 osmotolerance) and the relative ability to hydrolyze and utilize lipids. These properties help investigators determine which yeast strains merit investigation for a particular application (Glazer and Nikaido, 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 bilayer. Intracellular water is recruited from the va-cuole 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.

2.6.7 Inhibition of growth and fermentation by substrate

The production of high concentration of ethanol is frequently limited by the inhibitory effect on the 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 and 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 and Ingledew, 1986; D,Amore and Stewart, 1987) and cell viability is close to 95% compared to 40% for single batch run(Casey and Ingledew, 1986).

2.6.8 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 pathways 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, a 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 and Wilke, 1977). According to Kosaric and Vardar-sukan(2001),the 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).

2.6.9 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 and Tanaka, 2006).

Perspective techniques for yeasts immobilization can be divided into four categories: attachment or adsorption to solid surfaces (wood chips, delignified 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).

2.6.10 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 and Dijck, 2006). The common metals Copper, magnesium, Potassium, Zink and Iron have effect on ethanol production by Yeast.

2.7. Uses of Ethanol

Ethanol 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 the preservation of biological specimens.

Table 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

2.8. Process optimization

Production of bioethanol from lignocellulosic biomaterials requires a fermenting organism that can convert the raw materials into simple sugars so that yeast can ferment those lignocellulose derived sugars (Hägerdal et al., 2007). Different fermentation organisms among bacteria, yeasts and fungi were observed on their performance in lignocellulosic hydrolysis. One of the major issues is the selection of fermentation strategy such as batch culture, continuous culture with cell recycling and in situ ethanol removal. Process involved hydrolase and cellulase producing bacteria should be an integrated system. Inhibitory reactions, tolerance level and yield rate must be taken under consideration (Olsson and Hägerdal, 1996). High concentration of ethanol and sugars in fermentation broth can inhibit the growth of yeast cells and decrease the production

rate. Inhibitory effect of ethanol on yeast was reported I batch and continuous cultures, where growth was limited by sugar and ethanol (Ghose and Tyagi, 2004). Different parameters for optimization of the fermentation are essential for the high production rate.

CHAPTER 3.

Materials and Methods

This research work was carried out at the Microbiology and Biotechnology Laboratory of the, Department of Mathematics and Natural Sciences, BRAC University.

3.1 Materials

3.1.1 Equipment

1. Laminar airflow cabinet
2. Spectrophotometer
3. Incubator and shaking Incubator
4. Vortex machine
5. Autoclave machine
6. Glass wares, laboratory distillation apparatus- fractional distillatory set up, microscope.
7. pH meter petri dishes, slants, micro-pipettes, Bunsen burner, hot plate, clamp stands, electric balance, micro-burette, etc.

3.1.2 Samples

1. Yeast strains isolated from sugar cane juice, grapes & date juice.
2. Starch Hydrolyzing bacteria was isolated from soil.

3.1.3 Reagents

Dinitro Salicylic acid (DNS), Sulfuric acid, Sodium hydroxide, Sodium thiosulfate, Phenol red: phenol sulfonphthalein, Potassium Iodide, Potassium dichromate, 0.9% Sodium chloride solution, soluble Starch, Hydrochloric acid and Urea.

3.1.4 Media

Different types of media were used for selective growth, enrichment culture, indication of specific properties and fermentation. Media preparation and sterilization were done according to the protocol and standard recipe. For biochemical tests, specific mediums were prepared.

3.1.4.1 Agar media and broth

 Nutrient agar medium

NA is a common microbiological growth medium. Nutrient agar typically contains:

- 0.5%peptone
- 0.3% beef extract/yeast extract,
- 1.5% agar,
- 0.5% NaCl
- 97.2% distilled water.

 Yeast extract peptone dextrose medium (agar and broth)

YEPD is a complete medium for yeast growth. It contains:

- 1% yeast extract,
- 2% peptone,
- 2% glucose or dextrose and
- Distilled water.

It can be used as solid medium by including 2% agar.

 Starch agar medium

Starch agar is used for specific microbial growth and observation of starch hydrolyzing activity of the microorganism. It contains:

- 0.3% beef extract,
- 0.5% peptone,
- 0.2% starch and

- 1.5% agar.

3.1.4.2 Fermentation media

- Vegetable peels: 100gm waste materials (chopped and grinded green parts) in 1000 ml water boiled with 2 ml concentrated hydrochloric acid.

3.1.4.3 Biochemical test media

- Nitrate Broth
- Phenol red dextrose broth
- Phenol red lactose broth
- Phenol red sucrose broth

  Phenol red trehalose, maltose, starch, galactose and xylose broth.

3.2 Methods

3.2.1 Sample collection

Wild types of yeast strains were isolated from sugarcane, grapes and date juice.

Above-mentioned sources were collected from local market and kept for 1 week at room temperature for yeast growth.

3.2.2 Isolation of starch hydrolyzing bacteria from the soil

Starch hydrolyzing bacteria was isolated by mixing 100 g of soil with 100 g of distilled water. Serial dilution was done up to 10-5 and spread plated into nutrient agar fortified with 1% starch. Then the plates were kept for incubation at 37°C for overnight. The media which showed the clear zone was selected.

The inoculum was mixed with kitchen waste media prior to one day before starch hydrolyzing bacteria fermentation was done and was kept at room temperature.

3.2.3. Inoculum development

One mL liquid sample was serially diluted in sterile saline solution (0.9% NaCl) and inoculated onto the YEPD agar plate by spread plate technique. Incubation was done at 30°C for 48 hours.

Culture broth was made (0.3% yeast extract, 1% peptone, and 2% dextrose) and autoclaved at 121°C. Colonies from agar plates were inoculated into the broth. After 24 hours incubation at 30°C, 0.2 ml suspension from broth was again cultured (spread plating) on YEPD agar medium. This selective culture procedure was used to isolate pure yeast strains.

3.2.4 Observation and culture maintenance

Progressions of microbes were witnessed after 48 hour incubation and colony forming units (CFU) counted in each type of agar plate for specific dilution. The cultures of yeast were preserved by sub-culturing on YEPD plates, incubating for 48 hours at 30ºC and afterwards storing in a refrigerator at 4ºC. For long term preservation, -20 º C refrigerator was used.

3.2.5 Identification of the yeast

3.2.5.1 Morphological characterization

General procedures were done for the identification of yeast based on morphological (Kreger-VanRij, 1984; Mesa et al., 1999) and physiological characteristics. Forty eight hours old cultures were selected & inoculated on YEPD medium. Growth pattern was witnessed on that selective medium. Presences of the isolates on YEPD agar medium were observed. The texture, colour and surface of colonies were noted down. Shape of the cells was observed by compound microscope.

