

**SIMULTANEOUS SACCHARIFICATION AND  
FERMENTATION OF BIOETHANOL FROM STARCHY BASED  
KITCHEN FOODWASTE**

**A Thesis**

**submitted in the partial fulfillment of the requirement for the  
award of the degree of**

**MASTER OF SCIENCE  
IN  
MICROBIOLOGY**



**Under the guidance of  
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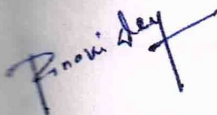
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**JULY 2014**

# CERTIFICATE

This is to certify that the thesis entitled "SIMULTANEOUS SACCHARIFICATION AND FERMENTATION AND PRODUCTION OF BIOETHANOL FROM STARCHY KITCHEN WASTE" being submitted by Rati Saraswat, Registration No. 301205011 in partial fulfillment of the requirements for the award of degree of Master of Science in Microbiology, Department of Biotechnology, Thapar university, Patiala, is a bonafide work carried out under by supervision and guidance. The thesis has not been submitted for award of any other degree or certificate in this or any other university.



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I hereby declare that the work which is being presented in the dissertation entitled, **"Simultaneous saccharification and fermentation of bioethanol from starchy based kitchen food waste"** in partial fulfilment of the requirement for the award of the degree of Masters of science in Microbiology, Department of Biotechnology, Thapar university, Patiala, Punjab; is an authentic record of my own work during a period of five months from January 2014 to June 2014, under the supervision of Dr. Pinaki Dey, Associate Professor, Department of Biotechnology, Patiala. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any other degree.

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# ACKNOWLEDGEMENT

I thank the Almighty for showering his blessings throughout the preparation of my thesis. I take this opportunity to acknowledge the warmth, guidance and cooperation of my teachers, colleagues, friends and family members in this study. I am pleased to have the opportunity to express my gratitude to those precious people who have contributed to this thesis.

I would like to express my deepest appreciation to all those who provided me the possibility to complete this report. A special gratitude I give to my Supervisors Dr Pinaki Dey, whose contribution in stimulating suggestions and encouragement, helped me to coordinate my project especially in writing this report. Without their kind and patient instructions, it is impossible for me to complete this thesis.

I express my sincere thanks to **Dr. Dinesh Goyal**, Professor and Head, Department of Biotechnology (DBTES), Thapar University, Patiala for providing the best laboratory facilities., and extended his helping hand toward me whenever needed.

I also wish to express my thanks to Mr. Babban, Mr. Mohinder, Mr. Surinder and Mrs. Lalita. I was fortunate enough to avail the ungrudging help and assistance from all our laboratory staff.

Last but not least I am very thankful to my wonderful family and friends for their encouragement.. Thanks to my dear friends **Neha Sharma, Neha kumari, Manish Sharma, Harjinder** and all the other **classmates**. I am forever indebted to my **Parents** for their support and love, who encouraged me in attaining my goal.

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# CONTENTS

List of Figures	(vii)
List of Tables	(viii)
List of Abbreviations	(ix)
List of Symbols	(x)
Abstract	1
Chapter I Introduction	2 - 3
Chapter II Literature Review	4
2.1 Environmental Issues Related to Fossil Fuels	4
2.2 Bio-energy	4
2.3 Bio-gas.....	5
2.4 Bio-diesel.....	5
2.5 Bio-ethanol.....	5
2.6 Production of Bioethanol.....	6
2.7 Producer microorganism of Bioethanol.....	7- 8
2.8 Feedstocks for Ethanol Fermentation.....	9
2.9 Sugars as a Feedstock for Ethanol Fermentation.....	10
2.9.1 Lignocellulosic material as feed stock.....	11
2.9.2 Starch as a Feedstock for Ethanol Fermentation.....	12 - 13
2.9.3 Fermentation.....	14
2.9.4 Simultaneous Saccharification and Fermentation.....	14 - 15
2.9.5 Novelty of the process.....	16
2.9.6 Potato.....	16 - 18
Chapter III Goal and Objectives.....	19
3.1 Goal.....	19
3.2 Objectives.....	19
Chapter IV Methods and Materials.....	20 - 21
4.1 Microorganism.....	20 - 21
4.2 Waste Potato Mash.....	21
4.3 Liquefaction and Saccharification process directly by microorganism.....	21

4.4. Moisture Analysis.....	21
4.5. Baseline Fermentation medium.....	21
4.6. Glucose estimation by DNS method.....	22
4.7. Biomass estimation.....	23
4.8. Ethanol estimation by Calorimetric method.....	23
4.9. Ethanol Purification.....	24
Chapter V Result and Discussion.....	26
5.1 Optimization of starch concentration.....	26
5.2 Hydrolysis of waste potato mash.....	27
5.3 Liquefaction and Saccharification during SHF process.....	27
5.4 Biomass weight.....	28
5.5 Ethanol production by SSF.....	30 - 31
5.6 Effect of pH on Ethanol production.....	31 - 32
5.7 Effect of inoculum size on Ethanol production.....	33
5.8 Effect of temperature on Ethanol production.....	34
5.9 Effect of Agitation rate on Ethanol production.....	35
5.10 Optimized experiment with different parameters.....	36
Chapter VI Conclusion.....	37
References.....	38 - 41
Annexure.....	42 - 44

## **LIST OF FIGURES**

Figure 2.1 Conversion of Glucose to Ethanol by <i>S.cerevisiae</i> .....	10
Figure 2.2 Ethanol production from lignocellulosic biomass.....	13
Figure 2.3 Structure of Amylose and Amylopectin.....	14
Figure 2.4 Ethanol production from Starchy biomass.....	15
Figure 4.1 <i>A.oryzae</i> in liquid medium and on petriplate.....	22
Figure 4.2 Methodology of the experiment.....	24
Figure 4.3 Ethanol samples showing colour change.....	26
Figure 4.4 Distillation column.....	27
Figure 5.a) <i>A.oryzae</i> at different starch concentrations.....	28
Figure 5.1 Graph of Glucose and Ethanol during SHF.....	31
Figure 5.2 Starch Vs Ethanol concentration.....	32
Figure 5.3 pH Vs Ethanol concentration.....	34
Figure 5.4 Inoculum size Vs Ethanol concentration.....	35
Figure 5.5 Temperature Vs Ethanol concentration.....	36
Figure 5.6 Agitation rate Vs Ethanol concentration.....	37

Figure 5.7 Optimized parameters Vs Ethanol concentration.....38

## **LIST OF TABLES**

Table 2.1 Ethanol production in the world.....	9
Table 2.2 Different feedstock for bioethanol production.....	12
Table 4.1 Fermentation medium constituents.....	22
Table 5.1 Glucose concentration of 4 four days at different starch concentration.....	29
Table 5.2 Biomass weight of different starch conc. of 4 days.....	30
Table 5.3 Starch concentration Vs Ethanol concentration.....	32
Table 5.4 pH Vs Ethanol concentration.....	33
Table 5.5 Inoculum size Vs Ethanol concentration.....	35
Table 5.6 Temperature Vs Ethanol concentration.....	36
Table 5.7 Agitation rate Vs Ethanol concentration.....	37
Table 5.8 Optimized different parameters Vs Ethanol concentration.....	38

## LIST OF ABBREVIATIONS

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et al.	Et alteri/et alii (and others)
A.	<i>Aspergillus</i>
S.	<i>Saccharomyces</i>
Z.	<i>Zymomonas</i>
SHF	Separate Hydrolysis and fermentation
SSF	Simultaneous Saccharification and fermentation
NaNO <sub>3</sub>	Sodium nitrate
MgCl <sub>2</sub>	Magnesium Chloride
Conc.	Concentration
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ATP	Adenosine tri- phosphate
O.D	Optical density

## LIST OF SYMBOLS

---

%	Percentage
$\alpha$	Alpha
$\beta$	Beta
$^{\circ}\text{C}$	degree(s) Celsius
g/L	gram / litre
Rpm	Revolutions per minute
wt%	Weight percent

## **ABSTRACT**

Ethanol is one of the renewable energy sources with high potential and low environmental impact. Various raw materials have been used as for ethanol production. In this study, waste potato mash was chosen for ethanol production; however, a pretreatment process named as Liquefaction and Saccharification is needed to convert starch of potato to fermentable sugar directly by using microorganisms *Aspergillus oryzae* and *Saccharomyces cerevisiae* instead of using expensive enzyme. This is a novel concept which was proposed by few scientists but much research is done on this subject. So, the process of SIMULTANEOUS SACCHARIFICATION AND FERMENTATION directly by microorganisms is being introduced. In order to obtain maximum fermentable sugar conversion, optimum parameters for the liquefaction and saccharification processes were optimized such as temperature, amount of potato mash, pH, agitation rate, length of fermentation. The optimum combination of temperature, and amount of potato mash was determined as 30°C, 5% of inoculum size, and 15 g dry-weight potato mash /100 ml DI water. The effect of pH, inoculum size and various sources to obtain maximum ethanol from waste potato mash was under batch fermentation. In conclusion, this study has given the potential for utilization of potato waste for ethanol production. Ethanol is basically produced by fermentation by well known fungal strain *Saccharomyces cerevisiae*. In our study we have used an economic and sustainable process of Simultaneous Saccharification and Fermentation by the two potent fungal strains *Aspergillus oryzae* and *Saccharomyces cerevisiae*.

## INTRODUCTION

In today's world, while the demand for energy for transportation, atomic and nuclear operations, and industrial processing is increasing day by day, environmental problems are a point of concern (Hahn-Hagerdal et al., 2006). Renewable sources gain attention not only to protect the environment, but also to supply energy needs by reducing dependence on fossil fuels. In recent years, bioenergy sources have become more important as an alternative and economical energy source. Ethanol is one of the bioenergy sources with high efficiency potential and low environmental impact. Ethanol, serves as a fuel enhancer, an oxygenate, and an octane enhancer. When blended with gasoline, ethanol increases the octane and the oxygen content of the fuel, which leads to more complete combustion and reduction in exhaust emissions such as carbon monoxide and unburned hydrocarbons. Furthermore, Reimelt et al., (2002) other benefits of bio-ethanol are: Lower carbon dioxide emission, higher octane number, lower dust emission, biodegradable, and renewable. The most commonly used types of raw material used for ethanol production are corn, sugar cane, and wheat (Balat et al., 2008). *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Aspergillus niger* and *Aspergillus oryzae* are microorganisms able to convert sugars to ethanol. Sugar and other feedstock, also act as a source for ethanol production by fermentation and are economically viable. Sugarcane, sugar beets, and molasses are widely used for ethanol fermentation and have been used. Less valuable feedstock should be utilized for ethanol production. In order for the low cost requirement, lignocellulosic biomass is another option for ethanol fermentation. However, lignocellulosic biomass is complex and requires costly pretreatments. Currently, potatoes are an alternative feedstock for ethanol production. Potatoes are starchy crops which do not require complex pretreatment process. Although it also is a potential crop, a significant amount of potato is lost during processing. Therefore, waste from the potato industry could be the carbon source for ethanol fermentation because it is relatively cheaper compared to other feedstocks.

By-products of potato industry are currently, utilized as animal feed, however ethanol production could be an alternative for the industry to utilize the waste. The mashed potato wafers also has vitamins A, D, E, C, folate, niacin, riboflavin, and thiamin. It also contains minerals, such as sodium, potassium, magnesium, calcium, phosphorus, zinc, and iodine. Based on the composition of potato flakes, potato mash can act as good feedstock for

microorganisms to produce ethanol. Ethanol production from waste potato is a relatively novel topic and limited research has been done about the utilization of potato waste for ethanol production. To reduce the use of fossil fuels, decrease the emission of carbon dioxide, and reduce dependency on fossils, ethanol is an alternative bioenergy source. The U.S. produces about 22 million ton of potatoes annually, with approximately 850,500 tons in Pennsylvania, during 2007 fall season (USDA, 2007). In the U.S., the breakdown of potato use is as follows: 34% frozen potato products, 28% fresh potato, 12% for chips, 10% dehydrated, 15% as potato seed and for consumption, and 1% canned potato products (NPC, 2008). In the potato chips industry, 18% of company production is starch waste, which could be a raw material for fermentative alcohol production (Fadel, 2000).

