Studies on Isolation of Yeasts from Natural Sources for Bioethanol production from Vegetable Peels and the Role of Cellulose Degrading Bacteria (*Bacillus subtilis*) on Ethanol Production

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

Salman Khan Promon
Student ID: 12136001

August 2015

Biotechnology Program
Department of Mathematics and Natural Sciences

BRAC University
Dhaka, Bangladesh

Dedicated To Humanity

DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled "**Studies on Isolation of Yeasts from Natural Sources for Bioethanol production from Vegetable Peels and the Role of Cellulose Degrading Bacteria (Bacillus subtilis) on Ethanol Production**" submitted by Salman Khan Promon, has been carried out under the supervision and able guidance of Associate Professor Dr. M. Mahboob Hossain, Microbiology Program, BRAC University in partial fulfillment of BS 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.

Candidate

____

Salman Khan Promon

Certified:

____

Dr. M Mahboob Hossain
Supervisor

Associate Professor
Microbiology Program
Department of Mathematics and natural Sciences
Dhaka, Bangladesh

// Acknowledgement

First of all, I am grateful to Allah who gives me opportunity of completing my project paper. The author obtained help from many individuals and institutions during conducting and completing this research work and as such he wishes to acknowledge them with gratitude and appreciation. I express my gratitude to the BRAC University Department of Mathematics and Natural Sciences (MNS) for providing the laboratory facilities.

I am grateful to Professor A. A. Ziauddin Ahmad, Chairperson, Department of Mathematics and Natural Sciences, BRAC University and Professor Naiyyum Choudhury, Former Coordinator, Biotechnology and Microbiology Programmes , for allowing me and encouraging me to complete my under graduation thesis.

I express my sincere gratitude and respect to my supervisor Dr. M Mahboob Hossain, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University for being a constant support all throughout my student life and guiding me during the research work which have enhanced my capability enormously.

I also wish to express my humble regards Akhtaruzzaman Khan and Abira Khan, Lecturer, Department of Mathmatics and Natural Science, BRAC University for their cordial and helpful guideline.

I am also thankful to laboratory assistants, laboratory officers and teaching assistants of Department of MNS for their cooperation in doing my research work.

I also appreciate my friends and colleagues Md. Rafid Feisal, Md. Wasif Kamal, Sreoshee Rafiq and Tribeni Ghosh for their help and support.

Finally I would be glad to extend my gratitude to the members of my family and to my friends for their prayerful concerns and supports.

Salman Khan Promon
August, 2015

Abstract

The requirement of an alternative clean energy source is increasing with the elevating energy demand of modern age. Microbial production of bioethanol can replace the conventional fossil fuel with green energy. In this study, local yeast isolates were used for the production of bioethanol using cellulosic vegetable wastes as substrate. This project were designed for the efficient bioconversion of lignocellulosic biomass into ethanol by microbial action. Wild-type yeast isolated form sugarcane juice (SC1) and date juice (DJ1) were used as ethanol producing organism. After proper isolation, identification and characterization of stress tolerance (thermo-, ethanol-, pH-, osmo- & sugar tolerance), detailed characterization and optimization of physiochemical parameters for ethanol production the strain is treating to be dubbed as an industrial strain. Very inexpensive and easily available raw materials (vegetable peel kitchen wastes) are used as fermentation media. The overall objective of this project is to meet the demand for an inexpensive and highly efficient integrated anaerobic *Saccharomyces spp*. fermentation process to produce ethanol as an energy source directly from insoluble lignocellulosic substrate (kitchen-waste). Fermentation was optimized with respect to temperature, reducing sugar concentration and pH. Analysis of fermentation characteristics under 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. Influence of *Bacillus subtilis* increased the alcohol production rate from the fermentation of cellulosic materials. The cellulolytic activity of this cellulose degrading bacteria converts cellulose into smaller sugars which will be easier to be fermented by yeast. Maximum ethanol production by yeast was 17.39% using vegetable peels as substrate at 48 hours (30°C and substrate was treated with *Bacillus subtilis)* under shaking condition.

Contents

Title	Page No.
Abstract	i
Content	ii
List of tables	iii
List of figures	iv
List of abbreviations	v
Chapter 1: Introduction	2-6
Chapter 2: Literature Review	7-18
Chapter 3: Materials and Methods	19-29
Chapter 4: Results	30-43
Chapter 5: Discussion	44-47
Chapter 6: Conclusion	48-49
Chapter 7: Reference	50-57
Appendices	58-60

List of tables

Content	Page
Chapter 2	
Table 2.1: Commonly used yeast species in food, beverage and chemical industries	10
Table 2.2: Taxonomic Classification of *Bacillus subtilis*	12
Chapter 4	
Table 4.1: Fermentation result of different carbohydrates for sugarcane juice (SC1) isolate	33
Table 4.2: Fermentation result of different carbohydrates for date juice (DJ1) isolate	34
Table 4.3: Nitrate reduction by yeast isolates	34
Table 4.4: Growth of strain SC1 and DJ1 at different temperatures	36
Table 4.5: Growth of yeast strain SC1 and DJ1 at different ethanol concentrations.	37
Table 4.6: Growth of yeast strain SC1 and DJ1 at different pH	38
Table 4.7: Alcohol production from defined sugars by yeast isolate SC1 and DJ1 at pH 6	40
Table 4.8: Alcohol production from defined sugars by yeast isolate SC1 and DJ1 at pH 5	40
Table 4.9: Alcohol production from vegetable peels by yeast isolate SC1 and DJ1 at pH 6	41
Table 4.10: Alcohol production from kitchen wastes by yeast isolate SC1 and DJ1 at pH 6	42
Table 4.11: Comparison between the results by Conway method and alcohol meter	43

List of figures

Content	Page
Chapter 2	
Figure 2.1: Yeast cell model	11
Figure 2.2: Sucrose hydrolysis and ethanol formation	13
Figure 2.3: Starch hydrolysis and ethanol formation	14
Figure 2.4: Cellulose hydrolysis	14
Figure 2.5: A scheme of ethanol production pathway by yeast.	15
Figure 2.6: Xylose and Glucose utilization pathway	16
Figure 2.7: Cellulase mediated hydrolysis	17
Chapter 3	
Figure 3.1: a) Kitchen waste media, b) Glucose and sucrose media, c) Sucrose and molasses media, d) Ethanol estimation by Conway unit, e) Culture of *Bacillus subtilis* and f) Staining of *Bacillus subtilis.*	28
Figure 3.2: a) Distillation procedure, b) Distilled product from fermented substrate	29
Chapter 4	
Figure 4.1: Colonies on YEPD agar medium after 24 hours incubation	31
Figure 4.2: The cell morphology under compound microscope (100X) a) From sugarcane juice , b) From date-juice	32
Figure 4.3: Results of physiological characterization of yeast isolates a. Fermentation of different carbohydrates, b. Result of nitrate reduction test, c. Test results of different carbohydrates fermentation using API kit	35
Figure 4.4: Result of tolerance to different conditions. a.Growth result at different temperatures, b. Growth result at different ethanol concentrations, c. Growth result at different pH, d. Culture of SC1 strain at 30°C	39
Figure 4.5: Alcohol production estimation by alcohol meter. a. Percentage result of re distilled product. b. Percentage result of first distilled product	43

List of abbreviations

mm: Millimeter

µm: Micrometer

mg: Milligram

gm: Gram

Kg: Kilogram

e.g.: For example

et al.: And others

pH: Negative logarithm of hydrogen ion concentration

CFU: Colony Forming Unit

spp.: Species

%: Percentage

°C: Degree Celsius

Chapter 1

Introduction

1. Introduction

1.1 Background

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. The issue regarding different drawbacks of traditional fossil fuels and their limited resources is compelling mankind to introduce an alternative source of green energy. Using biotechnology for creating such alternative is now a very significant step where innovative process of clean energy production will be implemented. Most common factors which are taken under consideration for the importance of new alternative of traditional energy source are the gradual diminution of geological resources, their negative effects on environment and organic life, expensiveness and several other reasons. Rapid population growth rate is a major concern for what a renewable, economical and ecofriendly energy source is needed.

Although fossil fuel has been using by human civilization for centuries as principal energy source but before being almost totally replaced by petroleum or chemical based raw material, natural derivatives and precursors were used for the production of many consumer products (Ragauskas *et al*., 2006; van Wyk, 2001). The application of petro-chemical products may have increased the efficiency and rate of production in many cases but considering the damaging impacts on organic life and environment, it has become an important concern to introduce a substitute with sustainable characteristics and environmental friendly features. An important priority can be given to bioethanol as such fuel alternative.

Using bioethanol as energy source has several advantages over conventional fossil fuel considering the limitations and harmful effects. These undesired influences on natural resources, ecology and global economy can be reduced by the replacement with biofuel. Also certain procedures those are associated with the bioethanol production have important roles at waste management system development and green fertilizer production. For these purposes, food waste can be used for the production of bioethanol (Matsakas and Christakopoulos, 2015) which process has the potential to be presented as sustainable green energy source. The escalating energy demand has adverse consequences and also the depletion of the reserves of fossil fuel may lead to many geographical

catastrophe where bioethanol can be produced from renewable sources like food wastes (Izmirlioglu and Demirci, 2012). Furthermore, the processed substrates of bioethanol production from food waste can be used as organic fertilizer.

1.1.2 Bioethanol as fuel

Fermentation derived ethanol (CH_3CH_2OH) or ethyl alcohol is commonly known as bioethanol. Ethanol can be produced chemically from petroleum and from biomass or sugar substrates fermentation. This organic chemical is a flammable, clear and colorless liquid which can be used fuel. Functions of ethanol as solvent, antifreeze and germicide are mostly observed (Licht, 2006).

For the protection of environment and reducing dependence on petroleum or nonrenewable energy sources, renewable energy attracts the attention with high importance. Bioethanol as a potential alternative to petroleum-derived fuel which can meet the increasing demand of energy. (Balat et al., 2008). Several processes of bioethanol production are currently present such as microbiological production from fermentable organic substrates or carbohydrates by yeast. Fermentation of cellulosic biomass, molasses, vegetable peels or food wastes can be mentioned as an economical process of bioethanol production. The utilization of bioethanol produced from cellulosic materials by direct conversion is in countries like Brazil, Canada and USA (Thenmozhi and Victoria, 2013).

The most consumed biofuel all over the world is ethanol. In Brazil, ethanol is the main biofuel for transportation. Ethanol blended with gasoline and also its different dilutions are used for various purposes. Most remarkably, bioethanol is produced from renewable non-fossil carbon like organic wastes that shows its potential as an efficient alternative fuel (RFA, 2011).

The emerging potential of ethanol as a biofuel can be demonstrated by its useful role at automobile fuel and electricity production. Current motor fuelling system can be replaced by ethanol with proper modification of engines. Ethanol shows a clean energy source for automobiles and industrial usages (Adarsha *et al*., 2010). Comparing to the gasoline combustion system, ethanol causes lower air pollution. As a complete combustion fuel, ethanol contains 35% oxygen which lowers the harmful gas emission. Statistics shows that USA uses 100 million gallons of ethanol per year as fuel (Wyman *et al*., 1992). Brazil was the main producer of ethanol for decades but recently USA has taken the place. Ethanol production was established by molasses utilization from

sugar industries but the growing demand during 80's made to add more alternative processes and substrates for ethanol fermentation (Basso and Rosa, 2010). About 19,534.99 millions of gallon of ethanol were produced worldwide in 2009 where 10,600.00 and 6577.89 million gallon were produced by USA and Brazil respectively (Izmirlioglu and Demirci, 2012).

1.1.3 Food waste as fermentation media

The economical production of bioethanol needs an easily available supply of inexpensive raw materials. Organic food waste is one of the top most suitable material for that process. Solid food wastes from household, restaurant or food processing industries can be obtained as substrate to be used as fermentation medium for bioethanol production. As organic solid waste, food waste contains higher percentage of moisture that can create a good fermentation condition (Wang *et al*., 2004). Food wastes can also be recycled as animal feed and fertilizer after specific treatment. (Yan *et al*., 2011).