3.2.5.2 Physiological characterization

Biochemical tests were done for physiological characterization of yeast based on fermentation of specific carbohydrates. Fermentation broth with Durham tube was used for testing of yeasts for carbohydrate fermentation. Carbohydrate utilization media were equipped with 10.0 gm peptone,5.0 gm NaCl, 0.018 gm Phenol red indicator and 5.0 gm carbohydrates (for1000 ml). After the addition of specific carbohydrates and adjusting the pH to 7.2, media spreading was done in 20 ml screw-cap test tubes with Durham tubes and then autoclaved.

The sugars used: The carbohydrates used were glucose (dextrose), sucrose, lactose, trehalose, maltose, starch, xylose and galactose. The principle of the sugar fermentation test is development

of (CO_2) gas in Durham tube and colour change of the medium from red to yellow due to the formation of acids (Warren & Shadomy, 1991)

3.2.6 Stress tolerance characterization

3.2.6.1 Detection of thermo-tolerance

YEPD broth was prepared and taken into test tubes (10 ml each) and then autoclaved. The media were then inoculated with 48 hours old selected yeast strain. One test tube was not inoculated, used as blank. Initial optical density of each tube was noted using spectrophotometer at 600 nm against the medium as blank. Test tubes were then incubated at various temperatures and the temperatures were 25°C, 30°C, 37°C and 44°C for 48 hours to observe thermo tolerance of yeast strain. After incubation, optical densities of the yeast suspensions were noted.

3.2.6.2 Detection of ethanol-tolerance

YEPD liquid medium was used for the method. Media (YEPD) was taken into test tubes and 1 ml absolute ethanol of different concentrations (5%, 10%, 15%, 20% and 25% by volume) were added into the test tubes and marked. Test tubes were then inoculated with the selected yeast isolates and negative control was not inoculated. The initial optical density of each test tube was taken at 600 nm against the medium as blank. The test tubes were incubated at 30°C for 48 hours and optical density was recorded again after the incubation at 600 nm.

3.2.6.3 Growth at different pH

Sterile YEPD broth was taken into two sets of 10 test tubes. Each test tube contained 10 ml of the media. YEPD broth of each test tube was adjusted to different pH (2 to 10). Then the broth having test tubes were inoculated by yeast and one blank media was used as a control. Initial optical density at 600 nm was measured against the control. After incubation at 30°C for 48 hours, optical density was taken.

3.2.7 Osmotolerence observation

YEPD broth was prepared containing 6%, 9%, 12%, 15%, 18% and 20% of NaCl. Each McCartney bottle contained 15 ml of YEPD liquid media with appropriate concentration of salt and blank media was used as a control. Then each was inoculated by half loop full of Yeast cell

and recorded the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hours cell density was further recorded at 600 nm.

3.2.8 Fermentation media preparation

3.2.8.1 Defined sugar media

Use of different sugars as fermentation substrate was studied. Molasses, glucose, sucrose were used as substrates.

a) Molasses: With ~20% reducing sugar molasses was used as fermentation media. It was prepared with 250 gm molasses, 0.10gm urea, and 0.30ml concentrated sulfuric acid. Tap water was added up to 1000 ml and the media was boiled. Concentration was conserved and distributed in conical flasks. Autoclave was done at 121°C and 15 psi.

b) Glucose and Sucrose: Glucose and sucrose were used as fermentation substrate at different proportions of their concentrations. Media of 10%, 15% and 20% concentrations of sugar were made by adding 10 gm, 15 gm and 20 gm of the sugars in 100 ml distilled water. After the distribution into conical flasks, they were autoclaved.

3.2.8.2 Cellulosic waste (vegetable peel) media

Lignocellulosic biomass was used as fermentation medium. Residual waste parts of potato, papaya, pumpkin, cucumber, lady's finger, basil were used as fermentation substrate. These vegetable peels were gathered from households and chopped into smaller pieces. In 1000 ml water 250 gm, 150 gm and 100 gm of solid wastes were crushed in an electrical blender machine. The blended material was transferred into a beaker and boiled for 10-15 minutes. Hydrochloric acid was added (2 ml) to decrease the pH to avoid bacterial contamination and convert calcium to calcium sulfate salts. Lower pH with high temperature can also produce a good fermentation state and primary hydrolysis of sugars.

3.2.9 Fermentation

Ethanol fermentation technique was done by inoculation of yeast in the prepared fermentation media. Different parameters were fixed to observe fermentation efficiency and ethanol production rate.

3.2.9.1 Preparation of cell suspensions

a) Yeast: Previously sub-cultured, 48 hours old yeast isolates were used for inoculation. Selected colonies were taken with sterile loop inside laminar air flow cabinet and transferred into the test tube (10 ml) containing 0.9% NaCl saline.

b) Starch hydrolyzing bacteria: A loop full of bacterial culture that showed clear zone was transferred from starch-nutrient agar slants to fermentation media, which served as enzyme source.

3.2.9.2 Fermentation of defined sugars

Inoculation of yeast in fermentation media was completed inside laminar air flow cabinet. One hundred and fifty milliliter fermentation media was taken into 500 ml Erlenmeyer conical flasks. The yeast cell suspensions were mixed well using a vortex machine and inoculated into the fermentation flasks. The flask was sealed with cotton and incubated at different temperature in both non-shaking and shaking condition.

3.2.9.3 Fermentation of cellulosic vegetable peel

i) One hundred and fifty milliliter of fermentation media was taken into 500 ml Erlenmeyer conical flasks and inoculated with yeast cell suspension the flasks were sealed with cotton and incubated in a rotatry incubator at 30°C in shaky condition (120rpm).

ii) One hundred and fifty milliliter fermentation media was taken into 500 ml Erlenmeyer conical flasks. Flasks of kitchen waste (peel of vegetables) media were aseptically inoculated with starch hydrolyzing bacterial suspension and incubated for 24 hours at 36°C in shaking condition (80 rpm). After the incubation, starch hydrolyzing bacterial suspension was inoculated with yeast cell suspension and the flasks were sealed with cotton and incubated in a rotary incubator at 30°C in shaking condition (120 rpm).

3.2.10 Estimation of ethanol

Ethanol manufacture percentage from specific amount of substrate was determined by titration method using Conway unit (Conway et al., 1994). After distillation, alcohol meter was used to evaluate the percentage of ethanol existing in the distilled product.