## **CHAPTER II**

### **LITERATURE REVIEW**

During the last decade, demand for energy has been increasing while environmental issues came in concerned. Society has realized that fuel is depleting and is also not an eco-friendly energy source. Therefore, renewable energy sources gain attention to protect the environment, and to supply our energy needs by reducing dependence on fossils and imported oils. Bioenergy, one renewable energy source, is a potential alternative to petroleum derived fuels and has the potential to meet the increasing demand for energy for industrial processes, atomic and nuclear operations, and transportation (Balat et al., 2008). Types of bioenergy are: Biogas, biodiesel, bio-ethanol, natural gas, etc. Biogas is formed by the digestion of the organic matter by anaerobic microorganisms under controlled conditions, whereas, biodiesel is a chemically processed fuel in which fatty acids are transferred to methyl esters and glycerin by transesterification. Natural gas is a fossil fuel, so while it is clean and safe, but it is not renewable instead of natural gas bio-ethanol as a fuel may be one of the solutions to reduce global warming and reducing dependency on foreign oil.

Microorganisms that are ethanol-fermentative, such as *Saccharomyces cerevisiae*, can use organic feedstock as a raw material to produce ethanol.

#### **2.1 Environmental Issues Related to Fossil Fuels**

Industrial revolution have caused environmental problems such as: Acid rain, air pollution, global warming, and ozone layer depletion. Flooding, drought, reduction of crop capacity, and have occurred more often than in the past from these climate changes. The causes of global warming are: Carbon dioxide (CO<sub>2</sub>), methane, nitrous oxide, deforestation, carbon in the atmosphere and ocean, (Ecobridge,2008). Carbon dioxide occurs in the atmosphere from the burning of fossil fuels (oil, natural gas, and coal), solid wastes, trees, wood products.

#### **2.2 Bioenergy**

Bioenergy is the production of products such as electricity, fuels, heat, chemicals, and other materials from renewable energy sources. Bioenergy is clean, pure, non-polluting energy. Bio-ethanol, biogas, biodiesel, and natural gas are examples of bioenergy.

### **2.3 Biogas**

Biogas is formed by the digestion of organic matter by anaerobic microorganisms under controlled temperature, moisture, and acidity conditions and the main component of biogas is methane (das Neves et al., 2009). Methane is a colorless, tasteless, and odorless gas. Biomass can be produced from different raw materials, e.g., animal manure, algae, kitchen waste material, dead plants and animal debries, residues of the food industry, etc.

Biogas is produced in four main steps which are: Hydrolysis, acidogenesis, acetogenesis, and methanogenesis das Neves et al. (2009). Hydrolysis is breakdown of insoluble compounds to soluble compounds. In the second step, fermentation of hydrozylate is taken place into hydrogen by acidogenic bacteria, carbon dioxide, and acidic compounds. Further, step is acetogenesis and acetogenic bacteria forming acetic acid and the last step is methanogenesis in which, methanogenic microorganisms convert hydrogen, carbon dioxide and acetic acid to methane.

### **2.4 Biodiesel**

Biodiesel is a renewable, eco- friendly, and energy efficient bioenergy. In this process of the production of biodiesel, fatty acids are transferred to methyl esters and glycerin by transesterification, in the presence of catalyst by an alkali or acid. Production of biodiesel from waste oil starts with pretreatment process of raw material, which includes filtration to remove dirt particles, food residues, and non-oil materials. After determining the concentration fatty acid, transesterification is performed to obtain biodiesel. Tranesterification is a reaction in which fatty acids convert to methyl esters and glycerin. Korus et al. (1993) reported that the ratio of alcohol to vegetable oil, temperature, agitation rate, moisture content in the reaction mixture are essential parameters in the transesterification process.

### **2.5 Bio-ethanol**

Ethanol, also known as pure alcohol, ethyl alcohol or bio-ethanol, is a colorless, flammable, volatile liquid with a strong odor. The melting point of ethnaol is  $-114.1^{\circ}\text{C}$ , and boiling point is  $78.5^{\circ}\text{C}$ . Due to the low freezing point of ethanol, it has been used in thermometers for temperatures below  $-40^{\circ}\text{C}$ , and automobile radiators as antifreeze (Shakhashiri, 2009). The chemical formula of ethanol is  $\text{C}_2\text{H}_5\text{OH}$ , contain a  $-\text{OH}$  group bonded to carbon. Ethanol can be produced synthetically and naturally by yeasts, molds and other bacterias. Ethanol fermentation has been used for the production of alcoholic beverages, and for the rising of

bread dough form centuries; recently, it has been produced to use industrially. Ethanol is also non-toxic, and non-contaminant to water sources. Bio-ethanol has been introduced as an alternative fuel to petroleum-derived fuels, corrosiveness, low flame luminosity, low vapor pressure (compared to gasoline), miscibility with water and low energy density are some of the disadvantages of bio-ethanol (Balat et al., 2008). Beside this ethanol has used for other various purposes Personal care products, cleaning agents, pharmaceuticals, and beverages, laboratory purposes.

Production of Bioethanol from cassava starch by Simultaneous Saccharification and Fermentation by co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae*. Ethanol production by co-cultures of *A. niger* (GS4) and *S.cerevisiae* (BK6) was studied using cassava starch as substrate. At 1% substrate concentration ethanol yield was 0.35g/100ml while the ethanol concentration increased to a maximum of 3.60g/100ml at 8% substrate concentration. When the culture conditions were optimized, the ethanol yield further increased to 4.30g/100ml at a temperature of 35°C, pH 5.0, 300rpm agitation rate and reduced fermentation period of 4 days.

## **2.6. Production of Bio-ethanol**

In 2006, worldwide bio-ethanol production was approximately 51.3 billion liters (Balat et al., 2008). An increase in fuel ethanol production is increased from the fact that many countries want to reduce dependency on import of oil and enhance air quality. Two countries which majorly contribute to ethanol production in the world are Brazil and the United States.

Bio-ethanol can be produced from different feedstock, such as corn, sugar cane, cellulose, potato, cassava starch, molasses etc. Sugar cane, as a raw material, is used for 60% of global ethanol production, while 40% of global production of ethanol comes from other crops. Corn grain is the main raw material of ethanol production in the United States (90% ) whereas in Brazil, sugar cane is the major source (Balat et al., 2008). Desirable raw materials for ethanol fermentation should have applicable sugars that can be fermented by microorganisms. Sucrose containing feedstock, starchy feedstock, and lignocellulose biomass can be used as raw materials for ethanol production.

Ethanol fermentation is summarized with the chemical equations:

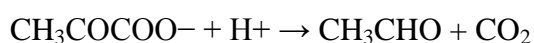
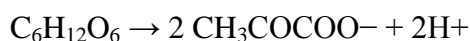


Table 2.1 Ethanol production in the world

**Ethanol Production (million liters)** (Sanchez and Cardona, 2008).

Country	Ethanol Production (million liters) Country 2005	2006
USA	16,139	18,376
<b>Brazil</b>	15,999	<b>16,998</b>
China	3,800	3,849
India	1,699	1,900
France	908	<b>950</b>
Germany	<b>431</b>	<b>765</b>
Russia	749	647
Canada	<b>231</b>	579
Spain	352	462

## **2.7 Producer Microorganisms of Bio-ethanol**

Microorganisms fulfill their energy demand by converting the carbon sources to by-products such as: carbon dioxide, lactic acid, ethanol, cellulose. Ethanol is the end product of fermentation, which can be performed by either bacteria or yeasts. Fermentation is an energy generation process with no electron transport mechanism (Shuler and Kargi, 2008). There are different pathways, which may be different from one microorganism to another one, such as the Entner-Doudoroff and the Embden-Meyerhof pathways (Shuler and Kargi, 2008). The Embden-Meyerhof pathway is used by yeast to convert glucose to ethanol under anaerobic conditions during fermentation, whereas the bacterium *Zymomonas mobilis* follow the Entner-Doudoroff pathway (Shuler and Kargi, 2008).

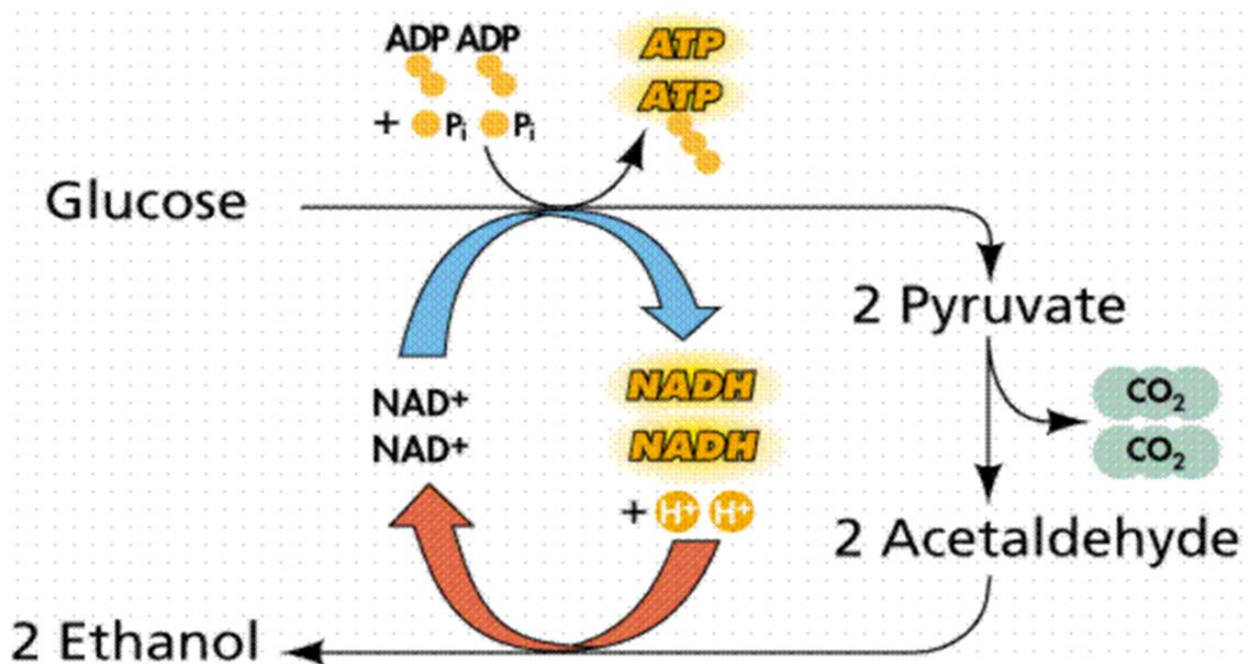
*Saccharomyces cerevisiae* has generally recognized as safe (GRAS) and is the most commonly used microorganism in the fermentation industry (Kunz, 2008) because it is well known for its efficiency to convert starch into ethanol.

Production of alcoholic beverages and bread dough rising are the two main utilities of *S. cerevisiae*. Alcohol production occurs by converting starch to sugar which is used as energy by *Aspergillus niger* or *A.oryzae*, and simultaneously *S. cerevisiae* meets its metabolic energy

need. Under anaerobic conditions, yeast ferments glucose, and ethanol and carbon dioxide are the by-products of the Embden-Meyehof (EM) pathway. Fermentation is carried out in an anaerobic environment, but *S. cerevisiae* needs small amounts of oxygen to synthesize fatty acid and sterols (Sanchez and Cardona, 2008). Although *S. cerevisiae* is the most common microorganism in ethanol fermentation, but it is not able to break down lignocellulosic and starchy material. One approach to solve this problem is hydrolysis before the fermentation process, which converts the unfermentable lignocellulosic sugars to glucose by hydrolyzing enzymes. In pretreatment for hydrolysis, either mixed cultures or genetically modified microorganisms or organisms sharing syngenetic relationship can be introduced. *S. cerevisiae* already has some modified strains to enhance the ethanol yield and assimilate pentoses (Cardona and Sanchez, 2007). Figure ` is an illustration of anaerobic fermentation of glucose to ethanol by *S.cereviciae*.