Fermentation of crops which are rich in sugar or starch is one of the ways to produce ethanol. Crops such as sugarcane, sugar beet, corn, cassava are being used by different countries for ethanol production. Bioethanol production based on the utilization of rotten potatoes are obtained from 5-20% of crops as by by-products in potato cultivation (Thenmozhi and Victoria, 2013). 60% of global ethanol produced form sugarcane as raw material. In USA, 90% of ethanol is produced from corn (Balat et al., 2008).

Frequent disposal of food waste may be resulted in environmental pollution has serious effects on ecology and organic life. Various methods for food waste disposal are used such as landfilling, incineration and recovery or recycle. Landfilling of food waste can increase groundwater contamination. Also, uncontrolled fermentation of organic food wastes results in greenhouse gas emission (Camobreco *et al*., 1999). Therefore, proper waste management and utilization of organic food wastes as fermentation medium to produce bioethanol is an efficient method to produce green energy (Churairat *et al*., 2013). In this project, enzymatic treatment and microbial fermentation process of organic food wastes for ethanol production are described briefly.

1.2 Objectives

Different yeast varieties are reported for the fermentation of lignocellulosic substrates to produce ethanol. The goal of the project is to establish a highly efficient microbial fermentation process by natural yeast isolates to produce ethanol as an energy source. It is to be mentioned that ethanol production rate from insoluble lignocellulosic biomass has not achieved the most economical state. Therefore, commonly available cellulosic kitchen wastes will be used as raw material and proper treatment of the substrate will be done to optimize the fermentation condition which will be resulted in a highly efficient and economical production rate.

- Isolation of ethanol producing wild type yeast strain from natural sources.
- Identification and characterization of isolated strain.
- Study of thermo-tolerance, pH-tolerance, and ethanol-tolerance of the yeast strain.
- To economically produce ethanol by using available and cheap raw materials (vegetable peels)
- Cellulose degrading microorganism will be used for the degradation of lignocellulosic fermentation media.
- Optimizing the fermentation condition for higher yield.

1.3 Hypothesis

Isolation of ethanol producing yeast from natural sources for bioethanol production by vegetable peels and observation of the role of cellulose degrading bacteria on ethanol production. Cellulose degrading bacteria (*Bacillus subtilis*) will be used for pre-treatment of the fermentation media. Specific enzymes from the cellulase-producing microorganism will degrade celluloses present in the lignocellulosic fermentation media and the degraded materials will be easier and more readily available to be fermented by yeast.

Potential wild type yeast strains (such as *Saccharomyces spp*.) will be isolated from date juice, sugarcane juice, grapes, and pineapples. The biochemical and physiological characterization will be done for identification. Wild type yeasts will be taken under comparative studies and experiments to obtain a strain with high productivity. To produce ethanol from the inexpensive raw material, fermentation process will be optimized for different physiochemical parameters.

Chapter 2

Literature Review

2. Literature Review

2.1 Overview

Processes those are involved in the conversion of biomass into fuel ethanol are getting attention in recent years. Fuel product of ethanol fermentation is considered as clean liquid fuel alternative of non-renewable energy sources. Ethanol fermentation technology has achieved significant advancement with its growing demand. The foremost focus of this ethanol production technology is optimized utilization of biomass resources and microbial action on fermentation. One promising technique is the fermentation of lignocellulosic biomass where hydrolysis action by specific microbial cellulase enzymes is involved. Ethanol can be derived from the fermentation process of sugar containing materials. Major raw materials those can be used in ethanol production must be converted into simple sugars to be fermented by the enzyme of specific microorganism such as yeast (Lin and Tanaka, 2005).

2.2 Fermentation of lignocellulosic biomass

Biochemical process by which sugars are converted into cellular energy and produce ethanol with carbon dioxide as metabolic products can be defined as fermentation. Ethanol production from sugars can be carried out by yeast in anaerobic fermentation which does not require oxygen (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).

Different forms of biomass resource those can be used as fermentation media are grouped into four major categories. The largest source of biomass is wood from many natural sources, industries or process mills. Second largest biomass source is municipal solid waste. Others are agricultural residues and dedicated energy crops like sugarcane or corn (Monique *et al*., 2003). Cheap fermentable carbohydrate, an efficient yeast strain, a few nutrients and simple culture conditions are required for an efficient ethanol production. Commonly molasses of sugar cane and molasses of sugar beet are the most important sources 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.

2.3 Microorganism: Yeast

Bioethanol production from fermentable raw materials requires organisms that converts the sugars present in the substrates into ethanol. It is reported that microbial action results in high yield with a high rate and yeast is the most common organism for such action. The aim is to develop industrial strains with the ability to ferment all lignocellulose-derived sugars (Barbel *et al*., 2007).

Yeast is a single cell fungi with a diverse phylogenetic grouping. Yeasts are eukaryotes as a member of kingdom Fungi (Kurtzman & Fell, 2006) that digest their energy sources externally and absorb the nutrient into cellular molecules. Specific taxonomical or phylogenetic groupings are not present in yeasts, they are a diverse assemblage of unicellular organisms that occur in 100 genera and divisions of Fungi those are *Ascomycotina* and *Basidiomycotina*. Budding is the asexual reproduction method of yeast and binary fission in some cases (Balasubramanian *et al.*, 2004). Yeasts that use budding for reproduction are known as ‘true yeasts’ and are classified in the order Saccharomycetales (SGD 2005). Yeasts belonging to *Saccharomyces cerevisiae* are most commonly used among various ethanol producing microorganisms (Laplace *et al*., 1992 and 1993). *Saccharomyces uvarum* (Detroy *et al*., 2004), *Schizosaccharomyces pombe* (Jong-Gubbels *et al*., 1996), *Kluyueromyces sp*. (Morikawa *et al*., 2004), *Pachysolen tannophilus, Candida Shehatae*, *Pichiastipitis* are the major yeast species currently used for industrial ethanol fermentation (Matsushika *et al*., 2008). Yeasts are chemoorganotrophs, organic compounds are used as their energy source and the fermentation takes place anaerobically. The principle carbon sources are

hexose sugars such as glucose, sucrose and maltose. Some species can metabolize other sugars such as fructose, as well as alcohols and organic acids. Yeasts are found primarily in sugar rich environments such as fruits or flower nectar since they derive energy from sugar fermentation. This ability to ferment sugars made them important for bioethanol production. Isolation of *Saccharomyces cerevisiae* from palm wine (Uma and Polasa, 1990) was reported for the production of an increased amount of ethanol in yeast extract peptone dextrose medium. Yeasts have been used to generate electricity (HUT 2006) and ethanol for biofuel industries.

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*

Table 2.1: Commonly used yeast species in food, beverage and chemical industries (Jacobson & jolly, 1989)

Yeasts are classified on the basis of the microscopic appearance of the cells, the mode of sexual reproduction, certain physiological features (especially metabolic capabilities and nutritional requirements) and biochemical features. The physiological features, that distinguish different yeasts, include the range of carbohydrates that a given organism can use as a source of carbon (Glazer & Nikido, 1995). Individually yeast cells appear colorless, but when grown on artificial solid media they produce colonies which may be white, cream colored, or tinged with brownish pigments. Colony characteristics are useful in the taxonomy of yeasts. Physiological characteristics are also used to a great extent in determining yeast species (Alexopoulos, 1962).

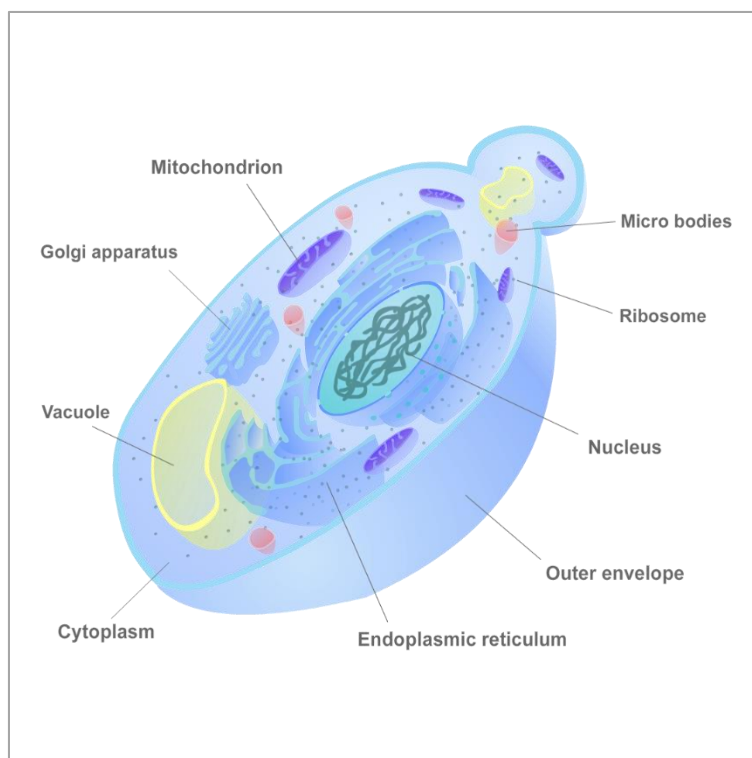


Figure 2.1: Yeast cell model
(Source: http://www.biocourseware.com/iphone/cell/img/ipad/cell.png)

2.4 Microorganism: *Bacillus subtilis*

Utilizing cellulosic biomass for ethanol production, thermotolerance strain of cellulase producing microorganism is preferred. In fermentation, thermotolerant strain of *Bacillus subtilis* can bring efficiency beside yeast because of its cellulolytic property (Yanase et al., 2010). Also it is reported that starch hydrolyzing enzymes such as α-amylase and gluco-amylase are produced by *Bacillus subtilis* (GTL, 2006).

Bacillus subtilis is a Gram positive, rod shaped and motile bacteria, commonly found in soil. *Bacillus subtilis* is an endospore forming bacteria with flagellum, and the endospore that it forms allows it to withstand extreme temperatures as well as dry environments. *Bacillus subtilis* is considered and obligate aerobe, but can also function anaerobically when in the presence of nitrates or glucose. There are several uses for *Bacillus subtilis* and the enzymes it produces. It can be used to create proteases and amylase enzymes (EBI 2009). Besides its many uses and applications, different strains of *Bacillus subtilis* are reported for enzymatic degradation of cellulose that plays

an important role in the bioconversion of cellulose and hemicellulose to soluble sugars. These sugars then can be fermented by targeted organism such as yeast (Kim *et al*., 2012). *Bacillus subtilis* AU-1 was found to produce carboxymethylcellulase (CMCase) activities (Chan and Au, 1987). Therefore, the fermentation includes converting cellulosic sugars into simple sugars, cellulase enzyme utilization in the hydrolysis of lignocellulosic biomass, immobilization of the microorganism, stimulation in simultaneous saccharification and production of bioethanol.

Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*
Species	*Subtilis*
Scientific name	*Bacillus subtilis*

Table 2.2: Taxonomic Classification of *Bacillus subtilis*
(Source: https://bch.cbd.int/database/record.shtml?documentid=103064)

2.5 Sugar utilization

Yeast has the ability to convert sugar into ethanol. The process starts by milling the substrate, then adding sulfuric acid or enzyme like α-amylase to break down the starches into complex sugars. To degrade the complex sugars further into simple sugars, gluco-amylase is added. Yeasts are then added for bioconversion of the simple sugars to ethanol which is distilled off to obtain higher concentration of ethanol (GTL 2006). Xylose, one of the major fermentable sugars present in cellulosic biomasses such as agricultural residues, paper wastes and food wastes which can be fermented by saccharomyces yeasts (Ho *et al*., 1998). Cellulase enzymes can be used for the conversion of cellulosic content of lignocellulosic biomass to fermentable sugars (Kadgar *et al*., 2004).

2.5.1 Bioconversion of different sugars

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.