3.2.10.1 Conway method

Oxidation-reduction titration principle with Conway unit was used to determine the ethanol percentage in the fermented broth and its distilled product. In this technique 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 produces iodine. Then the iodine is titrated with a standard solution of sodium thiosulfate (0.1N). The titration reading is used to analyze the ethanol content after fermentation (Ingram et al.,1987). Fermentation media were taken out of the incubator after specific incubation period. One ml supernatant was diluted up to 250x and 500x. One ml from these diluted samples was placed on the outer portion of Conway chamber. One Conway unit was used as blank by placing only distilled water. Then 1 ml potassium dichromate was placed into the Conway unit center.

After the addition of the supernatant and potassium dichromate, the unit was kept for 18 to 24 hours by covering them with glass plates. Petroleum jelly was used to make them air-tight. Oxidation transpires in the company of ethanol when it evaporates and reacts with potassium dichromate.

a) Procedure:

1 ml 0.05 N potassium dichromate solution was added in Conway unit center.

↓

1 ml fermented sample was added in outer chamber of Conway unit.

↓

Conway units were kept for 24 hours.

↓

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

↓

0.1N sodium thiosulfate was taken in Microburrete.

↓

Titration was done until the center becomes colourless and data was recorded.

b) Calculation: When the titration was complete, the data was calculated by using the given formula to recognize the percentage of ethanol (gm/100 ml) present.

Ethanol (%) = {(TR of blank sample- TR of FS) × 11.6 × 0.1 × DF × 100} ÷ (0.793 × 1000)

Here,

- Density of Ethanol: 0.793 g/ml
- DF: Dilution Factor
- FS: Fermented solution
- TR: Titration
- Volume of sodium thiosulfate used: 11.60 c

CHAPTER 4.

RESULTS

4.1 Identification of the yeast

4.1.1 Morphological characterization

The morphology of the vegetative cells of yeast was determined to confer 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 smooth white colonies on YEPD medium. The growing stage of the yeast isolates was seen under a microscope and established to be yeast.

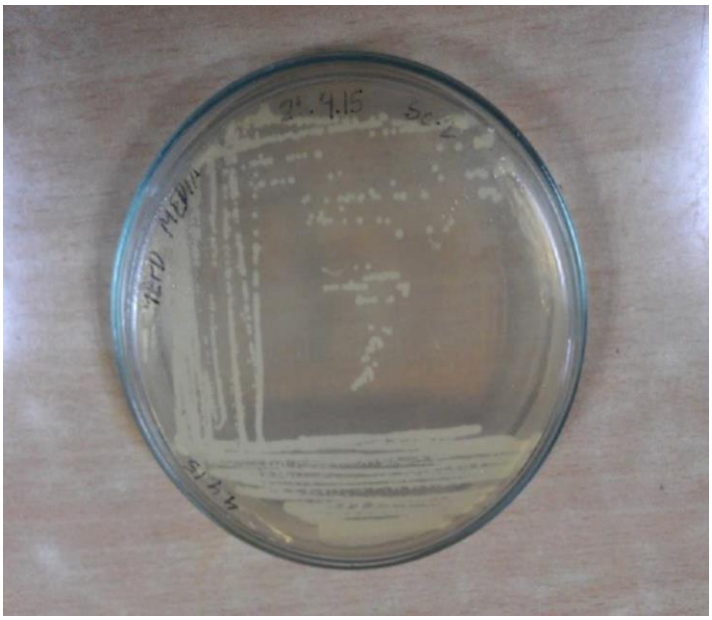
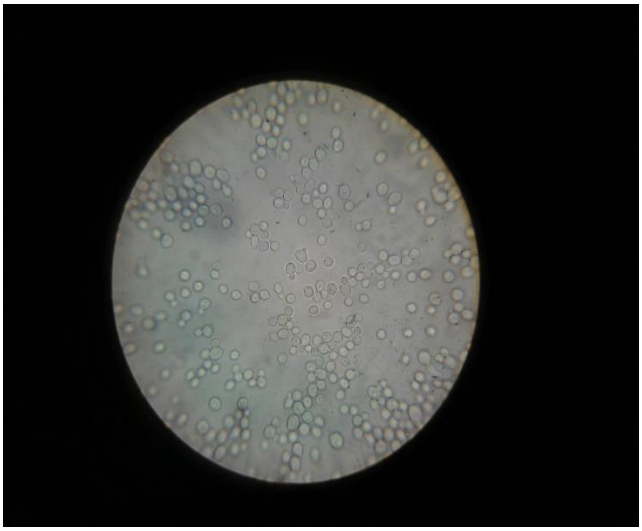


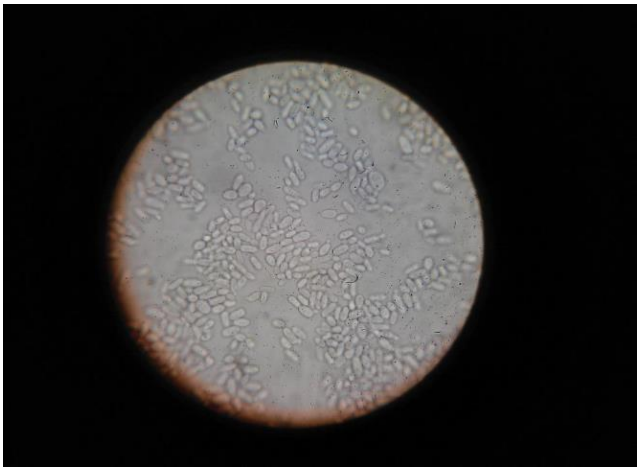
Figure 4.1: Colonies on YEPD agar medium after incubation of 24 hours at 30° C

4.1.2 Microscopic observation

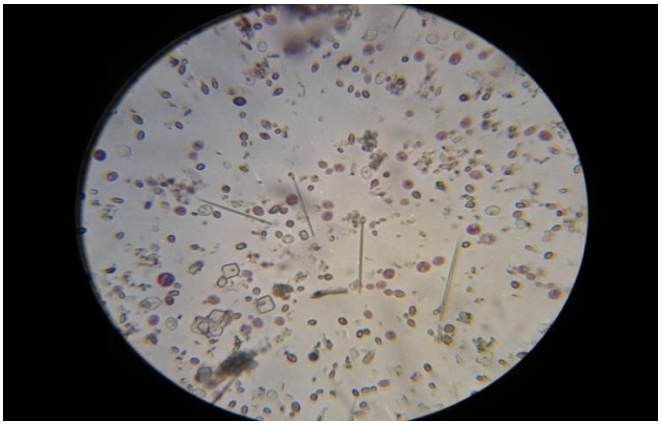
The cell morphology of the ethanol-tolerant yeast isolated strain was studied under the compound microscope. A cell to elongate has single, pairs, or triple budding cells. The isolates replicate vegetatively by budding.