Figure 2.1

**Anaerobic Conversion of glucose to ethanol by *S.cerevisiae***([www.biologie.uni-hamburg.de](http://www.biologie.uni-hamburg.de))



Two molecules of pyruvate and two ATP's are released. The major source for energy production in the yeast, *Saccharomyces cerevisiae*, is glucose and glycolysis is the metabolic pathway for conversion of glucose to pyruvate, whereby production of energy in form of ATP is coupled to the generation of intermediates and reducing power in form of NADH for biosynthetic pathways are evolved.

*Zymomonas mobilis*, gram negative facultative anaerobe, is able to metabolize glucose by the Entner-Doudoroff (ED) pathway. Additionally, *Z. mobilis* is an ethanol tolerable microorganism that can tolerate up to 120 g/L ethanol (Lin and Tanaka, 2006). *Zymomonas mobilis* is also able to ferment just glucose, fructose and sucrose (Classen et al., 1999). There have been recombinant *Z. mobilis* strains which have been modified to and is being used to transfer not only glucose, but also xylose to ethanol (Cardona and Sanchez, 2007). Another approach to utilize starchy or lignocellulosic materials for ethanol production by *Z. mobilis*, is various pretreatments. Although *Z. mobilis*, *Schizosaccharomyces pombe*, and some recombinant bacteria and yeast can ferment sugars to ethanol, *S. cerevisiae* is still the standard microorganism in the industry (Kunz, 2008; Lin and Tanaka, 2006).

## **2.8 Feedstock for Ethanol Fermentation**

Bioethanol can be produced from different feedstock such as sugar containing Feedstock (molasses, baggasses, sugarcane, grapes), starchy feedstock (potato, corn starch, cassava starch), and lignocellulosic feedstock (xyloses and pentoses sugars). For ethanol fermentation, raw material plays an important role in production costs (Cardona and Sanchez, 2007). Since 30% of costs affect the cost of product, media composition is very important (Lee et al., 1998). By reducing the cost of medium, cheap ethanol can be produced without sacrificing ethanol yield and biomass. The plant design and the process of fermentation is directly related to the type of raw material. Sugars can be transferred to ethanol without any pretreatment, however starchy and lignocellulosic materials need pretreatment prior to the fermentation process. The pretreatment of starch involves hydrolysis in which liquefaction and saccharification process takes place, whereas lignocellulosic material require more complicated treatments.

Table 2.2 Different feedstock for bio-ethanol production and their comparative production potential (\*Balat et al., 2008.)

<b>Raw Material</b>	<b>Potential of bio-ethanol production (L/ton)</b>
Sugar cane	<b>70</b>
Sugar beet	<b>110</b>
Sweet potato	<b>125</b>
Potato	<b>110</b>
Cassava	<b>180</b>
Maize	<b>360</b>
Rice	<b>430</b>
Wheat	<b>340</b>
Bagasse and other cellulose biomass	<b>280</b>

## **2.9 Sugars as a Feedstock for Ethanol Fermentation**

Sugars such as, hexoses and pentoses carbons, do not require pretreatment, such as hydrolysis, prior to being fermented. Thus bio-ethanol fermentation is easier, compared to starch or lignocellulosic feedstock, when the raw material itself is sugar. However, the limitation and major drawback of sugar is their high cost, because they are already valuable as a food source. In addition, availability and transportation costs and its high price of sugar containing raw materials increase the cost of ethanol production (Cardona and Sanchez, 2007). Sugarcane is the major sugar-containing feedstock for ethanol production in Brazil (Sanchez and Cardona, 2008). In Brazil, sugarcane juice is used to produce approximately 79% of total ethanol production, and 21% of ethanol is produced from cane molasses (Wilkie, 2000). In India, however, sugarcane molasses is the main raw material for ethanol production (Ghosh and Ghose, 2003) but the raised concentration of sugars and salts in the medium of cane molasses increases the osmolality, which is a disadvantage for the fermentation (Sanchez and Cardona, 2008). Sweet sorghum juice is another sugar containing feedstock for ethanol due to its high sucrose content (Cardona and Sanchez, 2007).

### 2.9.1. Lignocellulosic Biomass as a Feedstock for Ethanol Fermentation

Lignocellulosic biomass consist of feedstock of agricultural residues, wood, and energy crops (fast growing and low cost agricultural production). Rice straw is also another lignocellulosic waste material (Balat et al., 2008). Lignocellulosic biomass need to undergo very complex pretreatments prior to the fermentation process. Five main steps have been used to produce ethanol from lignocellulosic biomass: biomass pretreatment, cellulose hydrolysis, fermentation, separation and effluent treatment (Cardona and Sanchez, 2007). Figure 2.5 is a flowchart showing the basic steps of ethanol fermentation from lignocellulosic biomass. Pretreatment of lignocellulosic materials can be performed by physical methods (chipping, grinding, and milling), physicochemical methods (thermohydrolysis, etc.), chemical methods (applications of ozone, acids, alkalis, peroxides, and organic solvents), and biological methods (microbiological applications) (Sanchez and Cardona, 2008).

Simultaneous saccharification and fermentation (SSF), and separate hydrolysis and fermentation (SHF) are some of the processes studied to improve ethanol fermentation from lignocellulosic materials. Figure explains the pretreatments processes for lignocellulosic biomass to convert into ethanol and other useful products.

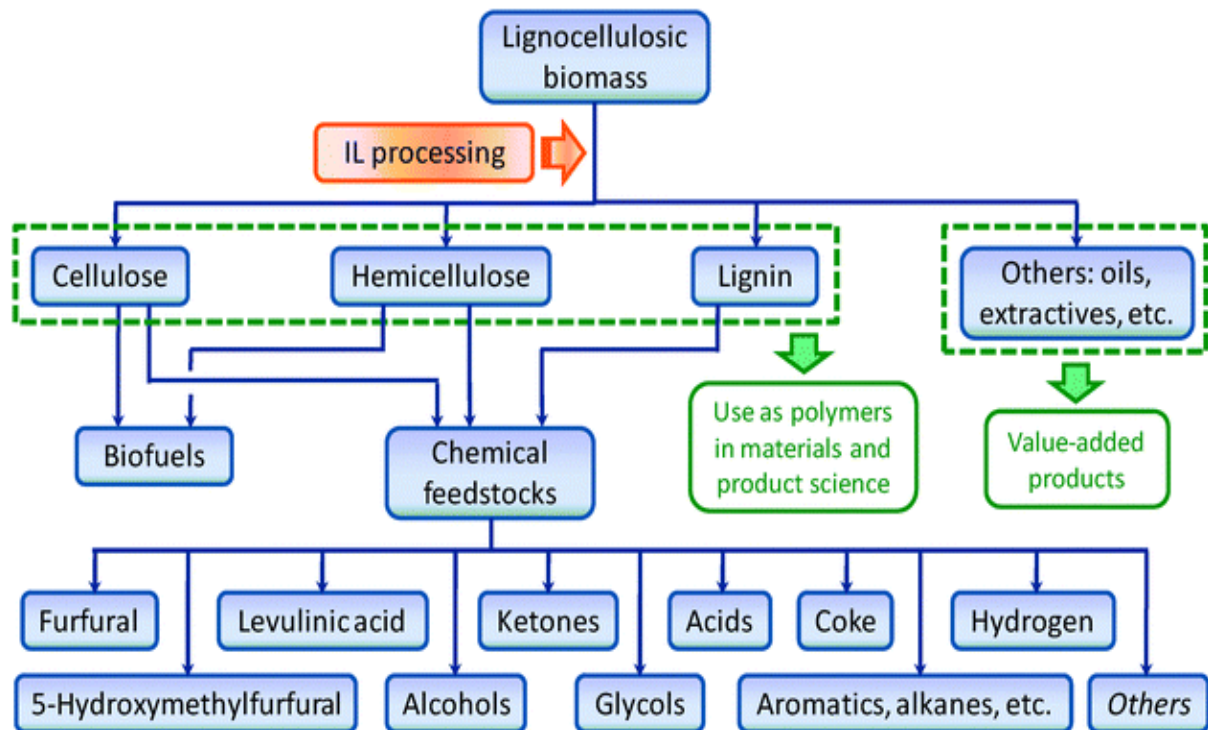


Figure- 2.2 Ethanol production and other useful products from lignocellulosic materials.

### 2.9.2. Starch as a Feedstock for Ethanol Fermentation

Starch is a basic nutrient and plays a major role in basic human diet. It is a polysaccharide composed of amylose and amylopectin, both of which are glucose units. Amylopectin, which is highly branched structure of  $\alpha$ -1, 4-linkage and side chains are branched with  $\alpha$ -1, 6-linkage, is 70-80% of starch by composition. Amylose, a linear polysaccharide formed by  $\alpha$ -1, 4-linked glucose residues is the minor component of starch (20-30%) (Eksteen et al., 2003). Hydrolysis is a process of breaking down amylopectin and amylose linkages into small glucose unit as fermentable sugars and is needed before the fermentation of starch materials. Hydrolysis is carried out at high temperature (90-110°C). At low temperatures, hydrolysis of starch is possible and can contribute to energy savings (Sanchez and Cardona, 2008). To convert starch into fermentable sugars, either acid hydrolysis or enzyme addition should be done but both are not an economical process. Both hydrolysis methods have disadvantages and advantages. The limitations of acid hydrolysis include the inhibition on growth of yeast by the by products (such as 5-hydroxymethylfurfural (5-HMF)), neutralization before fermentation, and expensive constructional material (Tasic et al., 2009). On the other hand, high prices of enzymes are not economical and increase the production cost and play a crucial role when feasibility is of concern for enzyme hydrolysis. Enzyme hydrolysis is chosen despite the high cost of enzymes and initial investment (Tasic et al., 2009) because of the high conversion yield of glucose from starch. Moreover, starch can be stored and a low transportation cost with the pretreatment cost of starch as compared to the pretreatment of lignocellulosic raw materials (Abouzieid and Reddy, 1986).