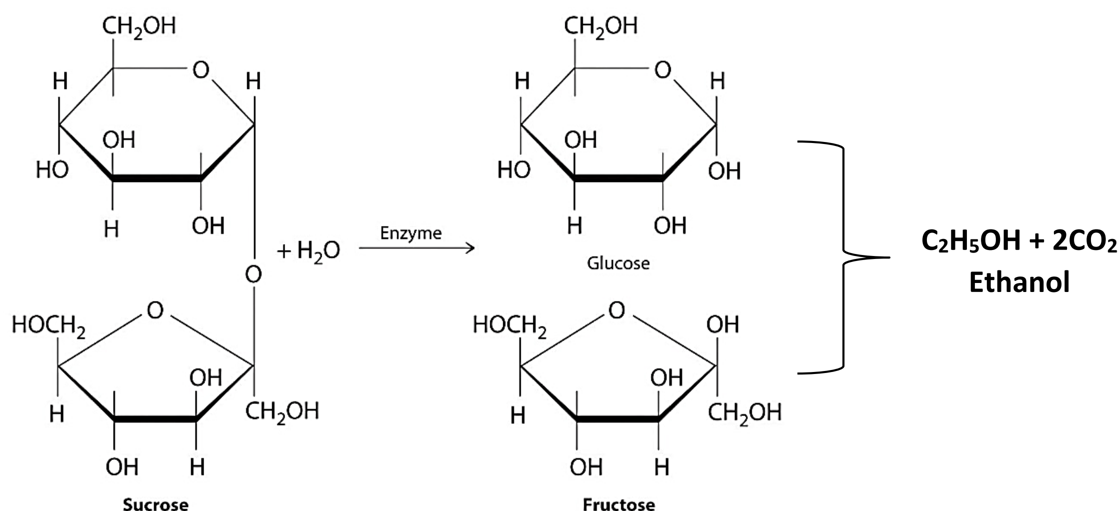


Figure 2.2: Sucrose hydrolysis and ethanol formation
(Source: https://acsundergrad.files.wordpress.com/2013/01/sfg.gif)

Starch molecules are long chains of α-D-glucose monomers 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 (Haissig *et al.,* 2006). Enzymes such as diastase and maltase have important role in starch hydrolysis.

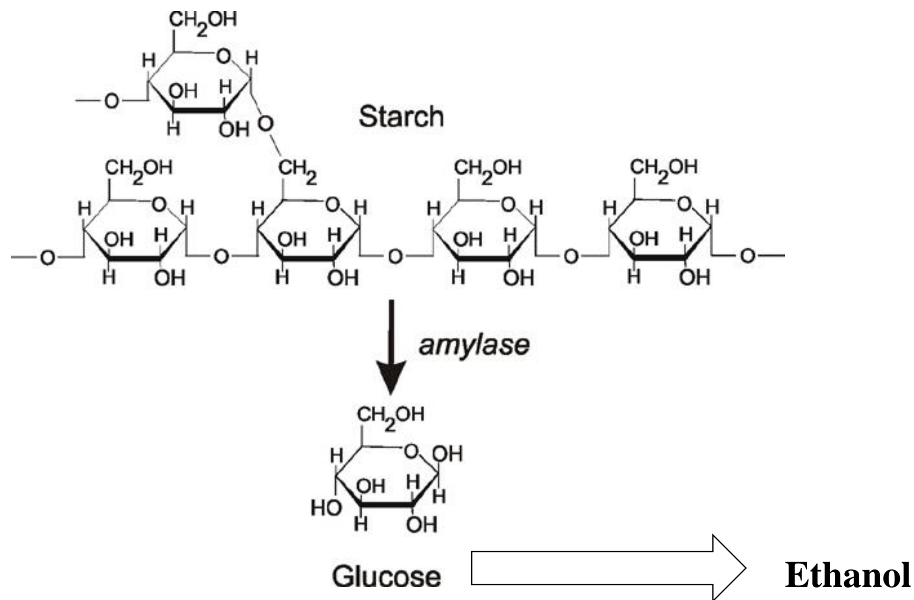


Figure 2.3: Starch hydrolysis and ethanol formation
(Source: http://www.biotek.com/assets/tech_resources/11087/figure1.jpg)

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, endo glucanase 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).

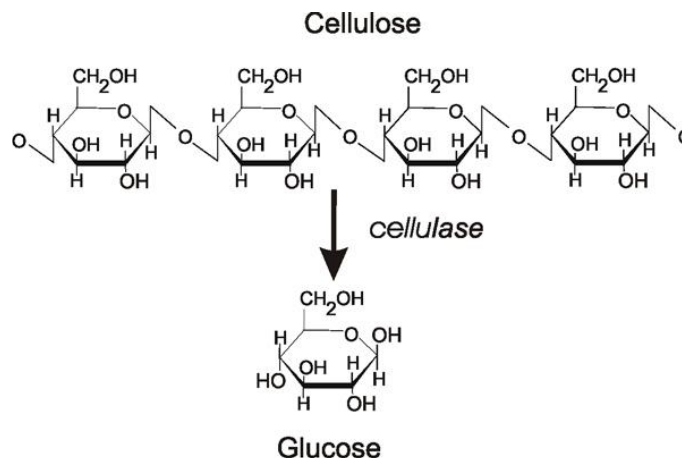


Figure 2.4: Cellulose hydrolysis
(Source: http://www.biotek.com/assets/tech_resources/11087/figure2.jpg)

2.5.2 Fermentation by yeast

Certain species of yeast (most importantly *Saccharomyces cerevisiae)* produce ethanol by metabolize fermentable sugars in absence of oxygen. Carbon dioxide is also produced in the overall chemical reaction. 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.

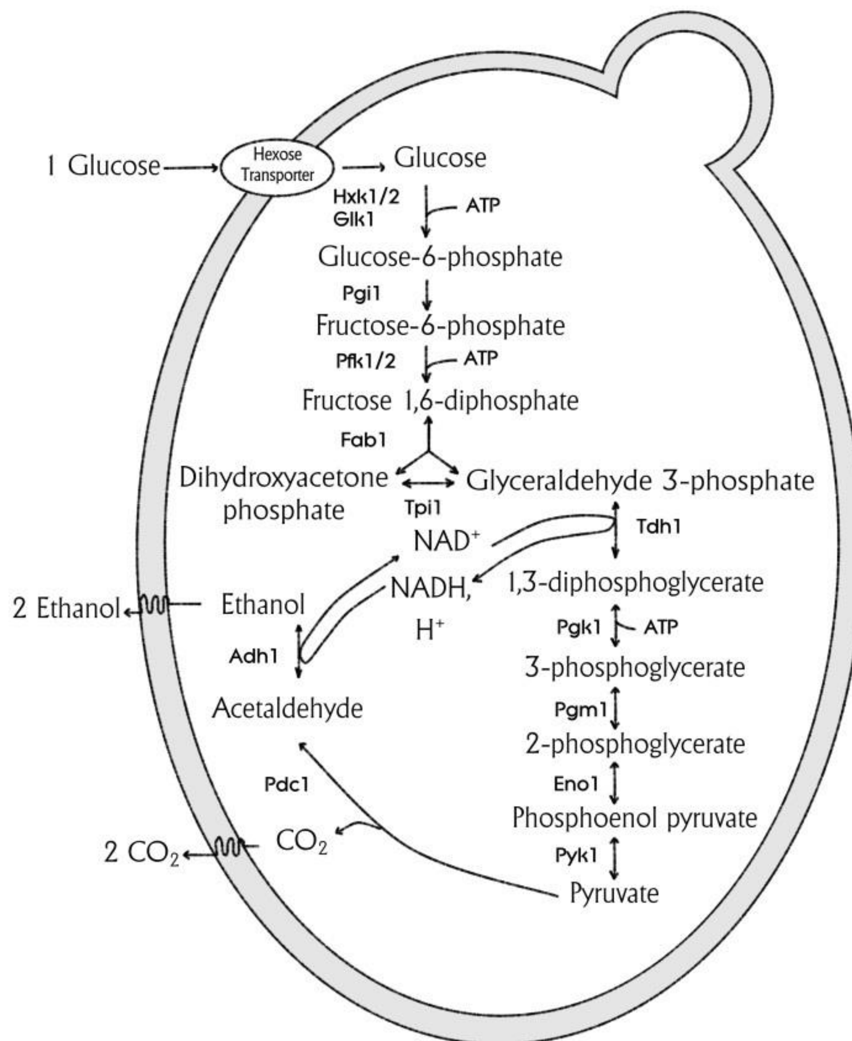


Figure 2.5: **A scheme of ethanol production pathway by yeast.**
(Source: http://www.intechopen.com/source/html/41685/media/image1.jpeg)

D-glucose and D-xylose are the major fermentable sugars from hydrolysis of most cellulosic biomasses. Naturally occurring saccharomyces yeast that are used for ethanol production can metabolize xylose and glucose (Sedlak and Ho, 2004). Besides biomass derived monosaccharides, *S. cerevisiae* can readily ferment glucose, mannose and fructose via Embden-Meyerhof pathway and galactose via Leloir pathway of glycolysis (Maris *et al.*, 2006).

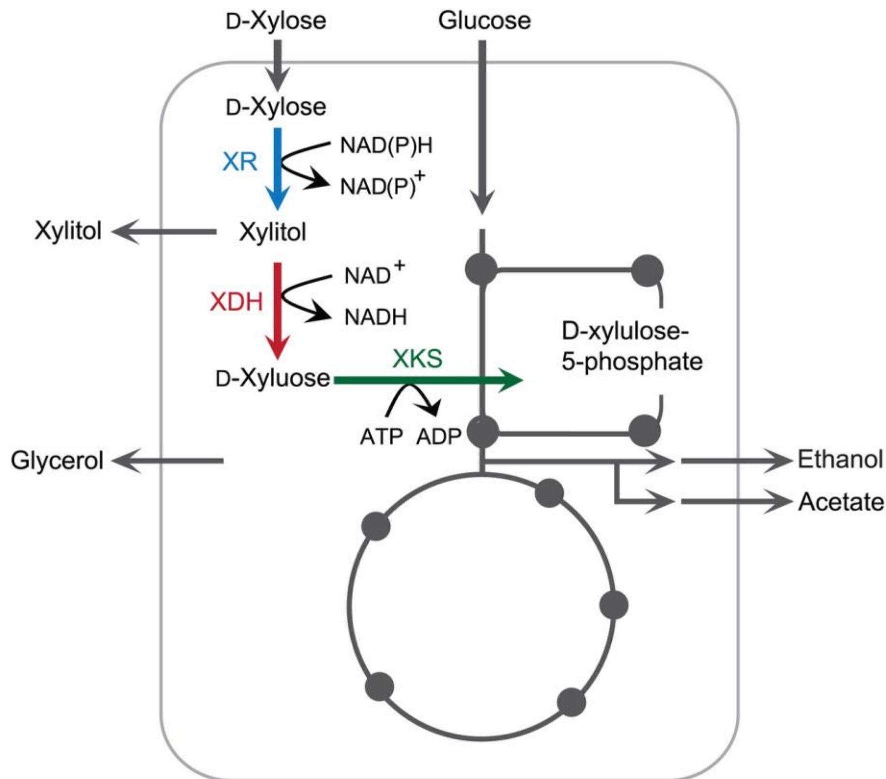


Figure 2.6: Xylose and Glucose utilization pathway
(Source: http://aem.asm.org/content/79/3/931/F1.large.jpg)

2.5.3 Enzymatic action by *Bacillus subtilis*

An efficient fermentation system can be developed by utilizing the ability of an organism to produce certain exoenzymes, including a-amylase, oligo-1, 6-glucosidase and cellulase that hydrolyze starch and cellulose. In this project, *Bacillus subtilis* (laboratory stock) was selected because of its aforementioned enzymatic properties. Successful utilization of cellulase and starch hydrolases depends on the enzyme source and function. Pre-treatment of cellulosic substrate with *Bacillus subtilis* has the potential to be resulted in the hydrolysis of starch and cellulose (FAO).