(a)



(b)



(c)

Figure 4.2: The cell morphology under a compound microscope. From (a) sugarcane juice, (b) Date juice (c) Grape juice

4.1.3 Physiological Characterization

4.1.3.1 Fermentation of carbohydrates

In this study, *Saccharomyces cerevisiae* showed a variation of consumption of eight different sugars (Table 4.1). The Sugarcane juice isolates consumed glucose, fructose, and galactose but failed to develop on lactose, xylose, trehalose. The isolates incompletely develop on sucrose and maltose. After 48 hours the following results were shown:

Table 4.1: Fermentation result of different carbohydrates for Sugarcane juice (SC.t) isolate (Positive:++, Variable:+-, Negative:--).

Carbohydrate	**Before Fermentation**	**After Fermentation**
Glucose/ Dextrose	Pink	+ +, gas formed
Sucrose	Pink	+ -
Maltose	Pink	+ -
Lactose	Pink	--
Fructose	Pink	+ +(yellow), gas formed
Xylose	Pink	- -
Trehalose	Pink	--
Galactose	Pink	**++,** gas formed

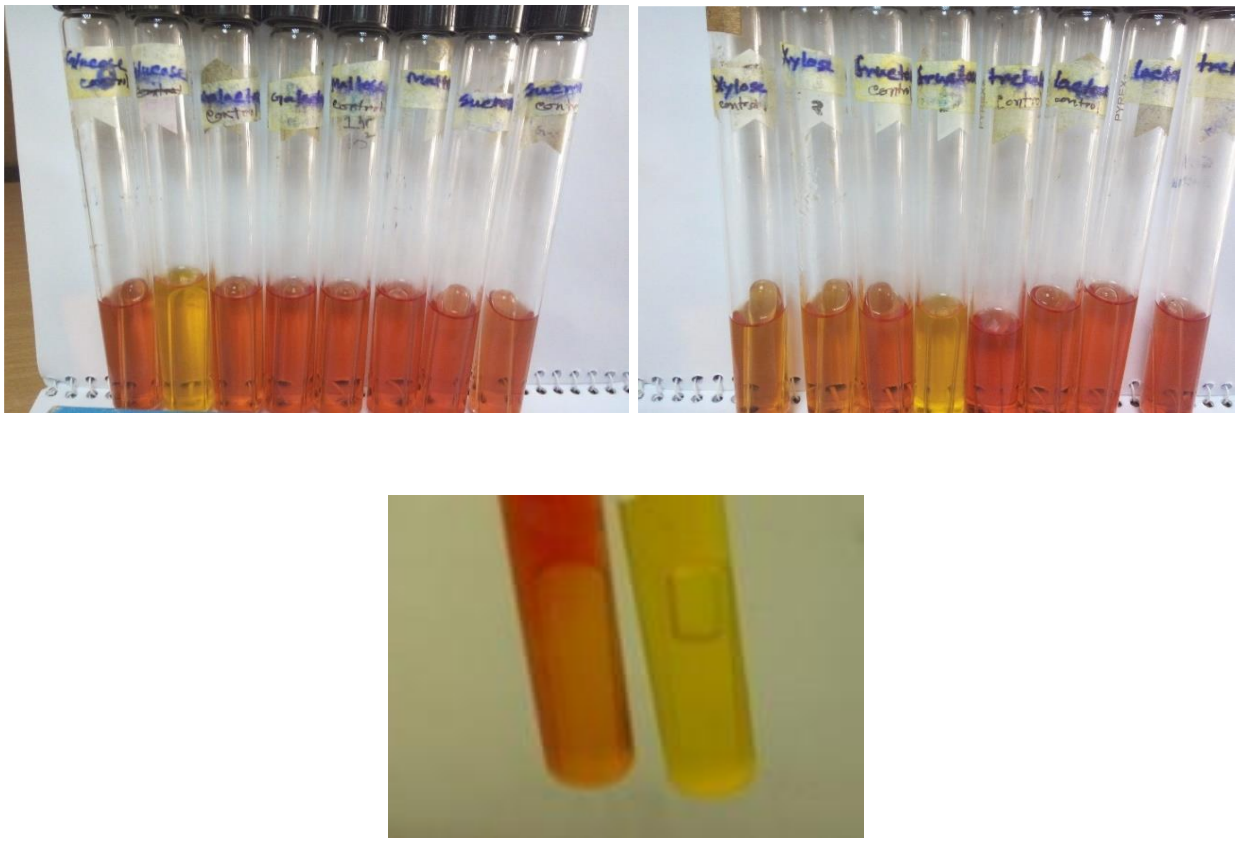


Fig 4.3: Media colour change after fermentation of the carbohydrate.

4.2 Stress tolerance outcomes

4.2.1 Thermo-tolerance

Five YPD Agar containing plates were streaked with Yeast cells and incubated for 48 hours at 25°C, 30°C, 37°C, and 44°C. Yeast isolate from sugarcane juice was able to grow at -25°C to 44°C, but the optimum temperature was at 37°C. To confirm the results obtained from solid media, the thermo-tolerance test was repeated again in liquid media. Development at liquid media is shown in the given table.

Table 4.2: Growth at different temperature in liquid media.

Temperature	O.D. At Inoculation	O.D. after 24 hours	O.D. after 48 hours	O.D. Change in 48 hours
25°	0.0575	0.3245	0.301	0.2435
30°	0.0635	0.361	0.3805	0.317
37°	0.056	0.3955	0.38	0.324
44°	0.022	0.2655	0.31	0.288

From the table, it is obvious that the sugarcane juice isolate is to some extent thermo-tolerant as it has the ability to grow at 44°C. And the most appropriate situation for developing of yeast was found to be 37°C.

4.2.2 Ethanol tolerance

The isolate was carefully chosen for selection of ethanol-tolerant yeast (Table 4.4).The isolate can develop up to 15% ethanol-containing liquid YEPD media. Maximum growth for the SC.t was found in 15% ethanol containing media. Growth was recorded at 5%, 10%, 12%, 15%, 18%, 20%, and 25% of ethanol containing liquid media and O.D was given gradually:

Table 4.3: Growth in different ethanol concentration containing media

Ethanol %	O.D. At Inoculation	O.D. after 24 hours	O.D. after 48 hours	O.D. Change in 48 hours
5	0.054	0.2765	0.367	0.313
10	0.018	0.2315	0.3485	0.3305
15	0.0365	0.2675	0.373	0.3365
20	0.07	0.292	0.1975	0.1275
25	0.085	0.0905	0.105	0.02

4.2.3 pH sensitivity

Yeast isolate SC.t had a flexible growth result at pH 2-10. The isolates had outstanding growth from pH 4 to 6. Though both of the isolates were able to grow in all the pH conditions, but pH lower than 3 and higher than 7 was not that much appropriate for a good growth.