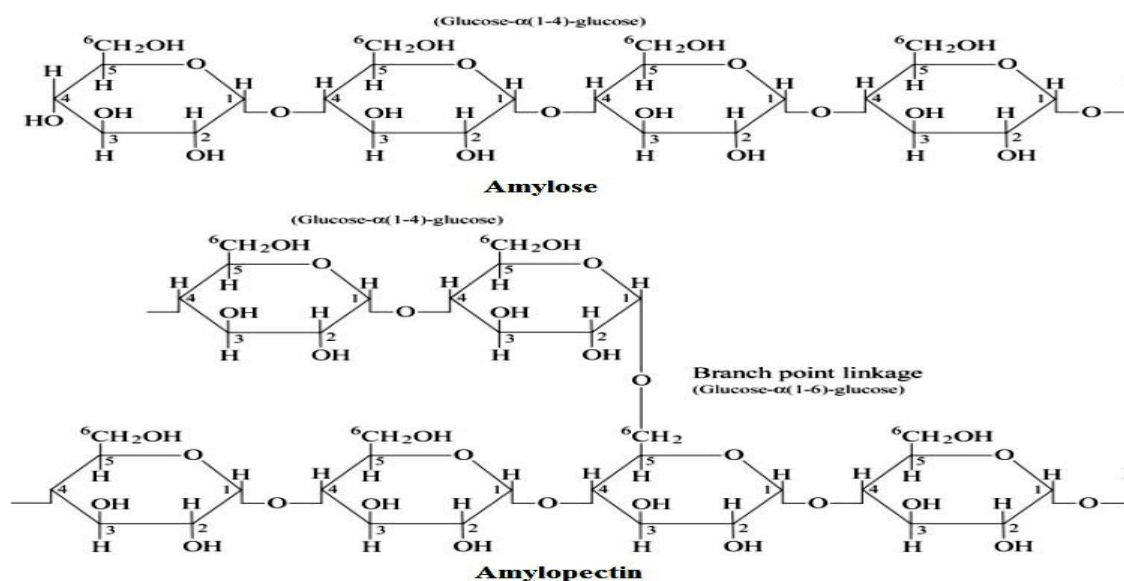
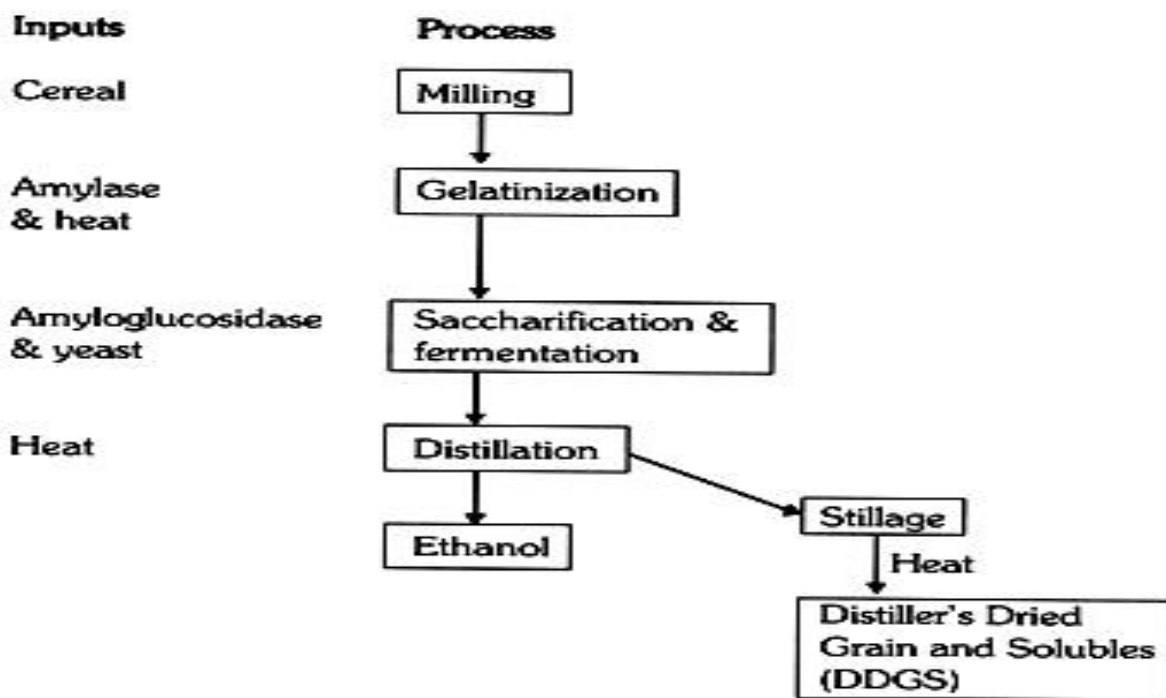


Figure 2.3 shows structure of Amylose and Amylopectin(www.intechopen.com)

Corn is used as the main feedstock for ethanol production in the U.S. Corn ethanol is obtained from corn syrup produced enzymatically after the milling process (Sanchez and Cardona, 2008). The last step of ethanol production from corn is fermentation at 30-32 °C and is accomplished by adding nitrogen sources (ammonium sulfate or urea or peptone) to medium (Sanchez and Cardona, 2008). However, costs of raw material, wet milling, and transportation expenses are the limitations of using corn crops. In addition, Nalley and Hudson (2003) state that “For each gallon of corn ethanol produced, about 160 gallons of waste water are produced”. Wheat as an other starchy raw material is also used for ethanol production in some countries like france (Sanchez and Cardona, 2008). To enhance ethanol production, some optimization of essential parameters has been done like temperature of fermentation (Wang et al., 1999; Sanchez and Cardona, 2002). Wheat as another starchy raw material is also used for ethanol production in some countries like france (Sanchez and Cardona, 2008). To enhance ethanol production, some optimization of essential parameters has been done like temperature of fermentation (Wang et al., 1999; Sanchez and Cardona, 2002). However, Montesinos and Navarro (2000) reported that a mix of *S. cerevisiae* and *Aspergillus niger* and enzyme used in saccharification process produced 67 g/L ethanol after the liquefaction with  $\alpha$ -amylase from raw wheat flour.



**Figure 2.4** Flowchart of Ethanol production from starchy materials (www.intechopen.com)

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of essential parameters has been don't like temperature of fermentation (Wang et al., 1999; Sanchez and Cardona, 2002). However, Montesinos and Navarro (2000) reported that a mix of *S.cerevisiae* and *Aspergillus niger* and enzyme used in saccharification process produced 67 g/L ethanol after the liquefaction with  $\alpha$ -amylase from raw wheat flour.

### **2.9.3. Fermentation**

Fermentation is a metabolic process of microorganisms to obtain energy by breaking down organic compounds and producing alcohol and carbon dioxide as end products. While microorganisms derive their energy, some by products are produced such as: lactic acid, butane, carbon dioxide, ethanol, cellulose. In ethanol Fermentation, energy is produced from sugars by either yeast or bacteria, and carbon dioxide and ethanol are produced. Because yeasts produce their energy without the need for oxygen, ethanol fermentation is a facultative anaerobic process. Fermentation methods are other important aspects of ethanol fermentation. Batch fermentation, semi-continuous, and continuous processes, SSF and SHF have been applied in the ethanol industry. There are also some other fermentation types, such as immobilized cultures.

### **2.9.4 Simultaneous Saccharification and Fermentation (SSF)**

There is no such process which can give the best productivity, yield, and sound economically good at the same time. To attain the maximum ethanol yield and reduce the cost and time, integration of processes is very effective. By integration of processes as in SSF, several operations combine and perform at the same unit. Since the pretreatments plays a crucial role in production of ethanol, most of the process involve integrated hydrolysis (liquefaction and Saccharification process) and fermentation. Simultaneous saccharification and fermentation (SSF) is one of the common processes. SSF found application to ethanol production in the starch-processing industry in the 1970's (Madson and Monceaux, 1995). After liquefaction, saccharification and ethanol fermentation are carried out simultaneously. The benefit of this process is prevention of of substrate inhibition. Because the glucose is transferred into ethanol right after its conversion from starch, no accumulation of glucose occurs in the media. In addition, the hydrolysis reactor is not needed (Cardona and Sanchez, 2007). The main drawback of SSF is that both saccharification and fermentation have different optimal conditionals to obtain maximum yield, and it is difficult to optimize parameters for both hydrolysis and fermentation. Optimizing temperature is an issue, because hydrolysis of starch

requires a high temperature, whereas high temperature is an inhibitor for ethanol production. Costs of enzyme is of concern because more enzyme is needed for a high yield (Cardona and Sanchez, 2007). The concept of the process for enzymatic hydrolysis of cellulose and simultaneous fermentation, so-called Simultaneous Saccharification and Fermentation (SSF) was introduced by Gulf Oil Company, US and the University of Arkansas (Gauss *et al.* 1976; Huff *et al.* 1976). The sequence of steps for the SSF is virtually the same as for the separate process, except that saccharification and fermentation steps are combined in one vessel with the co-cultures of syngenetic microorganisms. The presence of yeast or bacteria along with enzymes minimizes the sugar accumulation in the vessel, and because the sugar produced during starch breakdown slows down  $\alpha$ -amylase action, higher rates, yields and concentrations of ethanol are possible using SSF rather than SHF, at lower enzyme concentration. Additionally, the presence of ethanol makes the mixture less prone to contamination by unwanted microorganisms, (Montesinos and Navarro 2000a; Roble 2003). In this process, the saccharification of sugars released during starch hydrolysis (mainly maltose) is conducted simultaneously with fermentation. Immediately after liquefaction by  $\alpha$ -amylase, the enzyme glucoamylase is added to the slurry, concomitantly with yeasts, and the SSF is conducted in a single reactor (Montesinos and Navarro 2000b).

Various reports on bioethanol production have mentioned the superiority of ethanol yield and productivity using the SSF process, compared to the SHF process (Söderström *et al.* 2005; Neves *et al.* 2007)

Other promising integrated process is pentose fermentation in the SSF, process known as Simultaneous Saccharification and Co-fermentation (Sheehan and Himmel 1999; Cardona *et al.* 2007). In this process, it is necessary that both fermenting microorganisms shares syngenetic relationship i.e be compatible in terms of operating pH and temperature. A combination of *Candida shehatae* and *S. cerevisiae* was reported as suitable for the SSCF process (Cardona Co-fermentation is another promising application to improve ethanol fermentation. In this process, two or more microorganisms are inoculated to enhance the ethanol yield a mixed inoculum of these two or more organisms is formed first. While one of the microorganisms converts polysaccharides to glucose, the second microorganism produces the ethanol. Although, this process prevents substrate and/or metabolite inhibition, growth parameters of microorganisms are a problem. Temperature, pH range, requirement of oxygen, and agitation need to be optimized for both of the microorganisms (Cardona and Sanchez, 2007) .Genetically modified microorganisms are promising to solve these problems. By transferring the genes which allow yeast to hydrolyze the starch to yeast, one microorganism

can be obtained for a whole process. *S. cerevisiae* and *Z. mobilis* are the two microorganism that are mostly modified for this purpose. Simultaneous saccharification, yeast propagation and fermentation (SSYPF) is an application which is performed in the starch industry to produce ethanol (Cardona and Sanchez, 2007). In this process, active dry yeast incorporated in the fermenter during initial saccharification and cell growth is inhibited by lack of glucose because converted glucose transfers to ethanol rapidly (Cardona and Sanchez, 2007)..

### **2.9.5. NOVELTY OF THE SSF PROCESS**

Simultaneous saccharification and fermentation is the process which takes place in 3 main steps:

1. Liquefaction
2. Saccharification
3. Fermentation

In standard process of liquefaction enzyme  $\alpha$ -amylase is used which breakdown the polysaccharide into smaller monosaccharide and further complete breakdown takes place by Saccharification process in which enzyme glucomylase converts starch to glucose units, instead of using these expensive enzymes which is not commercially economical and a costly process few scientists earlier proposed the concept of using directly the microorganisms which releases enzyme  $\alpha$ -amylase extracellularly in the medium such as fungal strain *Aspergillus niger* and *Aspergillus oryzae* these organisms convert starch to glucose and right after its conversion yeast start producing ethanol. For this process a mixed inoculum of syngenetically grown microorganisms are used such as *A.niger* + *S.cereviciae* and *A.oryzae* + *S.cerevisiae* is prepared.

### **2.9.6 Potato**

The potato is a starchy, tuberous crop vegetable *Solanum tuberosum* of the Solanaceae family. Potato is composed of 80% moisture and 18% starch. Potato is a high value crop as a food source, and currently, 34% is frozen, 28% is fresh, 12% is chip, 10% is dehydrated, 15% is potato seed and on farm consumption, .The U.S. produced about 22 million tones of potatoes, with approximately 850,500 ton of potatoes produced in Pennsylvania during fall 2007 (USDA, 2007). Because of the high starch content, potato can be a raw material for ethanol production. Srichuwong et al., (2009) states that “According to available amount of fermentable sugars (soluble sugars and starch), 1 Kg of fresh tuber (potato) would yield

approximately 126 g or 160 mL of ethanol, if complete conversion of fermentable sugars to ethanol was accomplished.”. Although potato is a high-value product, the cost of bioethanol production can be decreased by using wastes from the potato industry and kitchen waste. Waste from processing of potatoes, an agro-industrial residue, can be an important feedstock for ethanol fermentation (Abouzied and Reddy, 1986).