For starch hydrolysis, several enzymes are observed to act by cleaving α-1,4 and α-1,6 glucosidic bonds and releasing oligosaccharides of different chain lengths. Amylase is the major starch hydrolase from microorganism. Amylases are classified according to the specific glucosidic bond they cleave as α-1,4-glucanases or α-1,6-glucanases. Endoglucanases act on interior bonds of starch while exoglucanases cleave the bonds successively from nonreducing ends of starch.

Three major components present in cellulase enzymatic system are endo-ß-glucanase, exo-ß-glucanase and ß-glucosidase. The mode of action of each of these enzymes are random scission of cellulose chains yielding glucose and cello-oligo saccharides, exoattack on the non-reducing end of cellulase with cellobiose as the primary structure and hydrolysis of cellobiose to glucose respectively (Verma *et al*., 2012)

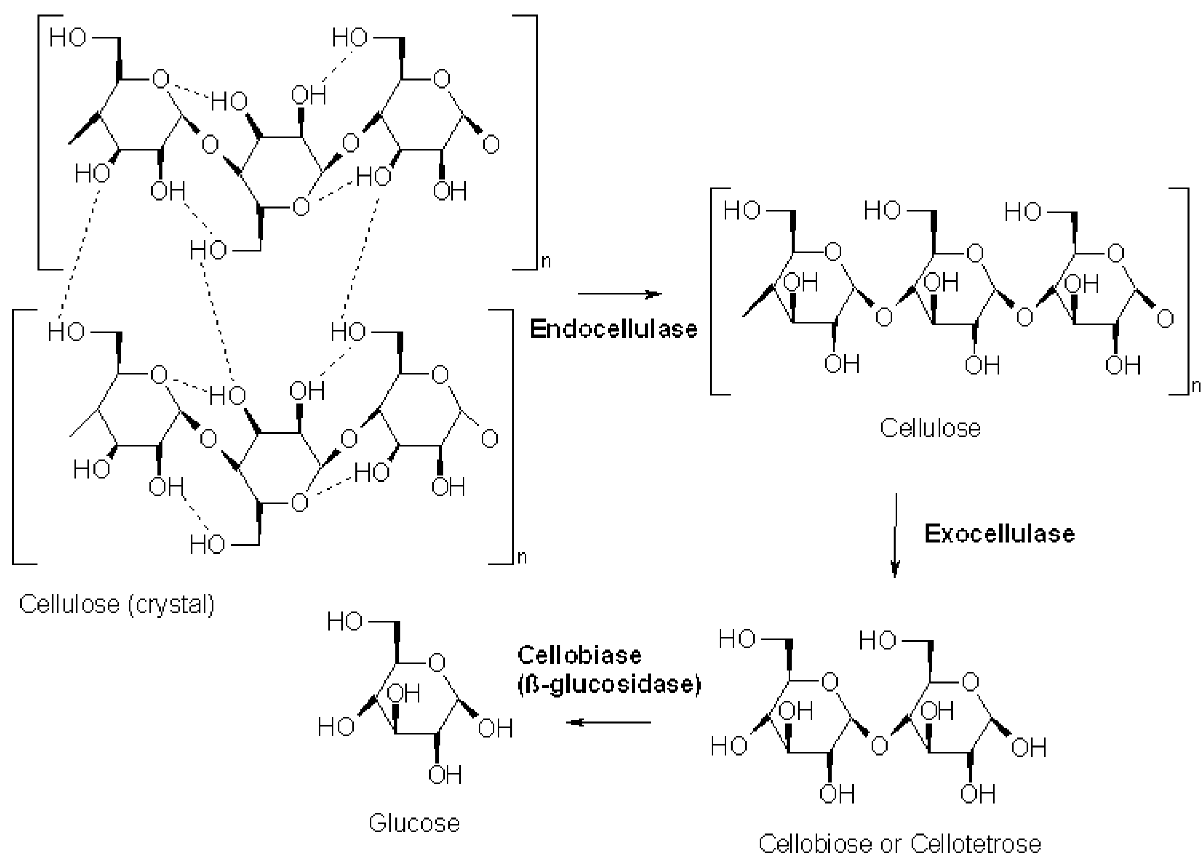


Figure 2.7: Cellulase mediated hydrolysis (Wyman et al., 2009)
(Source: http://docsdrive.com/images/academicjournals/jm/2011/fig1-2k11-41-53.gif)

2.6 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 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 is essential for the high production rate.

Chapter 3

Materials and Methods

3. Materials and Methods

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

3.1 Materials

3.1.1 Equipment

- Laminar airflow cabinet
- Spectrophotometer
- Incubator and shaking Incubator
- Vortex machine
- Autoclave machine
- Glasswares, laboratory distillation apparatus- fractional distillatory set up, microscope, pH meter petri dishes, slants, micro-pipettes, Bunsen burner, hot plate, clamp stands, electric balance, micro-burette, etc.

3.1.2 Samples

- Wild-type yeast strains isolated from different fruits.
- *Bacillus subtilis* from laboratory stock.

3.1.3 Reagents

DinitroSalicylic acid (DNS), Sulfuric acid, Sodium hydroxide, Sodium thiosulfate, Phenol red: phenolsulfonphthalein, 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 and API microbial identification kit were used.

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.
- **Carboxymethylcellulose agar medium**
  CMC is used to test bacteria (or fungi) for cellulolytic activity. It contains 1.5% peptone, 0.3% K_2HPO_4, 0.045% $MgSO_4$, 0.375% $(NH_4)_2SO_4$, gelatin: 0.3% 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 hydrochloric acid.
- **Defined sugar mediums:** Different composition of glucose and sucrose were used. Also molasses was used by boiling with the addition of 0.30ml concentrated sulfuric acid and 0.10gm urea (for 250gm molasses in 1000ml of distilled water)

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
- API microbial identification kit

3.2 Methods

3.2.1 Sample collection

Wild type of yeast strains were isolated from sugarcane juice, date juice and grapes. Aforementioned sources were collected from local market and kept for 1 week at room temperature for yeast growth.

3.2.2 Inoculum development

Liquid source sample (1ml) was serially diluted in sterile saline solution (0.9% NaCl) and inoculated onto the YEPD agar plate by spread palate technique. Incubation was done at 30°C for 48 hours. Culture broth was prepared (0.3% yeast extract, 1% peptone, and 2% dextrose) and autoclaved at 121°C and 15 psi. Colonies from agar plates were inoculated into the broth. After 24 hours incubation at 30°C temperature, 0.2 ml suspension from broth was again cultured (spread plating) on YEPD agar medium. This selective culture technique was used to isolate pure yeast strains.

3.2.3 Observation and culture maintenance

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

3.2.4 Identification of the yeast

3.2.4.1 Morphological characterization

General procedures were done for the identification of yeast based on morphological (Kreger-Van Rij, 1984; Mesa et al., 1999) and physiological characteristics. Selected 48 hours old cultures were inoculated on YEPD medium. Growth pattern was observed on that selective medium. Appearance of the isolates on YEPD agar medium were examined. The texture, color and surface of colonies were recorded. Shape of the cells were observed by compound microscope.

3.2.4.2 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 prepared with 10.0 gm peptone, 5.0 gm NaCl, 0.018 gm Phenol red indicator and 5.0 gm carbohydrates (for1000 ml). After addition of specific carbohydrates and adjusting the pH to 7.2, media distribution was done in 20 ml screw-cap test tubes with Durham tubes and then autoclaved. The sugar were used: The carbohydrate used were glucose (dextrose), sucrose, lactose, trehalose, maltose, starch, xylose and galactose. Tests for mannitol, inositol, sorbitol, mellibiose, arabinose, rhamnose, fermentation and urea hydrolysis were done by API kit. The principle of the sugar fermentation test is formation of (CO_2) gas in Durham tube and color change of the medium from red to yellow due to the formation of acids (Warren & Shadomy, 1991). Yeast suspension from 48 hours culture was inoculated into the broths and chambers of API kit containing specific carbohydrates.

Nitrate reduction test was performed to observe the nitrate utilization by yeast. Nitrate broth was prepared with 5.0 gm peptone, 3.0 gm beef extract and 5.0 gm potassium nitrate (1000 ml) and pH (7.0) was adjusted. Broth was then distributed into test tubes (10 ml screw cap test tubes) and autoclaved. Inoculation was done with an isolate from each sample plates and incubated for 48 hours. Then reagent A and reagent B were mixed carefully. If the organism produces nitrate reductase, the broth will turn a deep red within 5 minutes.

3.2.5 Stress tolerance characterization

3.2.5.1 Detection of thermo-tolerance

YEPD broth was prepared and autoclaved. The media was distributed into test tubes (10 ml each), and 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 recorded on 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, 40°C and 44°C for 48 hours to observe thermo tolerance of yeast strain. After incubation, optical density of the yeast suspensions were recorded.

3.2.5.2 Detection of ethanol-tolerance

YEPD liquid medium was used for the procedure. Ethanol of different concentrations were added with yeast growth medium (YEPD broth). After the media preparation it was sterilized. Media was distributed into test tubes and 1 ml absolute ethanol of different concentrations (5%, 10%, 15%, 20% and 25%) were added into the test tubes and marked. Test tubes were then inoculated with the selected yeast isolates and one test tube was not inoculated which was used as negative control. 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 measured again after the incubation at 600nm.

3.2.5.3 Growth at different pH

Sterile YEPD broth was distributed into two sets of 10 test tubes. Each test tubes contained 10 ml of media. YEPD broth of each test tube was adjusted to different pH (2 to 10). Then the broth containing 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 measured.

3.2.6 Fermentation media preparation

3.2.6.1 Defined sugar media

Utilization of different sugars as fermentation substrate was examined. 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 maintained 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 percentages of their concentration. Media of 10%, 15% and 20% concentrations were made by adding 10 gm, 15 gm and 20 gm of the sugars in 100 ml distilled water. After the distribution in conical flasks, they were autoclaved.

3.2.6.2 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 (individual and combinations). These vegetable peels were collected from households and chopped into smaller pieces. 250 gm of solid wastes were pulverized with 1000 ml water 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 create a good fermentation condition and primary hydrolysis of sugars.

3.2.7 Fermentation

Ethanol fermentation procedure was performed by inoculation yeast in the prepared fermentation mediums. Different parameters were fixed to observe fermentation efficiency and ethanol production rate.

3.2.7.1 Preparation of microorganism cell suspensions

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

b) *Bacillus subtilis*: Organism was taken from laboratory stock (MNS bioscience laboratory). To make cell suspension, 24 hours old culture from nutrient agar was placed into 10 ml NaCl (0.9%) saline. It was selected based on following characteristics (previously identified by lab authority):

- Gram staining, white colored colony and Rod shaped cell morphology.
- Sugar utilization tests were positive for glucose and sucrose but negative for citrate, xylose, mannitol and lactose.
- Urease, oxidase, catalase, nitrate reduction, casein hydrolysis and motility tests were positive and indole reduction was negative.

3.2.7.2 Fermentation of defined sugars

Inoculation of yeast in fermentation media was done inside Laminar biosafety cabinet. 150 ml 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 cotton plugged and incubated at different temperature in both non-shaking and shaking condition.

3.2.7.3 Fermentation of cellulosic kitchen wastes

150 ml fermentation media was taken into 500 ml Erlenmeyer conical flasks. Flasks of kitchen waste (peel of vegetables) media were aseptically inoculated with *Bacillus subtilis* suspension and incubated for 24 hours at 36°C in shaking condition (80rpm). After the incubation, yeast cell suspension was inoculated and the flasks were cotton plugged and incubated in a rotary incubator at 30°C in shaking condition (120 rpm).

3.2.8 Estimation of ethanol

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

3.2.8.1 Conway method

Oxidation-reduction titration principle with Conway unit was used to determine the ethanol content in the fermented broth and its distilled product. In this method ethanol is oxidized to ethanoic acid when ethanol react with excess of potassium dichromate solution (0.05 N) and unreacted dichromate is then determined by adding potassium iodide (50% KI) solution which is oxidized by the potassium dichromate. Potassium iodide reacts with potassium dichromate and creates iodine. Then the iodine is titrated with a standard solution of sodium thiosulfate (0.1N). The titration reading is used to calculate the ethanol content after fermentation (Ingram *et al.*, 1987). Fermentation media were taken out of 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 1ml potassium dichromate was placed into the Conway unit center.