Overall, pH 5 and 6 was optimum growth conditions where the isolate SC.t had its best growth at pH 6.

The result was constructed on the optical density at 600 nm. Cultures in YEPD liquid media was observed after 24 hours and 48 hours at 30°C incubation.

Table 4.4: Growth in different pH containing liquid media

pH	O.D. At Inoculation	O.D. after 24 hours	O.D. after 48 hours	O.D. Change in 48 hours
pH2	0.071	0.0965	0.115	0.044
pH3	0.0675	0.354	0.3665	0.299
pH4	0.0365	0.377	0.362	0.3255
pH5	0.035	0.3695	0.377	0.342
pH6	0.0265	0.379	0.375	0.3485
pH7	0.054	0.285	0.279	0.243
pH8	0.058	0.126	0.270	0.212
pH9	0.158	0.164	0.277	0.119
pH10	0.275	0.286	0.379	0.104

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 control was set using blank. Then each was inoculated by half loopful of Yeast cell and the initial optical density at 600 nm and incubated at 30°C for 48 h was measured. After 48 hrs 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 was given gradually :

Table 4.5: Growth in different NaCl containing liquid media

NaCl %	O.D. At Inoculation	O.D. after 24 hours	O.D. after 48 hours	O.D. Change in 48 hours
6	0.259	0.334	0.385	0.126
9	0.2215	0.334	0.3325	0.111
12	0.2135	0.2665	0.2945	0.081
15	0.224	0.236	0.251	0.027
18	0.13	0.088	0.059	0.0
20	0.071	0.167	0.167	0.096

The results showed both strains are resistant against higher osmotic pressure. Both strains had shown their highest growth in 6% NaCl containing media and gradually lowest number of growth at 12% and 15% NaCl containing media.

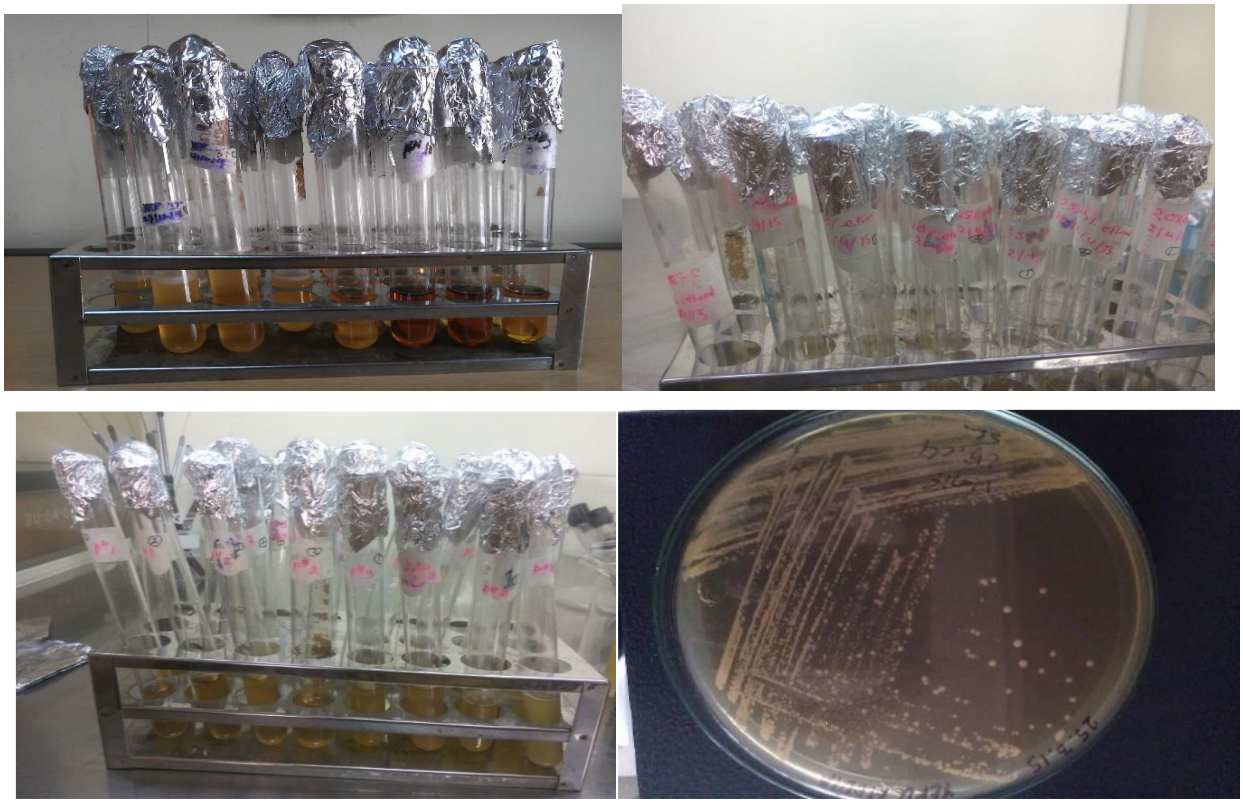


Figure 4.4: Result of tolerance to different conditions

a. Growth result at different temperatures, b. Growth results at different ethanol concentrations, c. Growth result at different pH, d. Culture of SC.t strain at 30°C

4.3 Ethanol Fermentation

4.3.1 Ethanol production from defined sugar mediums

Ethanol production was noticed from molasses, glucose, and sucrose after 24 and 48 hours fermentation .Ethanol estimation was done by titration method using Conway unit. The amount of ethanol produced was low. The ethanol production rate was recorded for the above-mentioned sugars in shaking condition at 30°C, pH 6. The production rate stretched from 1.71% to 6.23% from per 100 ml of substrate medium. Sugarcane isolate SC.t had the maximum rate of ethanol production (6.23%) from molasses and date juice isolate DJ.t had the lowest rate of ethanol production (1.71%) from sucrose. Ethanol production percentage was also determined for the same substrates in shaking condition at 30°C, pH 5. In this condition, sugarcane isolate SC.t had the highest rate of ethanol production (5.42%) from molasses and date juice isolate DJ.t had the lowest rate of ethanol production (2.17%) from sucrose.