Currently, waste potato is utilized as animal feed after a drying process, which is an energy demanding process. Without a drying process, waste potato could be used in the production of ethanol. Waste potato is a promising feedstock for ethanol fermentation and can provide growth of yeast. 73% available carbohydrate, vitamins and minerals such as sodium, potassium, magnesium, calcium, zinc, and iodine are included in potato flakes (FFCD, 2008).

Bio-ethanol, one of the renewable energy sources to reduce carbon dioxide emissions and dependency on foreign oil, has already been used in pure and/or blended with gasoline. About 51.3 billion liters of ethanol was produced worldwide in 2006 and expecting to increase further in future.

Corn is the major crop used for ethanol production in the U.S. (97%), but these productions of crops causes more soil erosion and leave the minerals in soil in less amount and requires more nitrogen fertilizer than other crops. In Brazil, sugar cane production also has the same environmental limitations (Balat et al., 2008). Cellulosic feedstock has to undergo expensive pretreatments to be effective for ethanol production. Because of the cost of ethanol, inexpensive raw material is important to produce cheap ethanol (Sanchez and Cardona, 2008). The main goals of most of the studies in bio-ethanol production are to produce cheap and highly potent ethanol compared to gasoline (Sanchez and Cardona, 2008). Because waste potato is neither a food source nor requires expensive pretreatments as compared to lignocellulosic raw materials, the waste from the potato industry could be utilized as a raw material for the ethanol industry to produce cheap and efficient bio-ethanol. However, the literature does not provide enough information about utilization of waste potato mash of the potato industry since many types of waste exist for the potato industry.

The present study, waste potato mash was evaluated for ethanol production to decrease the total cost. Waste potato mash was utilized as a fermentation medium after enzyme hydrolysis without any other pretreatment application. Ethanol production from waste potato mash is a cost effective process of ethanol production and could offer an alternative to animal feed which is the current use involving a costly drying process. Therefore, the aim of this study is to produce ethanol from waste potato mash while decreasing fermentation costs. Properties of waste potato mash, effect of pH for a high ethanol yield, effect of inoculum size for high ethanol and biomass yield, effect of nitrogen sources to obtain high ethanol yield.

## CHAPTER III

### GOAL AND OBJECTIVES

The energy demand of the world has been increasing due to rapidly increasing population. Bioenergy is an alternative source to energy to fulfill the demands of energy. Ethanol is, a type of bioenergy, has already occurred all over the world. However, the conventional feedstock are high value products, such as corn, wheat, cellulose, potato, and sugarcane. Feedstock, which have already found, use as food. Low value or waste byproducts should be utilized for ethanol production to meet the energy requirements. Therefore, the main goal of this research is to utilize potato mash waste as a carbon source for *Saccharomyces cerevisiae* for the fermentation of ethanol.

#### **3.1 Goal**

The main goal of this experiment is to produce ethanol from waste potato mash by evaluating starch hydrolysis directly by useful microorganisms following the method of Simultaneous Saccharification and Fermentation and controlling the fermentation conditions such as pH, inoculum sizes, temperature and Agitation rate,.

#### **3.2 Objectives**

The main goal of this research is to produce ethanol from waste potato mash by evaluating starch hydrolysis parameters and fermentation conditions such as pH, inoculum sizes, and four different nitrogen sources.

1. Determine the hydrolysis parameters of waste potato mash to obtain fermentable sugars from starchy materials.
2. Optimize the ethanol production in terms of inoculum size.
  - ⊙ It is proposed to study the effect of starchy food waste (cheap and renewable resource) as a fermentative media in the production process of bio-ethanol with the help of Simultaneous Saccharification and Fermentation technique
  - ⊙ Setup of the SSF process for ethanol production
  - ⊙ Optimization of the processes to improve ethanol production efficiency
  - ⊙ Analysis of ethanol production process by SSF technique
  - ⊙ Efficient separation of ethanol from fermentation broth by distillation technique.
  - ⊙ Optimization of inoculum sizes.

## CHAPTER IV

### MATERIALS AND METHODS

The purpose of this section is to describe the materials used, experimental designs, and the procedure of analysis to optimize ethanol production from waste potato mash by *Saccharomyces cerevisiae*

#### 4.1. Microorganism

The organisms which are used in process of Simultaneous Saccharification and Fermentation are *Aspergillus niger*, *Aspergillus oryzae*, and *Saccharomyces cerevisiae*. Mixed inoculum of organisms having syngenitic relationship was prepared of *A.oryzae* + *S.cerevisiae* a week before inoculation.:

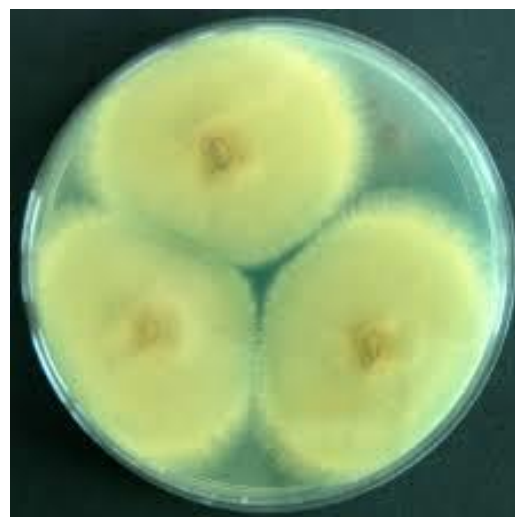


Figure 4.1 *Aspergillus oryzae*:

a) Growth in liquid medium

b) growth on petriplate

Table no: 4.1 Fermentation medium contains constituents as follows:

Constituents	g/L
Glucose	5
Yeast extract	12
Magnesium chloride	2
Calcium chloride	1
Ammonium phosphate	4
Ferrous sulphate	0.02

Petriplates of *Aspegillus niger* + *A.oryzae* was grown with variation in starch concentration of potato mash and it was observed that best growth was shown on 75/lt. This experiment shows that starch degradation had taken place and the fungal strain produces amylase enzyme extracellularly to hrdolyse potato starch. After this the experiment of starch degradation by iodine test was done. Where the starch degradation activity is done by amylase enzyme appears white in colour and this activity clears that starch degradation had taken place.

#### **4.2. Waste Potato Mash**

Waste potato mash was obtained from hostel kitchen from house kitchen. After determining moisture content, waste potato mash was stored in the freezer until use.

#### **4.3. Liquefaction and Saccharification process directly by microorganisms**

*A.niger* and *A. oryzae* is mainly used for converting polysaccharide starch into monosaccharide glucose units. These fungal strain releases extracellular enzyme amylase and glucoamylase in the medium which breakdown and hydrolyze the potato starch first into amylose and amylopectin by liquefaction and followed by Saccharification process in which glucose is formed and right after Saccharification process fermentation of potato begins and production of ethanol formation takes place.

#### **4.4 Moisture Analysis**

To determine moisture of waste potato mash, samples were weighed and the drying process was carried out at 105°C in an oven for 48 h until the weight of samples stabilized.

#### **4.5 Baseline Fermentation Medium**

The base-line fermentation medium used in this study as control contained 4g of glucose ,6 g of yeast extract , 2g of CaCl<sub>2</sub>.2H<sub>2</sub>O, 1g of MgCl<sub>2</sub>, 2g of Ammonium Phosphate, and Starch concentration various 10% to 50% in distilled water . For ethanol fermentation with hydolysed waste potato mash directly by microorganisms, the potato mash was used as the base medium supplemented with all of the other ingredients of baseline fermentation medium except glucose (glucose was replaced with starch as the carbon source).

## METHODOLOGY

### Flow chart of the experiment

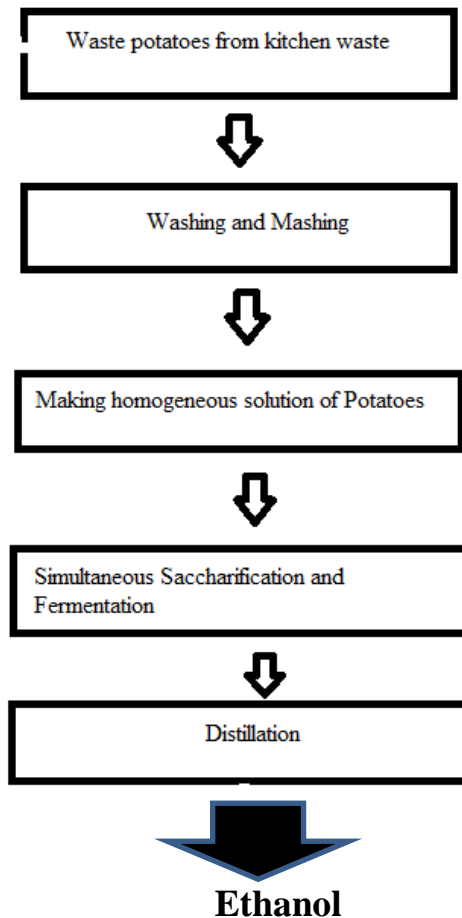


Figure 4.2

#### **4.6 Glucose estimation by DNS method**

Dinitrosalicylic method is widely used for glucose estimation. The microorganism which extracellularly releases amylase and glucoamylase in the medium which breaks down the starch into glucose units.

##### Procedure

1. After inoculation of fungus 5 samples of the medium is collected for 4 days.
2. Filter the samples and then centrifuge them so that residues of fungus are removed as pellet.
3. Collect supernatant of the sample
4. Make serial dilution of glucose as standard
5. Put samples with the standard and dilute the sample ten times.
6. Put 1 ml of DNS and boil it for 5-10 mins, immediately put it into ice cold water.
7. Take OD at 540 nm.

**4.7 Biomass Estimation** – In this process of estimation the fermented broth containing glucose, fungus and residues of potato starch is filtered and biomass is collected.

#### **PROCEDURE**

1. Take equal size filter paper.
2. Weigh all filter paper
3. Filter the fermentation broth and collect the residues of potato, cells.
4. Again weigh the filter paper along with biomass
5. Keep it in the oven for drying overnight at 90°C
5. Calculate the biomass

Calculations = 
$$\frac{\text{weight of biomass+ filter paper} - \text{weight of filter paper}}{\text{Total vol. of fermentation broth}}$$

#### **4.8 Estimation of Ethanol:**

**4.8a) By Colorimetric method:** This method was used for the estimation of ethanol in the sample. The supernatant was obtained by centrifugation of the fermentation broth which contains ethanol varying in concentration. The colour of solution became dark if the concentration of ethanol is more and colour was lighter when the concentration of ethanol is lesser.