After placing 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 occurs in the presence of ethanol when it evaporate and react 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 colorless and data was recorded.

b) Calculation:

After the titration, the data was calculated by using the following formula to detect 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 cm3

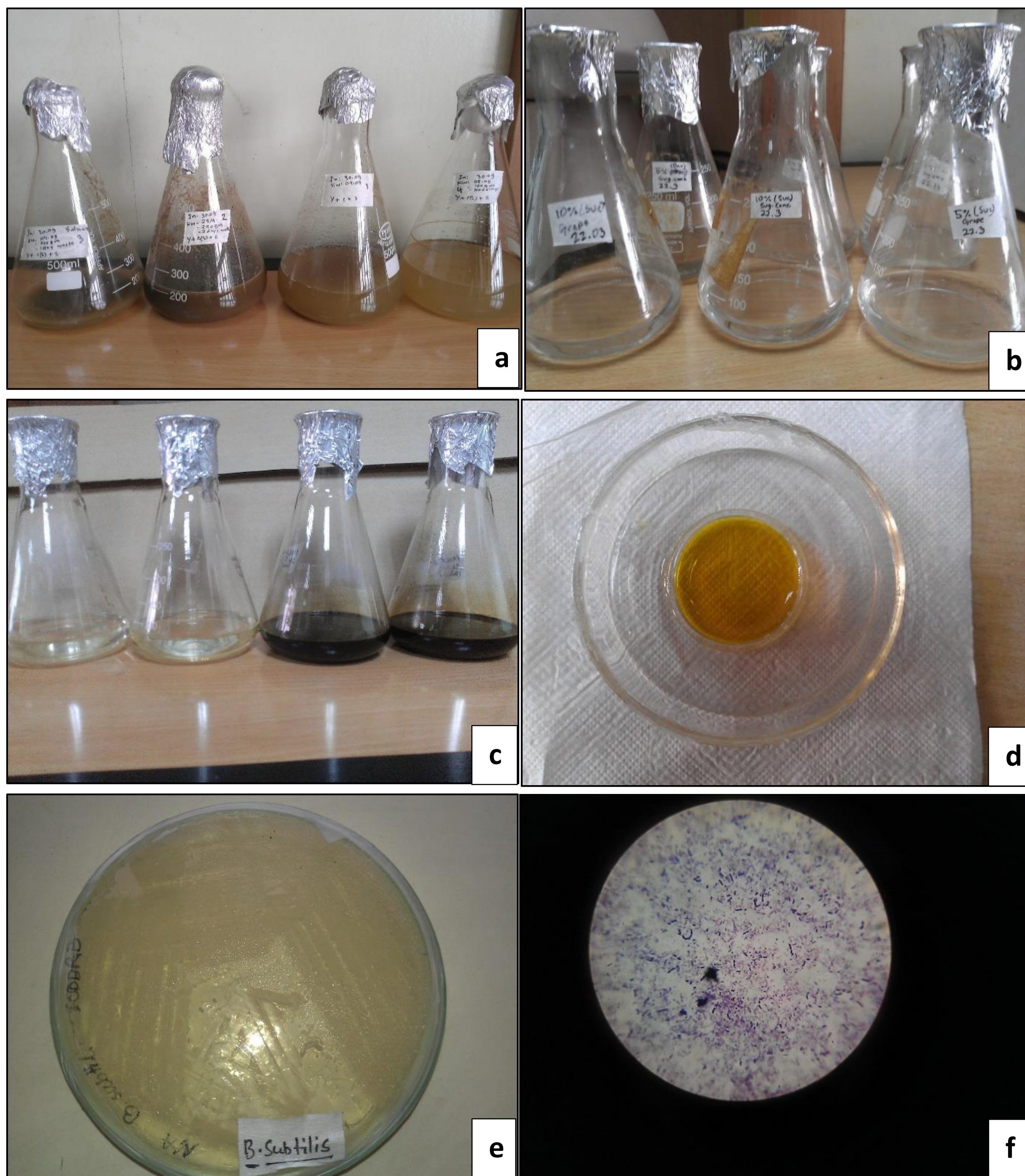


Figure 3.1: a) Kitchen waste media, b) Glucose and sucrose media, c) Sucrose and molasses media, d) Ethanol estimation by Conway unit, e) Culture of *Bacillus subtilis* and f) Staining of *Bacillus subtilis*.

3.2.8.2 Alcohol meter

Based on the result of Conway titration method, sample of higher production rate were selected for distillation. The distilled product was then examined by alcohol meter to detect the alcohol percentage.

a) Distillation:

Fractional distillatory set was used to separate ethanol from fermented broths. The fermented broth was placed onto the heating unit of the machine and water flow was connected. 78°C (evaporation temperature of ethanol) was maintained manually. The Distilled product was then collected.

b) Distillation vinometer

An alcoholmeter was used to measure the density of ethanol in distilled product as compared to water. Physical property based on specific gravity was used in this process. 50 ml distilled product was taken in a 50 ml measuring cylinder. The alcohol meter was placed in it and reading was recorded.

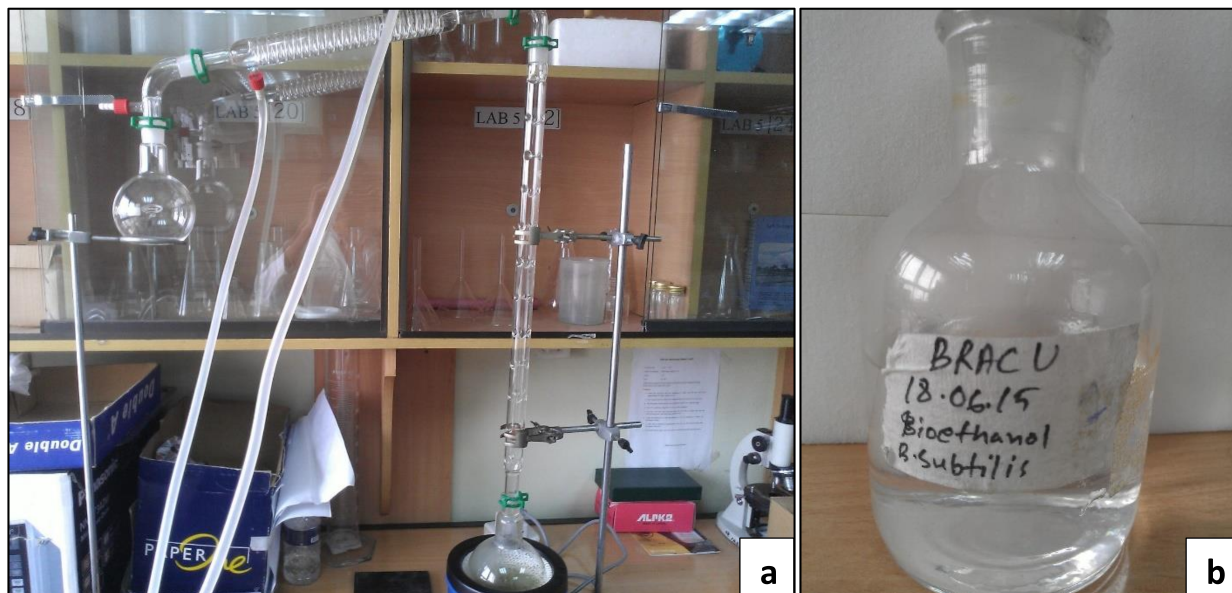


Figure 3.2: a) Distillation procedure, b) Distilled product from fermented substrate

Chapter 4

Results

4. Results

4.1 Identification of the yeast

4.1.1 Morphological characterization

Selected yeast cell morphology was detected on the basis of their growth on YEPD media. Also presence of yeast was confirmed by colony morphology and microscopic observation.

4.1.1.1 Growth on YEPD agar

Smooth, semi-white yeast colonies with butter like consistency were observed on YEPD agar plate. Colony shape and surface appearance were used for the confirmation.

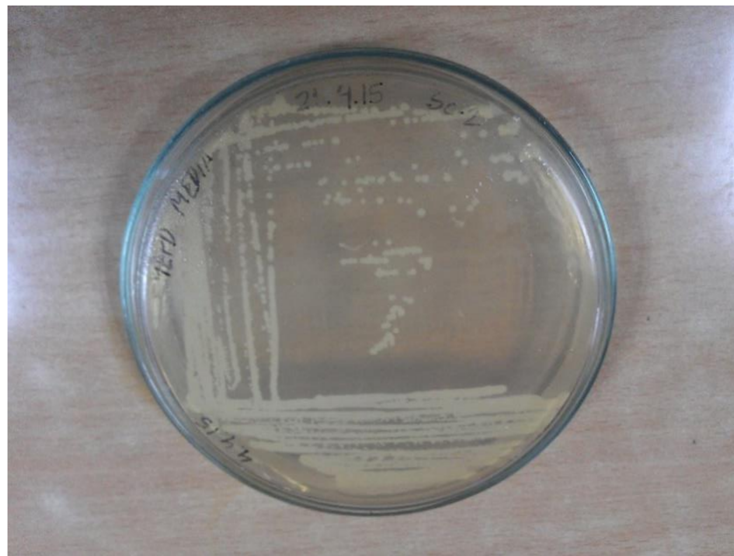
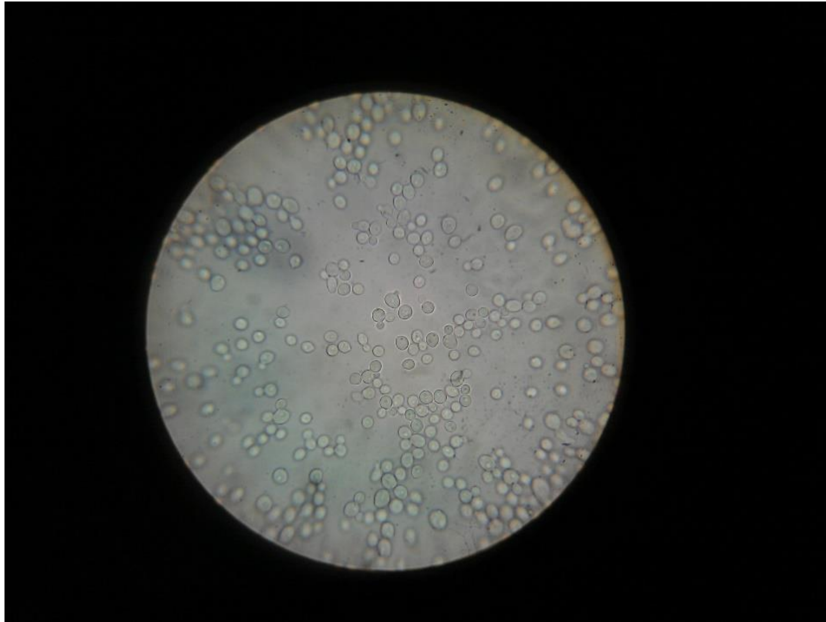


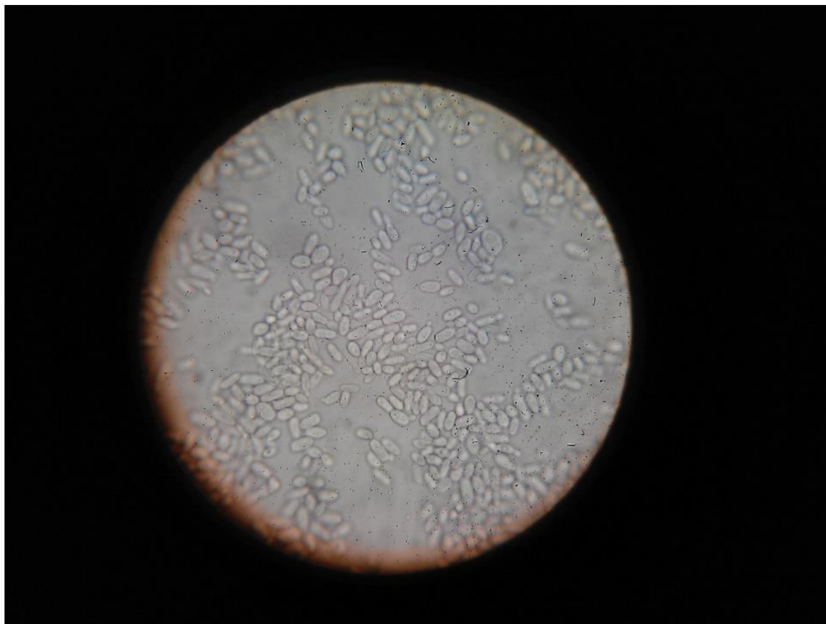
Figure 4.1: Colonies on YEPD agar medium after 24 hours incubation

4.1.1.2 Microscopic observation

Compound microscope was used to observe the cell morphology of yeasts isolated from date-juice & sugarcane-juice isolates. Vegetative reproduction via budding was detected. Yeast isolates from date juice was oval and strain isolated from sugarcane was round in shape.