Table 4.6: Ethanol production from defined sugars by yeast isolates SC.t and DJ.t at pH 6

Defined Sugar Medium	Isolate	Percentage of ethanol produced after 24 hours	Percentage of ethanol produced after 48 hours
Glucose	SC.t	2.24%	2.34%
	DJ.t	1.89%	1.94%
Sucrose	SC.t	2.17%	2.19%
	DJ.t	1.71%	1.80%
Molasses	SC.t	6.23%	5.89%
	DJ.t	3.24%	3.89%

Table 4.7: Ethanol production from defined sugars by yeast isolates SC.t and DJ.t at pH 5

Defined Sugar Medium	Isolate	Percentage of ethanol produced after 24 hours	Percentage of ethanol produced after 48 hours
Glucose	**SC.t**	**3.34%**	**2.96%**
	DJ.t	**3.79%**	**3.84%**
Sucrose	**SC.t**	**2.69%**	**2.77%**
	DJ.t	**2.17%**	**2.24%**
Molasses	**SC.t**	**5.12%**	**5.42%**
	DJ.t	**4.34%**	**4.93%**

4.3.2 Alcohol production from cellulosic wastes (media contain 250gm/ 150gm/ 100gm solid CW):

Ethanol production from cellulosic waste was determined. Different amount of cellulosic waste (250gm/ 150gm/ 100gm) was used as fermentation media. The ethanol production rate was recorded for the aforementioned media in shaking condition at 30°C, pH 6 and pH 5. The production rate ranged from 2.98% to 4.07% from per 100 ml of substrate medium for pH 6 and 2.19 to 3.25 for pH 5.

Table4.8: Ethanol production from cellulosic wastes (media contain 250gm/ 150gm/ 100gm solid kw) by yeast isolate SC.t at pH 6 (Substrates were not treated with starch hydrolyzing bacteria)

Media with different composition	Percentage of Ethanol produced at 24hr	Percentage of Ethanol produced at 48hr
Media (250gm CW)	2.98	3.12
Media (150gm CW)	3.52	3.56
Media (100gm CW)	4.05	4.07

Table4.9: Ethanol production from kitchen wastes (media contain 250gm/ 150gm/ 100gm solid kw) by yeast isolate S.tC at pH 5 (Substrates were not treated with starch hydrolyzing bacteria)

Media with different composition	Percentage of Ethanol produced at 24hr	Percentage of Ethanol produced at 48hr
Media (250gm CW)	2.19	2.22
Media (150gm CW)	3.20	3.23
Media (100gm CW)	3.21	3.25

4.3.3 Ethanol production from cellulosic waste (CW) at different parameter at pH6:

Ethanol production was recorded for media not treated with SC.t or starch hydrolyzing bacteria, media only treated with SC.t isolates, media in which SC.t isolates and starch hydrolyzing bacteria inoculated at the same time, media in which SC.t isolates was inoculated after 4hr and 24hr of starch hydrolyzing bacteria. The fermentation process was carried out under shaking condition at 30ºC isolates at pH 6. Ethanol production rate was estimated by the titration method using Conway unit. Highest ethanol production rate (10.41%) was recorded for media in which SC1 strain inoculated after 24hr of inoculation and lowest ethanol production rate (1.64%) for media without SC strain and starch hydrolyzing bacteria inoculation.

Table4.10: Fermentation of KW/CW by yeast isolate (SC.t) at different parameter at pH 6

Substrate	Percentage of Ethanol produced at 24hr	Percentage of Ethanol produced at 48hr
CW	1.64	1.66
CW + yeast	4.12	4.16
CW + yeast+ starch hydrolyzing bacteria	6.18	6.22
CW + starch hydrolyzing bacteria + yeast(add after 4hr of starch hydrolyzing bacteria inoculation)	9.10	9.14
CW + starch hydrolyzing bacteria + yeast(add after 24hr of starch hydrolyzing bacteria inoculation)	10.35	10.41

4.3.4. Ethanol production by using defined medium (potato and cucumber):

Ethanol production was recorded for media contain potato (150gm/1000ml) and cucumber (150gm/1000ml). Fermentation process was carried out under shaking condition at 30ºC isolates at pH 6. For the aforementioned process, SC.t, GRP.t, DJ.t yeast isolates were used and Cel 47 and Cel 66 (cellulose degrading bacteria and was collected from the lab and maintained in CMC Agar Media) were also used.

Table 4.11: Ethanol production from vegetable peels by yeast isolate SC.t and GRP.t and DJ.t pH 6 (Substrates were treated with *Cel-47)*

Substrate	**Isolate**	**Percentage of ethanol produced after 24 hours**	**Percentage of ethanol produced after 48 hours**
Potato (150gm/1000ml)	SC.t	7.19%	7.03%
	GRP.t	8.01%	8.46%
	DJ.t	6.67%	6.88%
Cucumber (150gm/1000ml)	SC.t	9.62%	9.95%
	GRP.t	11.18%	11.23%
	DJ.t	8.64%	8.83%

Table 4.12: Ethanol production from vegetable peels by yeast isolate SC.t, GRP.t and DJ.t at pH 6 (Substrates were treated with *Cel-66)*

Substrate	Isolate	Percentage of ethanol produced after 24 hours	Percentage of ethanol produced after 48 hours
Potato (150gm/1000ml)	S.C.t	6.19%	7.37%
	GRP.t	7.64%	8.02%
	DJ.t	5.81%	6.20%
Cucumber (150gm/1000ml)	SC.t	10.83%	11.49%
	GRP.t	9.67%	9.93%
	DJ.t	7.88%	8.01%

Table 4.13: Ethanol production from vegetable peels by yeast isolate SC.t, GRP.t, and DJ.t at pH 6 (Substrates were not treated with *Cel-47 and Cel-66)*

Substrate	Isolate	Percentage of ethanol produced after 24 hours	Percentage of ethanol produced after48 hours
Potato (150gm/1000ml)	SC.t	2.98%	3.55%
	GRP.t	2.23%	2.72 %
	DJ.t	1.66%	1.68%
Cucumber (150gm/1000ml)	SC.t	2.54%	3.51%
	GRP.t	2.49%	2.97%
	DJ.t	1.87%	2.16%

4.4 Optimization of fermentation process

4.4.1 Effect of sugar concentration

The effect of different sugar concentrations was studied through initial twenty-four hour fermentation in a volume of 250 ml media in a 500 ml conical flask in both shaking and non-shaking condition. 6.5% and 7% glucose concentration showed to be the optimum sugar concentration for Ethanol production. Date-juice strain seems to be the most productive strain than the Grape strain in this research. An additional 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 detected in the research.

4.4.2 Effect of pH

The Initial round of fermentation was carried out at pH 5.0, because that is the optimum growth condition for the microorganism. To compare the effect of pH, an additional round of fermentation was done at pH 6.0, as well as 5.0. Remarkably, pH 6.0 demonstrated to be a more suitable condition for the manufacture of Ethanol.