**Conclusion** – more the dark colour more is the concentration of Ethanol in the sample.

#### **Procedure**

1. Make a stock of 10% ethanol as 1%, 5%, 10%.
2. Take 1ml of working solution
3. Put 5ml of Sodium dichromate solution
4. Put 5ml of Acetate Buffer (pH – 4.3)
5. Add 25ml of 1N H<sub>2</sub>SO<sub>4</sub> mix it for 1 min
6. Incubate the solution for 120 minutes at room temperature
7. Take OD at 578nm

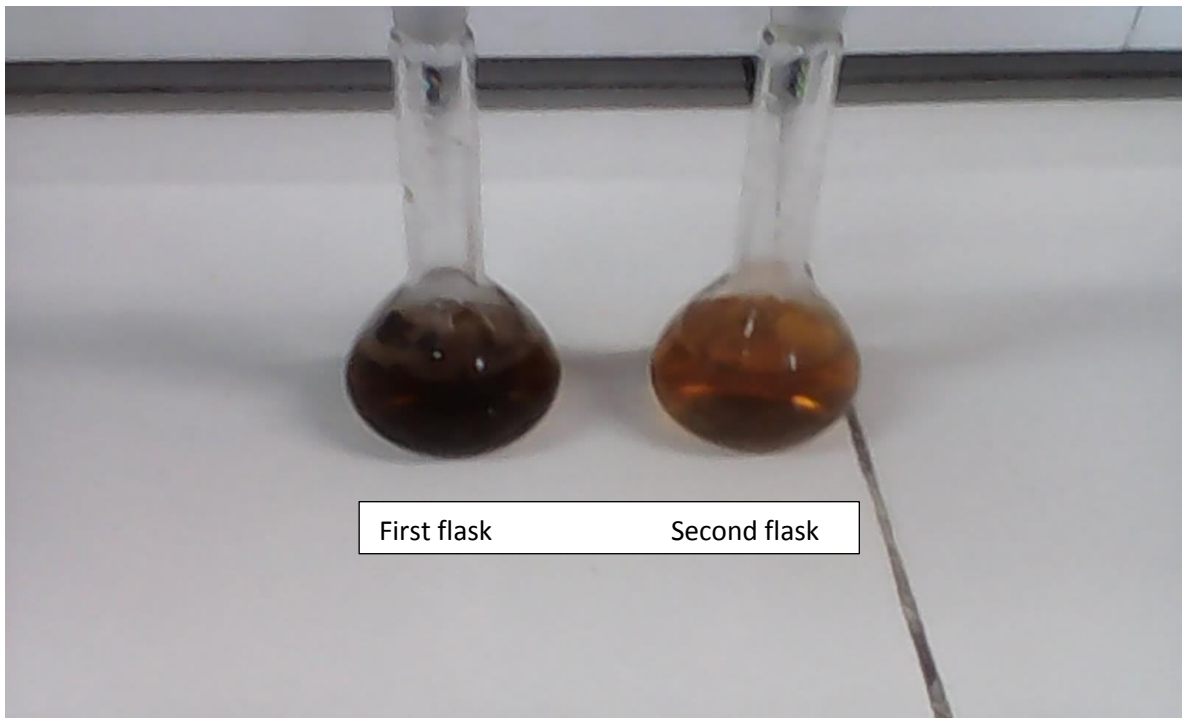


Figure 4.3 Ethanol Samples showing colour changes.

Above photo shows the colour change in first volumetric flask is more so concentration of Ethanol is more in first volumetric flask then the second flask.

#### **4.9 For Ethanol Purification.**

**4.9a) Distillation method** – Distillation is the process of obtaining pure ethanol by boiling it at five degree less then its boiling point  $78.03^{\circ}\text{C}$ . A feed composed of the two components to be separated,

- b. a source of energy to run the process (in most cases, this energy source is steam, either directly entering the base of the column or transferring its energy to the column contents through an indirect heat exchanger called are boiler),
- c. an overhead at the front of column, purified product consisting primarily of the feed component which have to be purified with the lower boiling point,
- d. a bottoms product containing the component of the feed possessing the higher boiling point,
- e. an overhead heat exchanger (condenser), normally water-cooled, to condense the vapor resulting from the boiling created by the energy input. The overhead vapor, after condensation, is split into two streams. One stream is the overhead product; the other is the

which is returned to the top of the tower to supply the liquid downflow required in the upper portion of the tower (R. Katzen)



Figure 4.4 This above picture shows the Distillation column used in laboratory for pure Ethanol extraction from samples.

## Chapter V

### RESULTS AND DISCUSSION

Ethanol was produced by combination of two fungal strains sharing a syngenetic relation among each other *Aspergillus oryzae* and *Saccharomyces cerevisiae* from waste potato mash by SSF in a single vessel. This study was designed to evaluate waste potato mash as a medium for ethanol production, but the hydrolysis of waste potato mash and fermentation parameters were investigated as well. Instead of using the expensive enzymes and other costly time consuming methods breakdown of starch is done directly by microorganisms which secretes extracellular  $\alpha$ -amylase and glucoamylase to hydrolyse starch and to get maximum glucose conversion, whereas effect of pH, inoculum sizes and temperatures were evaluated to optimize fermentation of ethanol. Because few studies were conducted on waste potato mash by SSF process using *S.cerevisiae* (Chapter 2), this study provides information about the fermentation conditions of ethanol from waste potato mash by *S. cerevisiae*.

**5.1 Optimization of Starch Concentration** – Petriplates having different starch concentrations was formed varying the concentration of starch as Potato mash from 10g/L,25g/L,50g/L,75g/L,100g/L along with the essential nutrients for 4 to 5 days at 37°C.



Figure 5. a) *A.oryzae* growth on 10g/L



b) *A.oryzae* growth at 75g/L

From the above experiment it is estimated and concluded that *Aspergillus oryzae* shows best growth at starch of concentration 75g/L and further the next steps of the experiment are followed.

### **5.2 Hydrolysis of Waste Potato Mash**

Hydrolysis is a process of breaking down amylopectin and amylose  $\alpha$ - 1,4 linkages into fermentable sugars and is needed before the fermentation of starchy materials. Because potato is a starchy feedstock, potato mash needs to be hydrolyzed before fermentation.

### **5.3 Liquefaction and Saccharification during SHF process**

Both these processes are done by fungus *Aspergillus oryzae* which secrete enzyme  $\alpha$ -amylase and production of glucose occurs. The parameters for these processes are setup. The optimum temperature for fermentation was 30° C, pH 6-6.5 and inoculum size 5% and glucose is obtained after 4 to 5 days. The standard curve of glucose is shows that with increase in glucose concentration the OD is increasing. Therefore, more the glucose concentration more is the ethanol production.

Table no. 5.1 shows glucose concentrations of 4 days at different starch concentration

Glucose concentration (g/L)	Day1 Glucose concentration(g/L)	Day 2 Glucose concentration(g/L)	Day 3 Glucose concentration(g/L)	Day 4 Glucose concentration(g/L)
10	1.98	2	2	2.6
25	2	2.7	3	3.01
50	5	4.5	4.9	8.01
75	6	6.7	7.8	10.52

Flasks with four different starch concentrations for experiment was setup for 4 days with concentration 10g/L,25g/L,50g/L,75g/L. Inoculation of *A.oryzae* was done, after 4 days different glucose concentration is obtained and the highest concentration at optimum parameter was seen in 75g/L.

#### 5.4 Biomass weight

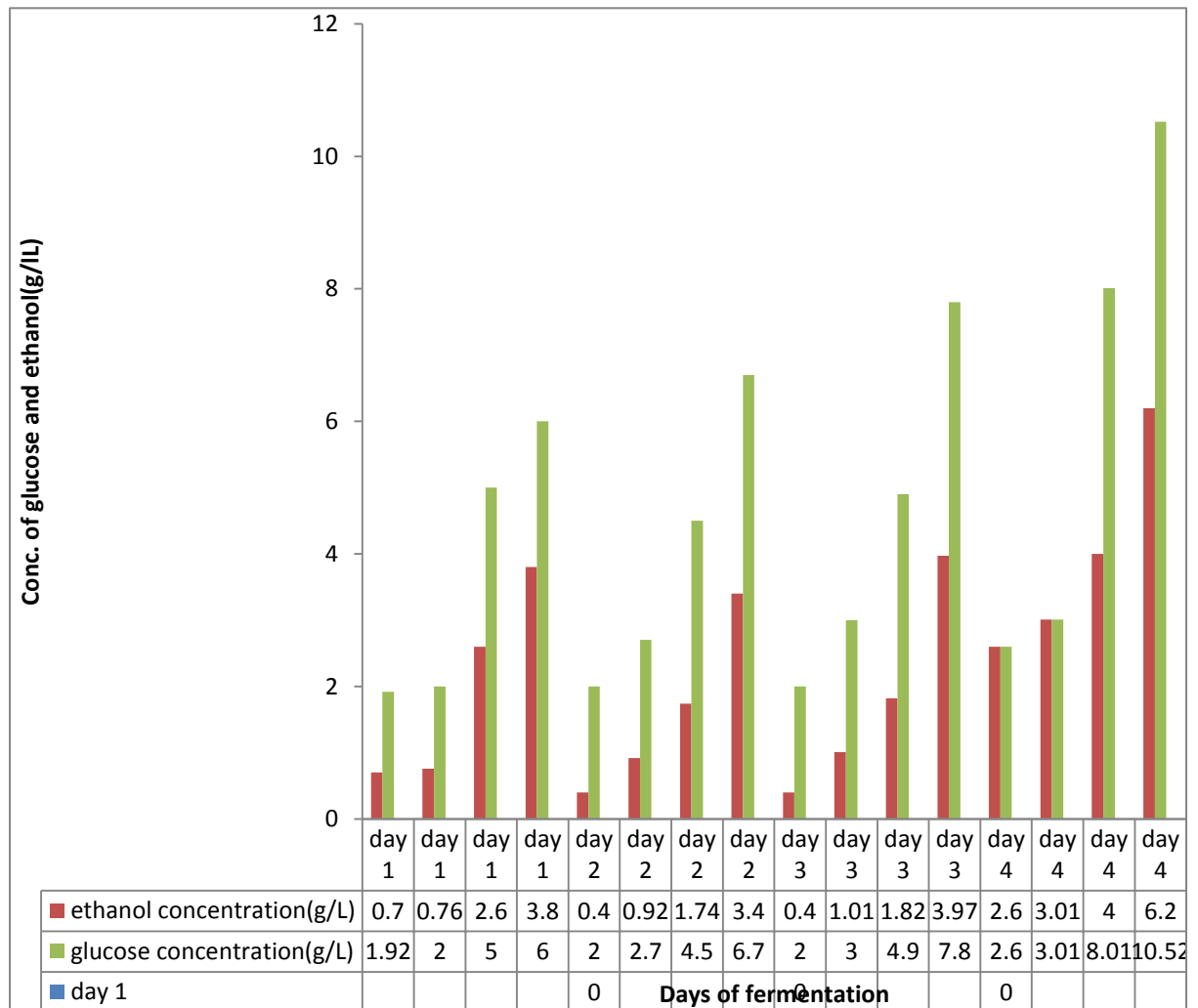
Biomass contains of fungus weight and the residues of potato waste obtained after fermentation of starch. The fungal cells are separated from the potato residues by centrifugation of biomass in Saline which is a solution of NaCl having concentration 9g/L.

Table : 5.2 Shows different biomass weight of 4 days

Starch concentration (g/L)	Biomass weight (g/L) Day 1	Biomass weight (g/L) Day 2	Biomass weight (g/L) Day 3	Biomass weight (g/L) Day 4
10	0.8	1.02	1.023	1.53
25	1.22	1.47	2.01	2.07
50	2.6	2.17	3.2	3.78
75	3.14	3.2	3.96	4.1

This above table depicts the increasing biomass weight as the duration of fermentation increases. This indicates that fungus weight was increasing by the uptaking of nutrients and minerals and starch present in the fermentation medium

**Graph representing glucose as well as ethanol concentration by SHF process**



**Figure 5.1** shows the concentration of glucose and ethanol fermentation.

*Saccharomyces cerevisiae* at temperature 30°C and pH 6 highest concentration of glucose and ethanol was attained at 75g/L.