(a)



(b)

Figure 4.2: The cell morphology under compound microscope (100X)
a) From sugarcane juice , b) From date-juice

4.2 Result of physiological characterization

4.2.1 Fermentation of carbohydrates

In the present study utilization of different sugars by yeast isolates were observed. Yeast isolates from sugarcane (SC1) utilized glucose, maltose, fructose, galactose, starch, sucrose, arabinose but failed to grow on sorbitol, mellibiose, mannitol, trehalose, inositol, xylose and lactose. Yeast strains from date juice (DJ1) utilized glucose, maltose, fructose, galactose, starch, but failed to grow on trehalose, xylose, Sucrose and Lactose. Results were taken after 24 hours incubation.

Carbohydrate	Fermentation
Glucose	+ + (gas)
Maltose	+ + (gas)
Galactose	+ + (gas)
Starch	+ +
Sucrose	+ -
Arabinose	+ -
Fructose	+ + (gas)
Trehalose	_ _
Mellibiose	_ _
Mannitol	_ _
Lactose	_ _
Xylose	_ _
Inositiol	_ _
Sorbitol	_ _

Table 4.1: Fermentation result of different carbohydrates for sugarcane juice (SC1) isolate (Positive: + +, Variable: + -, Negative: --)

Carbohydrate	Fermentation
Glucose	+ + (gas)
Maltose	+ + (gas)
Galactose	+ + (gas)
Starch	+ +
Sucrose	– –
Fructose	+ + (gas)
Trehalose	– –
Lactose	– –
Xylose	– –

Table 4.2: Fermentation result of different carbohydrates for date juice (DJ1) isolate (Positive: + +, Variable: + -, Negative: --)

4.2.2 Reduction of potassium nitrate

After 24 hours incubation in nitrate media, reagent A and B was mixed to observe the color change. Both yeast isolates from sugarcane juice (SC1) and date juice (DJ1) did not show the nitrate reductase activity as the color of the medium did not change to red after the addition of specific reagents.

Strain	Nitrate reduction Test
SC1	– –
DJ1	– –

Table 4.3: Nitrate reduction by yeast isolates
(Positive: + +, Variable: + -, Negative: - -)

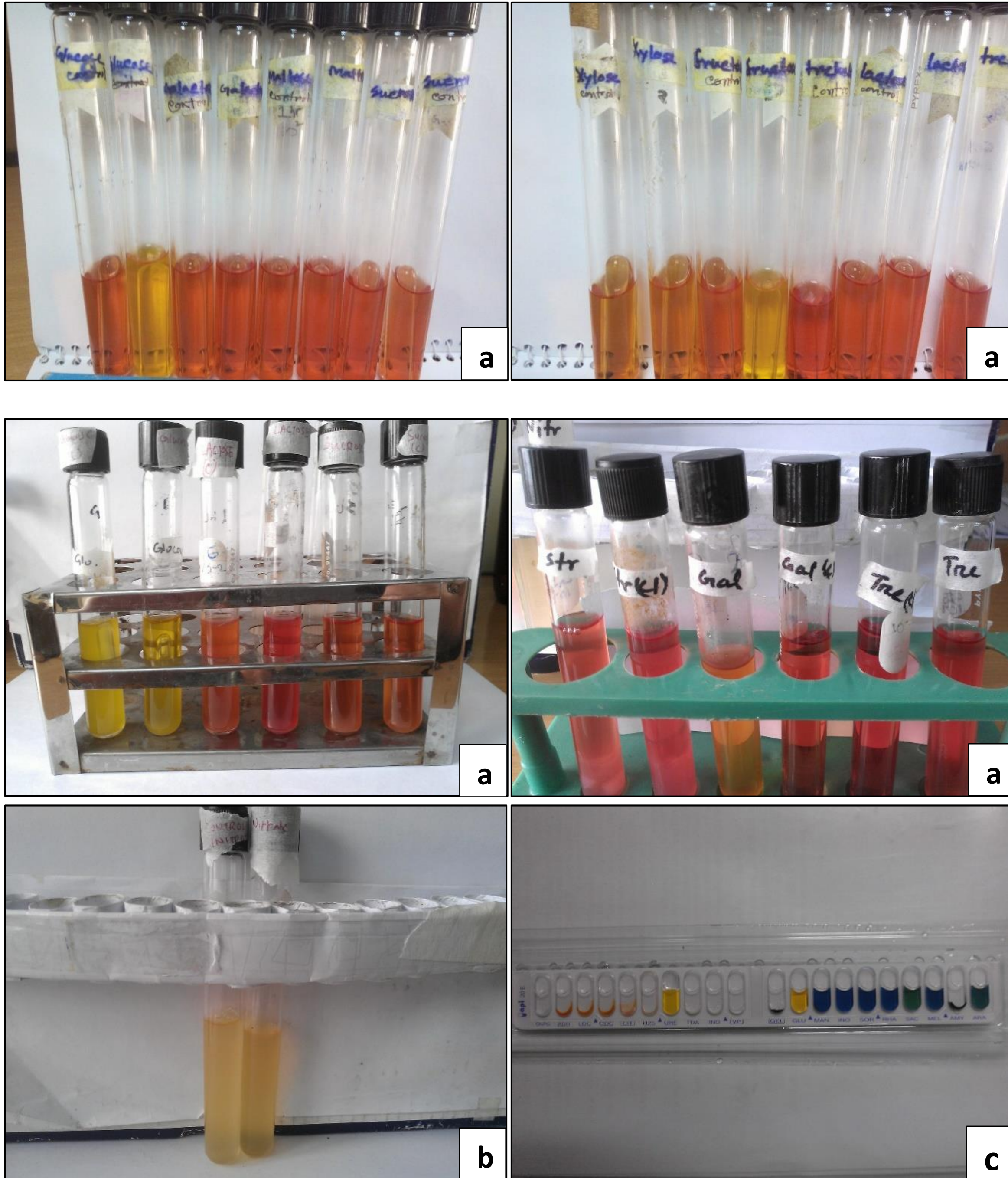


Figure 4.3: Results of physiological characterization of yeast isolates

a. Fermentation of different carbohydrates, b. Result of nitrate reduction test, c. Test results of different carbohydrates fermentation using API kit

4.3 Result of stress tolerance characterization

4.3.1 Thermotolerance test result

Thermotolerance range was observed from the growth at 5 different temperatures. Both of the yeast isolates (SC1 and DJ1) showed variations in their growth. Based on the optical density count, growth result at YEPD liquid media was used for thermotolerance range detection (table 4.4). Yeast isolates from sugarcane juice (SJ1) had a good growth at 25°C, 30°C, 37°C and 40°C but grew poorly at 44°C. Yeast isolates from date juice (DJ1) had a good growth at 30°C, 37°C and 40°C but grew poorly at 25°C and 44°C.

According to the result, yeast isolate SC1 has comparatively better tolerance than the isolate DJ1. But at 30°C, both of the strains did show best growth result.

Temperature	Isolate	Growth after 24 hours	Growth after 48 hours
25°C	SC1	+ +	+ -
	DJ1	+ -	+ -
30°C	SC1	+ +	+ +
	DJ1	+ +	+ +
37°C	SC1	+ +	+ +
	DJ1	+ +	+ -
40°C	SC1	+ +	+ -
	DJ1	+ -	+ -
44°C	SC1	+ -	- -
	DJ1	- -	- -

Table 4.4: Growth of strain SC1 and DJ1 at different temperatures

(Positive: + +, Moderate: + -, Negative: - -)

4.3.2 Ethanol tolerance test result

Ethanol tolerance range was observed from the 24 and 48 hours growth result of isolate SC1 and isolate DJ1 at different ethanol concentrations (5%, 10%, 15%, 20% and 25%). Optical density was taken from the inoculated YEPD culture broth after 24 hours and 48 hours. Isolate SC1 showed good growth at 5%, 10%, 15%, 20% ethanol concentration but failed to grow at 25% concentration Isolate DJ1 had good growth at 5%, 10% and 15% ethanol concentrations but failed to grow at 20% and 25% concentrations. Here, 10% ethanol concentration was the optimum condition for both of the isolates at 30°C incubation temperature.

Ethanol percentage	Strain	Growth after 24 hours	Growth after 48 hours
5%	SC1	+ +	+ +
	DJ1	+ +	+ +
10%	SC1	+ +	+ +
	DJ1	+ +	+ +
15%	SC1	+ +	+ +
	DJ1	+ +	+ -
20%	SC1	+ -	- -
	DJ1	- -	- -
25%	SC1	- -	- -
	DJ1	- -	- -

Table 4.5: Growth of yeast strain SC1 and DJ1 at different ethanol concentrations.

(Positive: + +, Moderate: + -, Negative: - -)

4.3.3 pH tolerance test result

Yeast isolate SC1 and DJ1 had a variable growth result at pH 2-10. Both of the isolates had excellent 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 suitable for a good growth.

Overall, pH 5 and 6 was optimum growth conditions where the isolate SC1 had its best growth at pH 6 and strain DJ1 had its best growth at pH 5.

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

pH	Strain	Growth after 24 hours	Growth after 48 hours
2	SC1	+ -	- -
	DJ1	+ -	- -
3	SC1	+ -	- -
	DJ1	+ -	+ -
4	SC1	+ -	- -
	DJ1	+ +	+ -
5	SC1	+ +	+ +
	DJ1	+ +	+ +
6	SC1	+ +	+ +
	DJ1	+ +	+ +
7	SC1	+ +	+ -
	DJ1	+ -	- -
8	SC1	+ +	+ -
	DJ1	+ -	+ -
9	SC1	+ -	- -
	DJ1	+ -	- -
10	SC1	+ -	- -
	DJ1	+ -	- -

Table 4.6: Growth of yeast strain SC1 and DJ1 at different pH

(Positive: + +, Moderate: + -, Negative: - -)

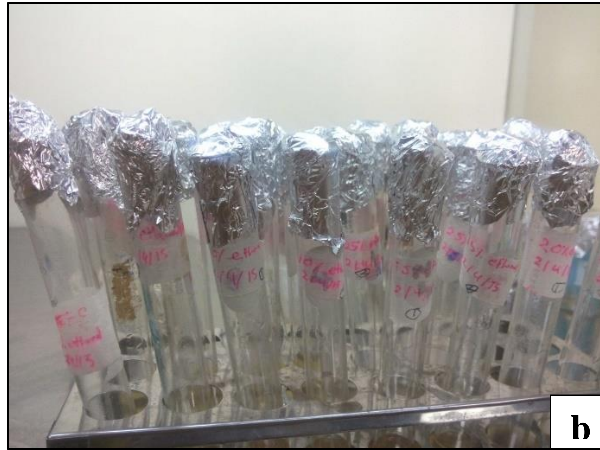


Figure 4.4: Result of tolerance to different conditions

a. Growth result at different temperatures, b. Growth result at different ethanol concentrations, c. Growth result at different pH, d. Culture of SC1 strain at 30°C

4.4 Ethanol fermentation

4.4.1 Ethanol production from defined sugar mediums

Ethanol production was detected 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 production was low. The ethanol production rate was recorded for the aforementioned sugars in shaking condition at 30°C, pH 6. The production rate ranged from 1.71% to 6.23% from per 100 ml of substrate medium. Sugarcane isolate SC1 had the highest rate of ethanol production (6.23%) from molasses and date juice isolate DJ1 had the lowest rate of ethanol production (1.71%) from sucrose. Ethanol production rate was also observed for the same substrates in shaking condition at 30°C, pH 5. In this condition, Sugarcane isolate SC1 had the highest rate of ethanol production (5.42%) from molasses and date juice isolate DJ1 had the lowest rate of ethanol production (2.17%) from sucrose.