In cellulosic wastes media fermentation, mostly the pH rises.

4.4.3 Effect of shaking

Fermentation carried out in both shaking (120 rpm) and non-shaking condition. Shaking condition demonstrated to be better than the stationary form.

Table 4.14: Fermentation at shaking and non-shaking condition at pH6:

	Percentage of Ethanol produced after 24hr	Percentage of Ethanol Produced after 48hr
Shaking condition	3.38	3.42
Non-shaking condition	2.04	2.10

CHAPTER 5.

DISCUSSION

In this study, three yeasts were isolated from sugarcane juice, date juice and grapes juice. Based on their colony characteristics (white and creamy texture), ovoid microscopic shape, the presence of budding pattern (multipolar), all isolates were found belongs to *Sacharomyces* type unicellular ascomycete according to Lodder (1971) and (Boekhout and Kurtzman, 1996).

The physiological characterization was studied by the process of fermentation of carbohydrates in which it is shown that *Saccharomyces cerevisiae*gave the variation on as an isolates of Sugarcane juice which consumed just only monosaccharaides (Table 4.1). The previous study recommended that that yeast isolate can be identified as *Saccharomyces spp* (Guimaraes *et al*., 2006; Vaughan-Martini and Martini, 1993).

For confirming the stress tolerance, a concise process like thermo tolerance test was performed which gave the result by showing thermo tolerant capacity of Sugarcane Juice strain (Table 4.2), but optimum temperature was at 37°C. It has been reported that, yeasts are mesophilic with upper limit growth temperature between 28°C and 38°C and thus the operating temperature must be maintained between 30 and 35 °C in typical yeast fermentation reactors (Ueno et al., 2001). From the thermo tolerance test report it was found that both of the yeast isolates (SC1 and DJ1) were able to grow at 30°C which is the optimum growth for *Saccharomyces cerevisiae* (Alexopoulos, 1962).

The ethanol concentrations are the noteworthy factors during the fermentation process. It has been shown that the high ethanol concentrations in the fermentation cultures can inhibit or depress the fermentation process. In a past review by (Casey and Ingledew, 1986), yeast strain TGY2 could endure up to 16% (v/v) ethanol. Practically Similar ethanol tolerance of 16.5% (v/v) has been watched for *Saccharomyces cerevisiae* by (Teramoto et al., 2005). In the present study it had been revealing that the sugarcane isolates can endure up to 20% ethanol but the maximum growth for the SC.t was found in 15% ethanol containing media (Table4.3).

Depending on temperature, the presence of oxygen, and the isolates of yeast, the optimal pH range for growth of yeast can defer from pH 4.0 to 6.0. 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). On the pH sensitivity test it was ensured that from all the isolates, the Sugarcane juice strain was grown at best level at pH 6 which confirms the pH sensitivity of this strain (Table 4.4).

Another test was done for testing the tolerance referred as Osmo-tolerance test which showed the resistance capacity of both strains at high osmotic pressure (Table 4.5). The previous study reported the changes in the growth dynamics of yeasts upon exposure to various osmotic stress conditions (D' Amore et al., 1988; Dombek and Ingram, 1986). The decrease in logarithmic growth rate constants of the test yeasts in relation to increasing osmotic pressure is therefore consistent with the views expressed by these workers (Osho, 2005). All tests for stress tolerance made Sugarcane juice strain of Yeast an effective candidate.

In the next step, study was performed on Ethanol fermentation for the successful production of ethanol (Table 4.5 and Table 4.6). For acquiring the product, different medium was used for the effective production. In different sugar medium Sugarcane juice isolates had the highest rate of ethanol production in comparison with Date juice isolates. The production rate ranged from 1.71% to 6.23%. Isolate SC.t had the highest rate of ethanol production (6.23%) from molasses and isolate DJ.t had the lowest rate of ethanol production (1.71%) from sucrose (Table 4.6). Ethanol production rate was also observed for the same substrates in shaking condition at 30°C, pH 5. In this condition, isolate SC.t had the highest rate of ethanol production (5.42%) from molasses and isolate DJ.t had the lowest rate of ethanol production (2.17%) from sucrose (Table 4.7).Five yeast isolates in Bangladesh were reported to use for the similar experiments where those 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 (Khan *et al*., 1989).

Based on the results of tolerance and ethanol production at different sugar concentration at 30°C temperature and pH 5 in shaking and non shaking condition SC.t isolate showed better result than DJ.t isolate. So a series of experiments had been conducted at different physico chemical conditions to optimize ethanol production by the SC.t isolate.

At first, different amount of cellulosic waste (250gm/ 150gm/ 100gm) was used as fermentation media (Table 4.8 and Table 4.9). The ethanol production rate was recorded for the

aforementioned media in shaking condition at 30°C, pH 6 and pH 5 without using starch hydrolyzing bacteria. The production rate ranged from 2.98% to 4.07% from per 100 ml of substrate medium for pH 6 and 2.19 to 3.25 for pH 5. Media contain 100gm cellulosic waste had produced the highest rate of ethanol (4.07% ethanol at pH6 and 3.25% ethanol at pH5).

Ethanol production was recorded for media not treated with SC.t or starch hydrolyzing bacteria, media only treated with SC.t isolates, media in which SC.t isolates and starch hydrolyzing bacteria inoculated at the same time, media in which SC.t isolates was inoculated after 4hr and 24hr of starch hydrolyzing bacteria (Table 4.10). Fermentation process was carried out under shaking condition at 30ºC isolates at pH 6. Ethanol production rate was estimated by the titration method using Conway unit. Highest ethanol production rate (10.41%) was recorded for media in which SC.t strain inoculated after 24hr of inoculation and lowest ethanol production rate (1.64%) for media without SC.t strain and starch hydrolyzing bacteria inoculation.

In the process of producing ethanol from cellulosic waste, the high rate of production was observed on the medium where SC.t strain was inoculated. On the process of production of ethanol, defined medium were used like Potato and Cucumber. Three isolates SC.t, GRP.t and Dj.t were used at pH level 6 for the production of Alcohol from vegetable peels. After 24 hours, the percentage of ethanol production was 7.19% and after 48 hours it was 7.03% for SC.t. By using isolate GRP.t, 8.01% and 8.46 % of ethanol production were found according to the interval of 24 hours and 48 hours which was higher than the SC.t Isolate. The lowest percentage of ethanol production was found for DJ.t isolates which was 6.67% after 24 hours and 6.88% after 48 hours. In Cucumber, a defined sugar medium, the highest percentage was also found where Grape Juice isolate was used (11.18% after 24 hours and 11.23 % after 48 hours). The lowest percentage was found for Date Juice isolate which was 8.64% after 24 hours and 8.83% after 48 hours.