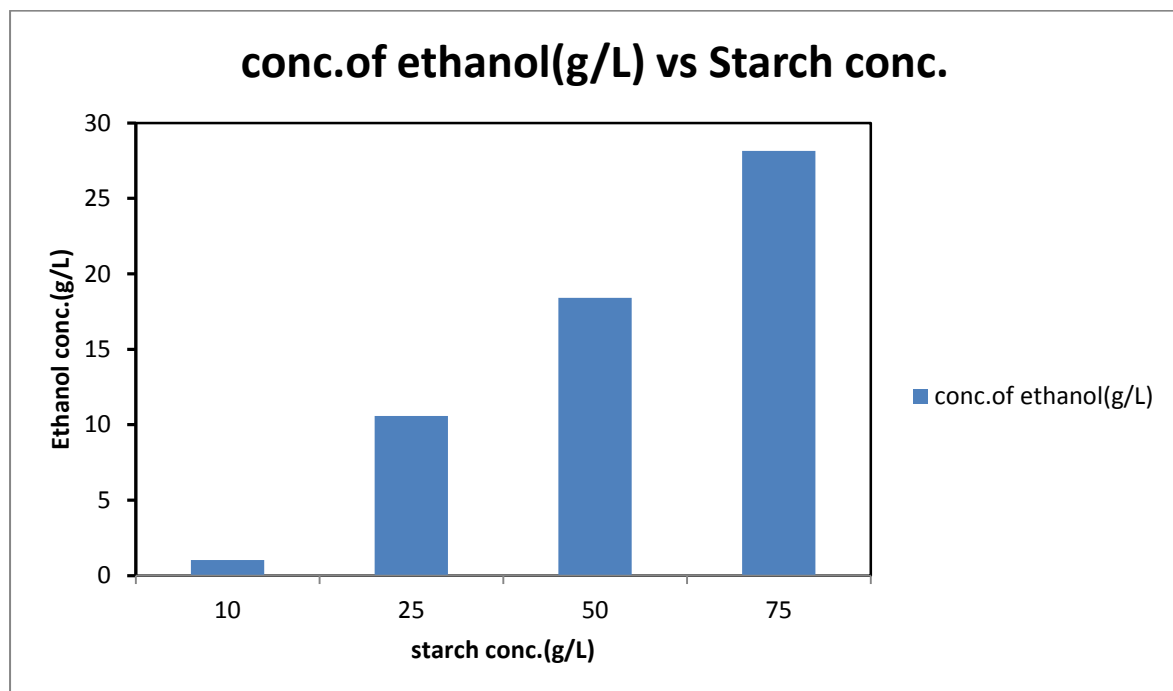
$$75\text{g/L} > 50\text{g/L} > 25\text{g/L} > 10\text{g/L}.$$

### **5.5 ETHANOL PRODUCTION BY SSF PROCESS:**

In simultaneous saccharification and fermentation process *A.oryzae* and *S.cerevisiae* plays key roles and these microorganisms shares syngenetic relationship i.e (can actively work on same temp.,pH, agitation rate). *A.oryzae* which hydrolyse the starch into amylose and amylopectin and further into glucose units which are called as fermentable sugars used for fermentation and production of ethanol. As soon as *A.oryzae* converts starch into sugars simultaneously *S.cerevisiae* starts its activity to convert sugar into ethanol. This whole process takes place in a single vessel in this process emd product inhibition had not taken place. The graph below shows the standard curve of ethanol:

Table no.5.3 The table below shows the ethanol concentration at different starch concentrations:

Starch concentration(g/L)	% of Ethanol	Ethanol Concentration (g/L)
10	0.13	1.027
25	0.23	10.58
50	0.40	18.4
75	0.72	27.2



**Figure 5.2**

**Figure 5.2** Above graph indicates the concentration of ethanol increases as the concentration of starch increases upto certain level i.e 75g/L shows the maximum concentration of Ethanol production. In SSF process the substrate inhibition does not takes place as the amount of glucose generated right after Saccharification is used by *S.cerevisiae* in fermentation and production of Ethanol.

### OPTIMIZATION OF PARAMATERS:

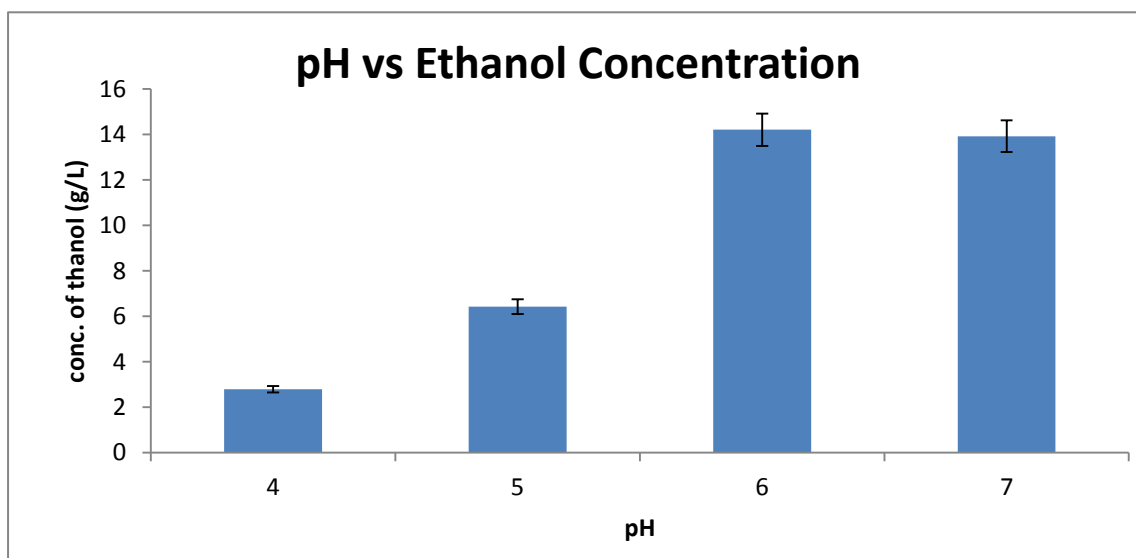
#### **5.6 Effect of pH on ethanol Production**

Ethanol fermentation was examined at different pH profiles to determine the effect of pH: at pH 4, pH 5 pH 6, pH 7 containing 75g/L of starch. At 6 pH and 7 pH, glucose concentration of the fermentation medium began to increase during the first few hours of fermentation instead of reduction, which might be because saccharification was still in progress. This increase of glucose enhances ethanol yield and growth of biomass. Glucose conversion was simultaneously during the fermentation, and after 24 h of fermentation was completely consumed in. After consumption of glucose, both production of ethanol and growth of biomass slowed as expected. At pH 6-6.2 best results are obtained.

Table no :5.4

**pH vs Ethanol concentration**

pH	% of Ethanol	Ethanol concentration(g/L)
4	0.045	2.79
5	0.127	6.43
6	0.331	14.21
7	0.329	13.92



**Figure 5.3** Above graph indicates the maximum concentration of Ethanol at pH 6 .The fungal syngenetic co-cultures have optimum range from 6-6.3 as suggested in the literatures and by optimization. Slightly acidic medium is optimum for fungal co-culture to perform the activity efficiently.

### 5.7 Effect of Inoculum Sizes on Ethanol Production

Four different inoculum sizes (4%, 8%, and 12% , 16% (v/v)) were investigated to determine the effect of inoculum size on ethanol fermentation from waste potato mash having 75g/L of starch. As the inoculum size increase the ethanol concentration increases but after a certain concentration it decreases because of high biomass and product inhibition takes place. Table no. shows the ethanol production (g/L), after 4 days of fermentation.

Table no.5.5

Inoculum size (%)	% of Ethanol	Ethanol Concentration (g/L)
4	0.151	7.749
8	0.295	12.01
12	0.427	16.24
16	0.327	13.27

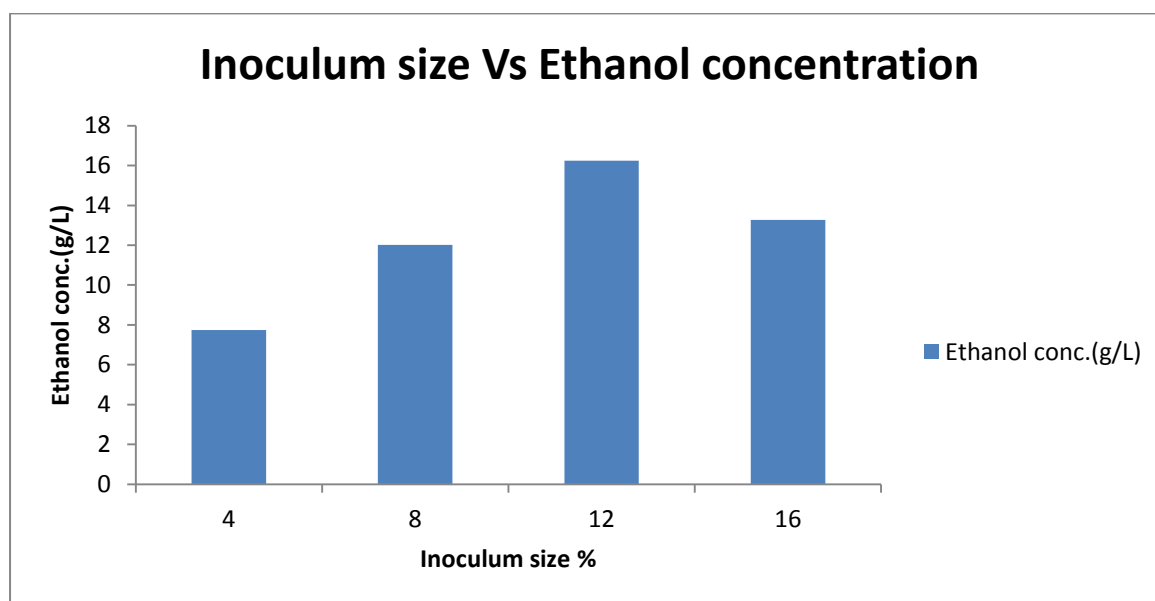


Figure 5.4 shows maximum concentration at 12%

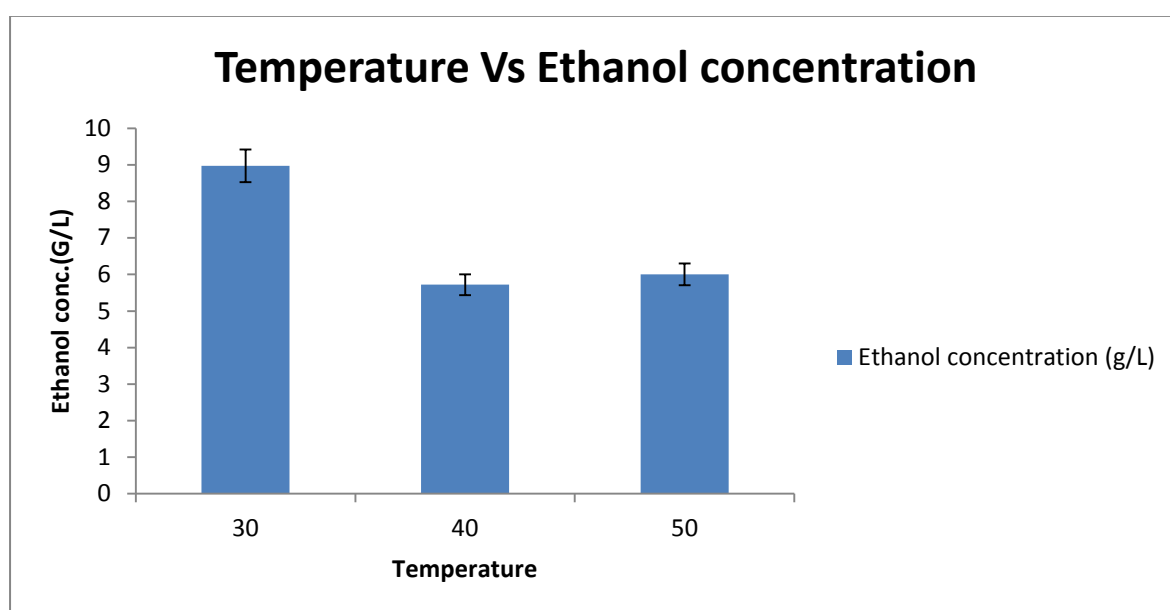
Among all these four inoculation size the optimum results are shown on 12% which is best suited to the fungal culture .At 16% the production is decreased because of high biomass and product inhibition enzymes which are secreted by fungal co-cultures itself block the active sites or change the active sites where the substrate have to bind. So, it it decreases the Ethanol concentration.

### 5.8 Effect of temperature on Ethanol production :

Three different temperatures were taken to evaluate the effect on ethanol production from potato mash at different temperatures. Optimum temperature for ethanol production as the literatures suggest is  $30 \pm 32$  °C.