Defined Sugar Medium	Isolate	Percentage of ethanol 24 hours	Percentage of ethanol 48 hours
Glucose	SC1	2.24%	2.34%
	DJ1	1.89%	1.94%
Sucrose	SC1	2.17%	2.19%
	DJ1	1.71%	1.80%
Molasses	SC1	6.23%	5.89%
	DJ1	3.24%	3.89%

Table 4.7: Alcohol production from defined sugars by yeast isolate SC1 and DJ1 at pH 6

Defined Sugar Medium	Isolate	Percentage of ethanol 24 hours	Percentage of ethanol 48 hours
Glucose	SC1	3.34%	2.96%
	DJ1	3.79%	3.84%
Sucrose	SC1	2.69%	2.77%
	DJ1	2.17%	2.24%
Molasses	SC1	5.12%	5.42%
	DJ1	4.34%	4.93%

Table 4.8: Alcohol production from defined sugars by yeast isolate SC1 and DJ1 at pH 5

4.4.2 Ethanol production from cellulosic vegetable peels

Pretreatment of vegetable peels with *Bacillus subtilis* increased the rate of alcohol production. Ethanol production rate was estimated by the titration method using Conway unit. Two sets of substrates/combination of peels of substrates were used as medium. In a fermentation condition of 30°C incubation temperature with a pH of 6, the highest rate of alcohol production from pretreated medium (papaya + potato) was 17.39% per 100 ml by yeast isolate SC1. Lowest rate of alcohol production from pretreated medium (basil) was 5.17% per 100 ml by yeast isolate DJ1. On the other hand, at same fermentation condition, highest rate of alcohol production from untreated medium was 3.25% by SC1 (from papaya). Lowest alcohol production rate recorded from untreated medium was 1.15% (from potato+ cucumber) by yeast strain DJ1.

Defined Sugar Medium	Isolate	Percentage of ethanol 24 hours	Percentage of ethanol 48 hours
Potato (150gm/1000ml)	SC1	8.27%	7.11%
	DJ1	6.95%	6.38%
Papaya (150gm/1000ml)	SC1	11.65%	12.05%
	DJ1	10.02%	10.18%
Cucumber (150gm/1000ml)	SC1	13.61%	13.23%
	DJ1	9.42%	8.96%
Lady’s finger (150gm/1000ml)	SC1	5.88%	5.89%
	DJ1	5.27%	5.48%
Basil (150gm/1000ml)	SC1	6.35%	6.37%
	DJ1	**5.17%**	5.21%
Potato+ Papaya (75gm+75gm/1000ml)	SC1	16.88%	**17.39%**
	DJ1	13.69%	12.24%
Potato+ Cucumber (75gm+75gm/1000ml)	SC1	7.19%	6.92%
	DJ1	6.57%	6.55%
Cucumber + Papaya (75gm+75gm/1000ml)	SC1	15.29%	16.25%
	DJ1	11.12%	11.78%

Table 4.9: Alcohol production from vegetable peels by yeast isolate SC1 and DJ1 at pH 6 (Substrates were treated with *Bacillus subtilis*)

Defined Sugar Medium	Isolate	Percentage of ethanol 24 hours	Percentage of ethanol 48 hours
Potato (150gm/1000ml)	SC1	2.98%	3.12%
	DJ1	1.66%	1.68%
Papaya (150gm/1000ml)	SC1	3.21%	**3.25%**
	DJ1	2.19%	2.22%
Cucumber (150gm/1000ml)	SC1	2.89%	2.82%
	DJ1	1.57%	1.36%
Lady's finger (150gm/1000ml)	SC1	2.52%	2.25%
	DJ1	2.31%	2.38%
Basil (150gm/1000ml)	SC1	2.19%	2.96%
	DJ1	1.87%	1.85%
Potato+ Papaya (75gm+75gm/1000ml)	SC1	3.20%	3.23%
	DJ1	2.12%	2.48%
Potato+ Cucumber (75gm+75gm/1000ml)	SC1	3.16%	3.18%
	DJ1	1.17%	**1.15%**
Cucumber + Papaya (75gm+75gm/1000ml)	SC1	3.07%	3.19%
	DJ1	2.93%	3.01%

Table 4.10: Alcohol production from kitchen wastes by yeast isolate SC1 and DJ1 at pH 6 (Substrates were not treated with *Bacillus subtilis*)

4.5 Distillation

Fermented media with the highest percentage of alcohol (17.39%) was distilled by a fractional distillatory set. This highest percentage was achieved by the yeast isolate SC1 in a fermentation condition of 30°C, pH 6 at 120 rpm. Using an alcohol meter, the percentage of alcohol in the distilled products were measured. The pretreated fermentation media (potato+ papaya) was distilled after the fermentation and the distilled product (one time distillation) had an ethanol percentage of 32% (approximate). Re-distillation of the first distilled product had an alcohol percentage of 54% (approximate). Ethanol percentages in the same distilled products were also estimated using Conway method. From the first distillation the percentage was 34.74% and from the re-distilled product, the alcohol percentage was 52.29%.

Figure 4.5: Alcohol production estimation by alcohol meter.

a. Percentage result of re distilled product. b. Percentage result of first distilled product

Estimation technique	Percentage first distilled product	Percentage of re-distilled product
Conway method	34.74%	52.29%.
Alcohol vinometer	32%	54%

Table 4.11: Comparison between the results by Conway method and alcohol meter

Chapter 5

Discussion

5. Discussion

Despite the availability of several industrial strains of yeasts, local isolates are usually more adapted to their own climatic condition. In this study, yeasts were isolated from local resources. The isolates were subjected to be screened via different sets of parameters. The utilization of isolated yeasts is an important strategy for the production of bioethanol.

On the basis of white and creamy appearance of selected isolates on solid media with butyrous colony texture, polar budding and oval cellular shape it can be assumed that isolates are members of *Saccharomyces spp* (Boekhout and Kurtzman, 1996).

Fermentation of different sugars by the selected yeast isolates were observed. Yeast isolate from sugarcane (SC1) utilized glucose, maltose, fructose, galactose, starch, sucrose, and arabinose but failed to grow on sorbitol, mellibiose, mannitol, trehalose, inositol, xylose and lactose (Table 4.1). Yeast isoltae from date juice (DJ1) utilized glucose, maltose, fructose, galactose and starch, but failed to grow in trehalose, xylose, sucrose and lactose (Table 4.2). The selected isolates also gave negative results in nitrate reduction test where no reduction of potassium nitrate took place (Table 4.3). Recorded results suggested that those yeast isolates can be identified as *Saccharomyces spp* (Guimaraes *et al*., 2006; Vaughan-Martini and Martini, 1993). Furthermore, from the microscopic observation (Figure 4.2) the yeast isolates were found to belong *Saccharomyces* type species.

From the thermotolerance test report it was found that both of the yeast isolates (SC1 and DJ1) were able to grow at 30°C which is optimum growth for *Saccharomyces cerevisiae* (Alexopoulos, 1962). Yeast isolate SC1 and DJ1 showed a good growth from 30°C to 37°C (Table 4.4).

Ethanol tolerance test is a significant criteria to compare similarities with *Saccharomyces spp*. Both yeast isolates (DJ1 and SC1)) were tested for ethanol tolerance and up to 20% ethanol tolerance ability was observed in YEPD liquid growth media (Table 4.5). In the present study it has been observed that growth became slower over 10% ethanol concentration. *Saccharomyces spp* can tolerate the highest concentration of ethanol (up to 20%) among the eukaryotic organisms. Teramoto *et al*. reported that *Saccharomyces cerevisiae* can tolerate up to 16.5% ethanol (2005).

Different growth factors affects the pH tolerance of yeast. Depending on strain, the optimum pH range for ideal growth does vary from 4-6 (Ivorra *et al*., 1999). The cellular structure of yeast has

diverse mechanism to endure pH. In this experiment, yeast isolates SC1 and DJ1 had a variable growth result from pH 2-10. Both of the isolates had excellent growth from pH 4 to 6. However, those isolates were able to grow at all the pH condition, but pH lower than 3 and higher than 7 was not that much suitable for a good growth. Overall, pH 5 and 6 were optimum growth conditions (Table 4.6) where isolate SC1 had its best growth at pH 6 and isolate DJ1 had its best growth at pH 5.

The ethanol production rate was recorded from the fermentation of molasses, glucose and sucrose after 24 and 48 hours fermentation. The production rate ranged from 1.71% to 6.23%. Isolate SC1 had the highest rate of ethanol production (6.23%) from molasses and isolate DJ1 had the lowest rate of ethanol production (1.71%) from sucrose. Ethanol production rate was also observed for the same substrates in shaking condition at 30°C, pH 5. In this condition, isolate SC1 had the highest rate of ethanol production (5.42%) from molasses and isolate DJ1 had the lowest rate of ethanol production (2.17%) from sucrose. Other author observed that 7.8% percent of (m/v) ethanol production from sugarcane molasses using *Saccharomyces cerevisiae* has been reported (Nofemele *et al*., 2012). 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).

The primary purpose of the present study was to increase the ethanol production rate from the fermentation of cellulosic kitchen waste (vegetable peels) using cellulose degrading bacteria. *Bacillus subtilis* was used for its cellulolytic activities and pretreatment by this bacteria increased the production rate. In a fermentation condition of 30°C incubation temperature with a pH of 6, the highest rate of alcohol production from pretreated medium (papaya + potato) was 17.39% per 100 ml by yeast isolate SC1 (table 4.9). The lowest rate of alcohol production from pretreated medium (basil) was 5.17% per 100 ml by yeast isolate DJ1. On the other hand, at same fermentation condition, the highest rate of alcohol production from untreated medium was 3.25% by SC1 (from papaya). The Lowest alcohol production rate was recorded from untreated medium was 1.15% (from potato+ cucumber) by isolate DJ1. Estimation of ethanol was done by titration method using Conway unit. The fermented media with the highest ethanol production rate was distilled for ethanol recovery. After two times distillation, approximately 54% (Table 4.11) ethanol was obtained (according to the reading of alcohol meter).

Therefore, yeast isolate from sugarcane juice showed the highest percentage of alcohol production from cellulosic kitchen wastes. Vegetable peels pretreated by cellulolytic bacteria is detected as a suitable fermentation substrate. The fermentation condition was optimized, this procedure may be used for large scale bioethanol production form cellulosic wastes.

Chapter 6
Conclusion

6. Conclusion

From microscopic observation and morphological features, yeast like cells was recognized. From the biochemical and physiological characteristics, the isolates can be considered as *Saccharomyces spp.* From two yeast isolates, isolate from sugarcane (SC1) showed the highest ethanol productivity.

For isolate SC1 the optimum fermentation temperature was 30°C with a pH of 6.0 where the highest percentage of alcohol was produced from papaya and potato peels. Within optimum fermentation period (24 and 48 hours), the ethanol production by isolate SC1 was up to 17.39%. *Bacillus subtilis* was used for its cellulolytic activity by which it could make cellulosic materials into smaller sugars that can be easily fermented by yeast.