After treating substrate with Cel-66, on the Potato medium, the highest percentage of ethanol production (7.64% after 24 hours and 8.02% after 48 hours) was also found where Grape Juice isolate was used. The lowest percentage (5.81% after 24 hours and 6.20% after 48 hours) was noticed for Date Juice isolate in there. But in Cucumber medium, there is slightly different result was recorded. In this medium, the highest percentage of ethanol was produced when using

Sugarcane isolate. The percentage was 10.83% after 24 hours and 11.49 after 48 hours. The Date juice was remaining as the similar lowest percentage of ethanol producing isolates.

In the process of alcohol production from kitchen waste, substrates were treated without Cel-47 and Cel-66. In the Potato medium, the highest percentage of ethanol was produced using Sugar cane isolate which was 2.98% after 24 hours and 3.55% after 48 hours.

Lowest percentage was found for Date Juice isolate. In the case of Cucumber medium, the rate of ethanol production was also high for Sugar Cane isolate which was 2.54% after 24 hours and 3.51% after 48 hours. The rate of ethanol production was also low for Date juice isolate which was 1.87% and 2.16%.

From this record, it was confirmed that in the production of Alcohol from vegetable peels, there was slightly different result found in between Potato Medium and Cucumber medium after treating with both Cel-47 and Cel-66. But in the process of producing Alcohol from kitchen waste, for both Potato and Cucumber medium, the high rate of production was found when using Sugar cane juice as isolates in comparison with other isolates after treating without Cel-47 and Cel-66.

In this total study, different level of pH was used to determine and analyze the effect of pH on fermentation process and it was found that pH 6 is the much suitable level for manufacturing the ethanol. There was also shaking effect in the process of fermentation. In the shaking condition (120 rpm), the rate of fermentation was 3.38 in 24 hours and 3.42 in 48 hours. On the other hand, during non-shaking condition the fermentation rate was lower than shaking condition which was 2.04 in 24 hours and 2.10 in 48 hours.

CONCLUSION

In this work, the fermentation process was conducted according to various parameters. The different environmental condition was also followed to do the whole process of fermentation. Anaerobic *Saccharomyces spp*. fermentation process was used to produce ethanol as cost effective energy source which was derived from the cellulosic waste. After successful experimentation and the analysis of fermentation process with the enhancement of temperature, reducing sugar concentration and different level of pH, it was demonstrated that the yielding of maximum ethanol was found at the optimum temperature 30°C and at the pH level 6.0. The percentage of maximum ethanol production was 11.49% by using substrate (vegetable peels) confirms the future effective replacement of the conventional fossil fuel with the environment - friendly fuel as ethanol.

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APPENDIX

Appendix-A

Microbiological Media

Media used were prepared methods using appropriate compositions. Components used high grade. All media were sterilized by autoclaving for 20 minutes. The compositions used for different media have been shown below:

1) Composition of YMM media

Molasses	2%
Agar	2%
Peptone	0.50%
Yeast extract	1%
Malt extract	1%

2) Composition YEPD agar media

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

3) Composition YEPD liquid media

Yeast extracts	3.0 g
Peptone	10.0 g
Dextrose	20.0 g
Distilled water	1.0 L

4) Composition YPD liquid medium

Glucose	20 g
Peptone	20g
Yeast extracts	10g
Water	1 L

Reagents & Chemicals:

Reagents which were used in different methods together with their sources are mentioned below:

1) DNS (3, 5-Dinitro Salicylic acid) reagent:

Mix: 1 .Distill water: 141.6 ml

2. NaOH (analytical grade):2.0gm

3. DNS: 1.06gm

Dissolved above then added: 4 K-Na-Tartarate: 30.6gm

5. Phenol: 760µl

6. Na-metabisulphite: 0.83gm

The solution was filtered and stored in room temperature in dark bottle.

2) 0.1 N Sodium thiosulfate solution:

Add 25 g Sodium thiosulfate and make the volume up to 1000 ml with Distilled water.

3) 0.05 N Potassium Dichromate solution:

9.808 g dry Potassium Dichromate

↓

Pour in 500 ml 10 N sulfuric acid (No. 1-solution)

↓

62.5ml no.-1 solution dilute again in 500ml 10 N sulfuric acid

↓

0.05 N Potassium Dichromate (prepared)

4) 10 N sulfuric acid:

140 ml sulfuric acid and make the volume up to 500 ml with Distilled water.

5) 50% Potassium Iodide:

50 g KI and make the up to 100 ml with Distilled water.

APPENDIX-B

Instruments name	Model no.	Country
Autoclave(18 L)	CL-32L,APL Co.Ltd,Japan	Japan
Autoclave	Model:WAC-47, Daihan Scientific, Korea	Korea

Balance(Core series)	Adam, UK	UK
Balance(pw series)	Adam, UK	UK
Distilation plant(merit water still)	Mo-W4000 ,EURO	EURO
Freeze (-20°C)	Siemens	Japan
Haemocytometer		
Incubator	Model:DSI300D,Taiwan	Taiwan
Laminerairflowcabinet	SAARC	
Microscope	Model-CX-21,Olympus	
Microscope	Model-XSZ-107 BN	
Microscope	Model-H.903,Optima,Taiwan	Taiwan
Oven(Universal drying oven)	Model:LDO-060E,Labtech,Singapore	Singapore
pH Meter	Model-E 201-C,Shanghai RuosuaaTechnologycompany,China	China
pH Meter (Pocked-sized)	pHep,HI-98107,Hanna instruments	
Refrigerator	Model-0636,Samsung	
Refrigerator	Toshiba	
Spectrophotometer	Model-TGOU, UK	UK
Shaking Incubator	Model-WIS-20R, Daihan Scientific, Korea	Korea
Vortex Mixture	VWR International	
Water bath		Korea

APPENDIX-c

SERIAL	NAME OF THE GLASSS WARE AND OTHERS

NO.	
1	Beaker
2	Conical flasks
3	Disposable micropipette
4	250 ml Erlenmeyer flask
5	Inoculating loop
6	Knife and forceps
7	Micro pipette 100µl
8	Micro pipette 1000µl
9	Micro pipette 5000µl
10	Measuring cylinder
11	Mackartny bottle
12	Pipette 10ml
13	Petri dishes , disposable
14	Screw cap bottle
15	Spreader
16	Slide
17	Screw cap bottle
18	Screw cap test tube
19	Cotton plug
20	Spatula
21	Scotted bottle