Table no. 5.6                      **Temperature vs Ethanol**

Temperature	% of Ethanol	Ethanol concentration(g/L)
30	0.14	8.97
40	0.119	5.72
50	0.123	6.002



**Figure 5.5** shows maximum ethanol concentration at 30°C.

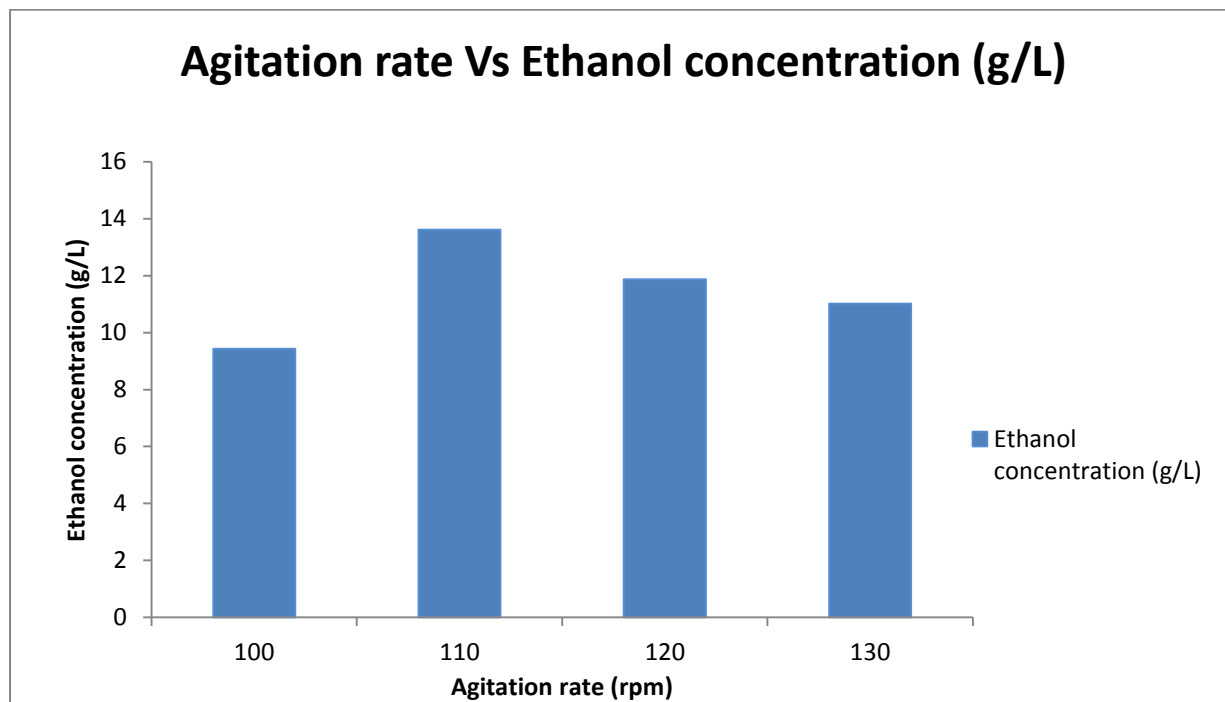
After studying each and every aspects it is evaluated that the best ethanol concentration is generated at temperature  $30 \pm 32$ , pH 6- 6.5, inoculum size 12%, 100-110 rpm agitation rate. These parameters are suitable for the simultaneous saccharification and fermentation of ethanol from mash potato waste directly by using microorganisms. Hence, it is a economical and alternative method.

### 5.9 Effect of Agitation rate on Ethanol production

Four flasks containing starch concentration 75g/L and with all essential nutrients and minerals at 30°C at controlled pH 6.2 was kept varying the agitation rate 100 rpm, 110 rpm, 120 rpm, 130 rpm.

Table no : 5.7 shows Agitation rate Vs Ethanol concentration

Agitation rate (rpm)	Ethanol %	Ethanol concentration (g/L)
100	0.18	9.43
110	0.33	13.62
120	0.28	11.87
130	0.24	11.02



**Figure 5.6** Shows the maximum concentration of ethanol at 110rpm.

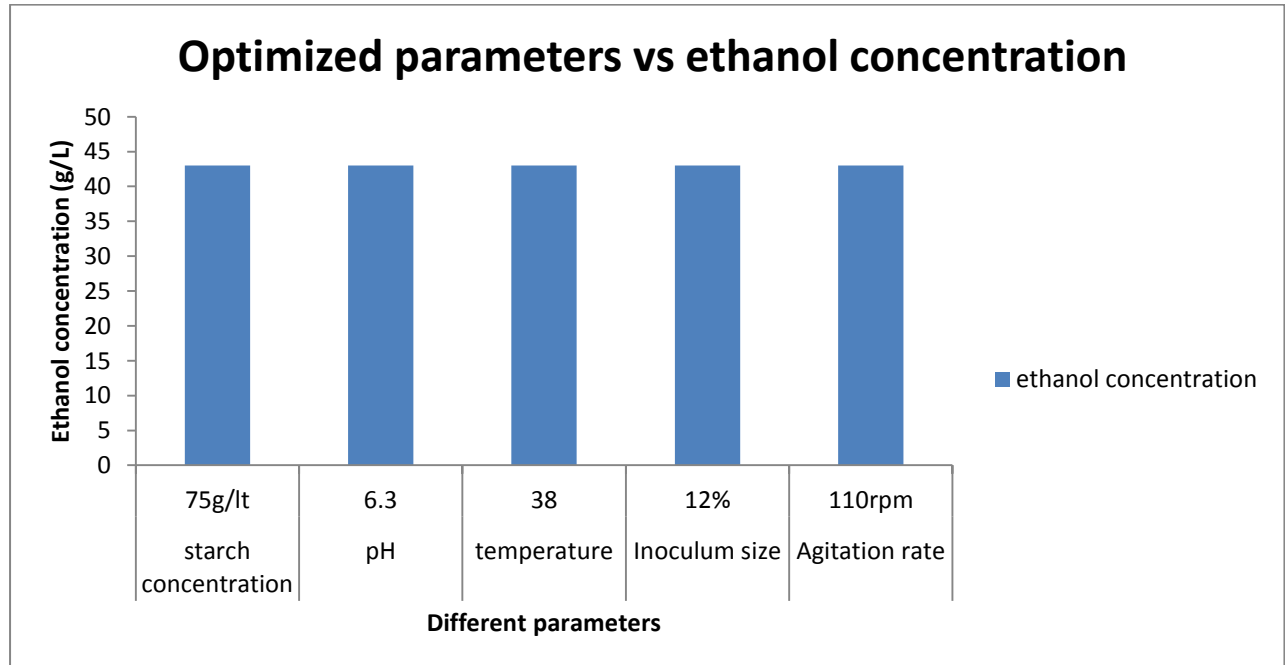
After doing the experiment at the end it was concluded that maximum concentration of ethanol was obtained at 110rpm. The optimum agitation rate for the fermentation by the fungal co-culture was 110 rpm also stated in the literatures.

### 5.10 Optimized Experiment with different parameters

In this experiment every possible parameter was optimized to get the maximum ethanol yield at temperature 37°C, pH 6.2, Agitation rate 110 rpm, Inoculum size 12%, Starch concentration 75g/L.

Table no.5.8 Optimized parameters Vs Ethanol

Parameters		Ethanol concentration(g/L)
Starch concentration(g/L)	75	43.07
pH	6.3	
Inoculum size	12	
Agitation rate	110	
Temperature	38	



**Figure 5.7** Ethanol concentration at different parameters

Above graph shows the result of optimized experiment with ethanol concentration of 43.7g/L which is drastically more than when done uncontrolled and with SHF process. Hence, it states that SSF is more proficient process then SHF.

## **CHAPTER VI**

### **CONCLUSION**

This research successfully demonstrated that waste potato mash can be used for ethanol production by *Aspergillus oryzae* and *Saccharomyces cerevisiae* by SIMULTANEOUS SACCHARIFICATION AND FERMENTATION figure- 5.7 shows the overall results for ethanol at various stages of optimization studies.

Between SHF and SSF, SSF turns out to be better because in SHF separate hydrolysis of starch has been done which is a more time consuming, more costly because use of commercial enzymes, more the apparatus required not an efficient process. In SHF there are more chances of end product inhibition because  $\alpha$ - amylases are often inhibited by the sugars. Whereas, in SSF both process LIQUEFACTION and SACCHARIFICATION takes place simultaneously and lacks the chances of end product inhibition because it is taken place in single vessel and directly consumed by *S.cerevisiae*, and its more proficient process, less time consuming, cost effective process. In SHF highest ethanol concentration was 6.2g/L whereas, in SSF it is 43.7g/L. So, it is a novel process and more research on this process in future can be done.

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## ANNEXURE

### Fermentation media composition:

Constituents	g/L
Glucose	5
Yeast extract	12
Magnesium chloride	2
Calcium chloride	1
Ammonium phosphate	4
Ferrous sulphate	0.02

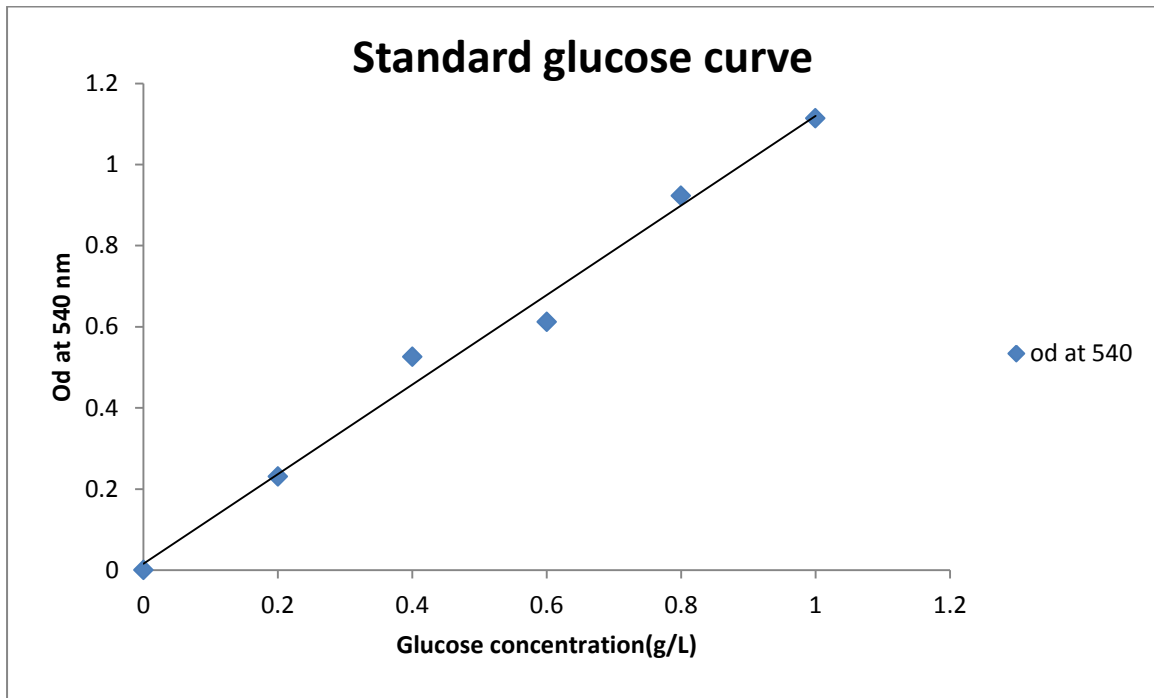
### YPD media composition

Constituents	g/L and L
Yeast extract	3
Peptone	10
Dextrose	20
Distilled water	1

### **Czepak media composition**

Constituents	g/L
NaNO <sub>3</sub>	3
K <sub>2</sub> HPO <sub>4</sub>	1
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5
KCl	0.5
Ferrous sulphate	0.01
Sucrose	30

### Standard glucose curve during SHF



### Standard Ethanol Curve