The experiment was done in a laboratory controlled condition. Scaling up of the experiment can be beneficial for the power generation as this bioethanol can be used as an alternative of fossil flues. The raw materials required for the production of bioethanol is cheap and available. As a fuel, it will decrease the environment pollution, will make an opportunity to develop a proper waste management system and also fertilizers can be produced from the used substrates.

Chapter 7
References

7. References

Adarsha, A Asha DL and Balaji,RR (2010). African journal of Microbiology Research. 4(12), 1340-1342.

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

Balat M, Balat, H Oz, C (2008). Progress in bioethanol processing. Prog. Energ. Combust. 34, 551–573.

Balasubramanian M E. Bi, and M. Glotzer (2004). Comparative analysis of cytokinesis in budding yeast, fission yeast and animal cells. Curr Biol 14(18): R806-18. PMID 15380095.

Basso LC and Rosa CA (2010). Sugar cane for potable and fuel ethanol. In: Worldwide Distilled Spirits Conference - New Horizons: energy, environment and enlightenment. Walker GM and Hughes PS (eds). Nottingham: Nottingham University Press, p.1-7.

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

Camobreco V, Ham R, Barlaz M, Repa E, Felker M, Rousseau C, Rathle J (1999). Life-cycle inventory of a modern municipal solid waste landfill. Waste Manage. Res. 17:394-408.

Chan KY, Au KS (1987). Studies on cellulase production by a *Bacillus subtilis*. Antonie Van Leeuwenhoek; 53(2):125-36. PMID: 3116921

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

Conway, Roger K, McClelland J, and Shapouri H (1994). Comments Concerning the Environmental Protection Agency's Regulation of Fuels and Fuel Additives: Renewable Oxygenate Requirement for Reformulated Gasoline Proposed Rule, Public Document A-93-49. U.S. Department of Agriculture,Office of Energy

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

EBI: European Bioinformatics Institute (2009). "Bacteria Genomes – Bacillus Subtilis." Available from: http://www.ebi.ac.uk/2can/genomes/bacteria/Bacillus_subtilis.html

FAO Corporate Document Repository. Renewable biological systems for alternative sustainable energy production. Agriculture and Consumer Protection. Retrieved on: July 21, 2015. Available from: http://www.fao.org/docrep/w7241e/w7241e08.htm.

Guimaraes TM, Moriel DG, Machado IP, Picheth CF, Bonfim T (2006). Isolation and characterization of Saccharomyces cerevisiae strains of winery interest. Brazilian Journal of Pharmaceutical Sciences. vol. 42, n. 1, Jan. /mar.

Genomics: GTL (2006). Fuel ethanol production. Genomics: GTL. Yeast. New World Encyclopedia. Available from: http://www.newworldencyclopedia.org/entry/Yeast.

Ghose T.K, Tyagi R.D. (2004). Rapid ethanol fermentation of cellulose hydrolysate. II. Product and substrate inhibition and optimization of fermentor design. Biotechnology and Bioengineering. Volume 21, Issue 8, pages 1401–1420, August 1979. DOI: 10.1002/bit.260210808.

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

Hägerdal BH et al. (2007). Towards industrial pentose-fermenting yeast strains. Applied Microbiology and Biotechnology. Vol: 74, Issue 5, pp 937-953. Doi: 10.1007/s00253-006-0827-2

Haissig BE and Dickson RE (2006). Starch Measurement in Plant Tissue Using Enzymatic Hydrolysis. Physiologia Plantarum. Volume 47, Issue 2, pages 151–157. DOI: 10.1111/ j.1399-3054.1979.tb03207.x

Helsinki University of Technology (2006). Biofuelcell. Helsinki University of Technology. Retrieved December 24, 2006. Available from: www.newworldencyclopedia.org/entry/Yeast

Ho, NWY, Chen Z, and Brainard AP (1998). Genetically engineered Saccharomyces yeast capable of effective cofermentation of glucose and xylose". Applied Environmental -Microbiology 64(5): 1852-1859

Huang D et. al (2011). Fructose impairs glucose-induced hepatic triglyceride synthesis. Lipids Health Dis. Available from: http://joelbergerdc.com/tag/glucose-vs-fructose.

Ingram LO, Conway T, Clark DP, Sewell GW and Preston JF (1987). Genetic Engineering of Ethanol Production in Escherichia coli. Applied and environmental microbiology. p. 2420-2425. Vol. 53, No. 10.

IVORRA, C.; PEREZ-ORTIN, J. E.; OLMO, M. (1999). An inverse correlation between stress resistance and stuck fermentations in wine yeasts. A molecular study. Biotechnol. Bioeng. v. 64, p. 698-708.

Izmirlioglu G and Demirci A (2012). Ethanol Production from Waste Potato Mash by Using Saccharomyces Cerevisiae. Applied Sciences, 2, 738-753; doi: 10.3390/app2040738

Jackman EA (1987). Industrial alcohol. In: Bu'lock JD, Christiansen B (eds) Basic biotechnology. Academic, London, pp 309–336

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

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

Kádár Zs, Szengyel Zs, Réczey K (2004). Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. Industrial Crops and Products. Volume 20, Issue 1, Pages 103–110. doi:10.1016/j.indcrop.

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

Kim YK, Lee SC, Cho YY, Oh HJ and Ko YH (2012). Isolation of Cellulolytic Bacillus subtilis Strains from Agricultural Environments. ISRN Microbiology. Volume 2012, Article ID 650563, 9 pages. doi:10.5402/2012/650563

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

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

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

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

Lin Y, Tanaka S (2005). Ethanol fermentation from biomass resources: current state and prospects. Applied Microbiology and Biotechnology. Volume 69, Issue 6, pp 627-642; doi: 10.1007/s00253-005-0029x

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

Matsakas L, Christakopoulos P (2015). Ethanol Production from Enzymatically Treated Dried Food Waste Using Enzymes Produced On-Site. Sustainability, 7, 1446-1458; doi: 10.3390/su7021446

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

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

Monique H, Faaij A, van den Broek R, Berndes G, Gielen D, Turkenburg W (2003). Exploration of the ranges of the global potential of biomass for energy. Biomass Bioenergy 25:119–133

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

Moukamnerd C, Kawahara H, Katakura Y (2013). Feasibility Study of Ethanol Production from Food Wastes by Consolidated Continuous Solid-State Fermentation. Journal of Sustainable Bioenergy Systems, 3, 143-148

Nofemele Z, Shukla P, Trussler A, Permaul K and Singh S (2012). Improvement of ethanol production from sugarcane molasses through enhanced nutrient supplementation using Saccharomyces cerevisiae. Journal of Brewing and Distilling Vol. 3(2), pp. 29-35. DOI: 10.5897/JBD12.003

Olsson L, Hägerdal BH (1996). Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme and Microbial Technology. Volume 18, Issue 5, Pages 312–331. doi:10.1016/0141-0229(95)00157-3.

Piškura J, Rozpędowskaa E, Polakovaa S, Mericob A, Compagnob C (2006). How did Saccharomyces evolve to become a good brewer? Trends in Genetics. Volume 22, Issue 4.

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T. (2006). The path forward for biofuels and biomaterials. Science, 311: 484-489.

R. Thenmozhi and J. Victoria (2013). Optimization and improvement of ethanol production by the incorporation of organic wastes. Pelagia Research Library, 4(5):119-123

REBEL WP7 (2009). Bioenergy. Starch-hydrolysis and required enzymes. Access Jul 20 th 2015. Retrievedfrom:www.responsiblebusiness.eu/display/rebwp7/Starchhydrolysis

RFA (2011). Renewable Fuels Association. In: Statistics. Access Feb 17th 2011. Available from: http://www.ethanolrfa.org/pages/statistics

Roy L. Whistler, James R. Daniel (2000). Starch. Encyclopedia of chemical technology. DOI: 10.1002/0471238961.1920011823080919.a01

S. Yan, P. Wang, Z. Zhai and J. Yao (2011). Fuel ethanol production from Concentrated Food Waste Hydrolysates in Immobilized Cell Reactors by Saccharomyces cerevisiae H058. Journal of Chemical Technology and Biotechnology, Vol. 86, No. 5, pp. 731-738. doi:10.1002/jctb.2581

Saccharomyces Genome Database (SGD). 2005. Yeast. New World Encyclopedia. Available from: http://www.newworldencyclopedia.org/entry/Yeast

Sadhu S and Maiti TK (2013). Cellulase Production by Bacteria: A Review. British Microbiology Research Journal. 3(3): 235-258,

Sedlak M, Ho NW (2004). Production of ethanol from cellulosic biomass hydrolysates using genetically engineered saccharomyces yeast capable of cofermenting glucose and xylose. Applied Biochemistry and Biotechnology. Volume 114, Issue 1-3, pp 403-416.

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

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

Van Maris AJ, et al. (2006). Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie van Leeuwenhoek. Volume 90, Issue 4, page: 391-418

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

VAUGHAN-MARTINI, A.; MARTINI, A. A (1993.) taxonomic key the genus Saccharomyces. Syst. Appl. Microbiology. v. 16, p. 113-119.

Verma V, Verma A and Kushwaha A (2012). Isolation & production of cellulase enzyme from bacteria isolated from agricultural fields in district Hardoi, Uttar Pradesh, India. Pelagia Research Library, 3 (1):171-174.

Wang QH, Ma HZ, Wang XM, Ji YZ (2004). Resource recycling technology of food wastes. Modern Chemical Industry 24:56-59.

Wei N, Oh J, Million G, Jamie H. D. C, and Jin Y.S (2015). Simultaneous Utilization of Cellobiose, Xylose, and Acetic Acid from Lignocellulosic Biomass for Biofuel Production by an Engineered Yeast Platform. ACS Synthetic Biology doi: 10.1021/sb500364q

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

Wyman CE, Spindler DD and Gromann K (1992). Biomass. Bioenergy. 1992, 3(5), 301-307.

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

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

Yanase S, et al. (2010). Direct ethanol production from cellulosic materials at high temperature using thermotolerant yeast K. marxianus displaying cellulolytic enzymes. Applied Microbial Biotechnol. 88:381-388. Doi: 10.1007/s00253-010-2784-z

Zhao XQ, Zi LH, Bai FW, Lin HL, Hao XM, Yue GJ and Ho NWY (2011). Bioethanol from Lignocellulosic Biomass. Springer. 128: 25–51. DOI: 10.1007/10_2011_129

Appendices

Appendix I

Reagents

Nitrate reagent

- Solution A, Sulfanilic acid (1gm of sulfanilic acid was dissolved in 125 ml of 5N acetic acid).
- Solution B, Alpha-napthylamine (0.625 gm of α-napthaylamine dissolved in 120ml of 5N acetic acid.)

Dinitrosalicylic acid (DNS)

About 1g of DNS was dissolved in 50ml of distilled water. To this solution, 30g of sodium potassium tartarate tetrahydrate was added. Then 20ml of 2N NaOH was added, which turns the solution to transparent orange yellow color. The final volume was made to 100 ml with the distilled water. This solution was stored in an amber colored bottle.

Phenol red indicator

Phenol red indicator solution was formed by dissolving 0.1 g of phenol red in 14.20 ml of 0.02 N NaOH and diluted to 250 ml with deionized water.

Sodium hydroxide solution

Solution was made by adding 4 g of sodium hydroxide pellets in 50 cm3 of cold water and made up to 1 litre with water.

0.5 M potassium iodide solution

Solution was made by adding 8.3 g potassium iodide in distilled water and make up to 100 ml with water.

0.l M sodium thiosulfate solution

To make the solution, 15.8 g of anhydrous sodium thiosulfate was dissolved in boiled deionized water and make the solution up to 1 liter using a volumetric flask

Appendix II

Instruments

List of the important equipment used through the study.

Instrument	Manufacturer
Autoclave	SAARC
Freeze (-20°C)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200µl)	Eppendorf, Germany
Oven, Model:MH6548SR	LG, China
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Refrigerator (4oC), Model: 0636	Samsung
Safety cabinet Class II Microbiological	SAARC
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Water bath	Korea
Weighing balance	ADAM EQUIPMENT™, United Kingdom