OPTIMIZATION OF PRE-TREATMENT FOR ENHANCED SACCHARIFICATION AND BIOETHANOL PRODUCTION FROM RICE STALKS USING SSF THROUGH FREE AND IMMOBILISED *Saccharomyces cerevisiae*

Project Report

Submitted for the fulfillment of the requirement for award of degree of

Master of Technology in Biotechnology

Submitted by:

Nitika Dhap (Reg no. 11210080)

Under the supervision of

Mr. Himanshu Singh

Assistant Professor

SCHOOL OF BIOENGINEERING AND BIOSCIENCES
LOVELY PROFESSIONAL UNIVERSITY
PHAGWARA, PUNJAB-144411

DECLARATION BY CANDIDATE

I hereby declare that the project entitled "Optimization of pre-treatment for enhanced saccharification and bioethanol production from rice stalks using SSF through free and immobilised *Saccharomyces cerevisiae*" is an authentic record of our own work carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, for the partial fulfillment of the award of Master of Technology in Biotechnology under the guidance of **Mr. Himanshu Singh (**Assistant Professor, Domain of Biotechnology, and Lovely Professional University).

This work is our original work and has not been submitted for any degree/diploma in this or any other University. The information furnished in this report is genuine to the best of my knowledge and belief.

Place: **Nitika Dhap (11210080)**

Date:

CERTIFICATE

This is to certify that Nitika Dhap (11210080) have completed her project, entitled "Optimization of pre-treatment for enhanced saccharification and bioethanol production from rice stalks using SSF through free and immobilised *Saccharomyces cerevisiae*" under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study.

No part of the Dissertation has ever been submitted for any other degree or diploma at any University. The Report is fit for the submission and the partial fulfillment of the conditions for the award of B.Tech-M.Tech dual degree in Biotechnology.

Date:

Mr. Himanshu Singh
Assistant Professor
Lovely Professional University
Phagwara, Punjab

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I Nitika Dhap express my deep sense of gratitude to my parents and God, whose support, love, blessing have strengthened me to carry out my studies unhindered as they bore all load of life for me.

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Nitika Dhap

ABBREVIATION

A. Niger	*Aspergillus niger*
AFEX	Ammonia Fiber Explosion
CBP	Consolidated Bio Processing
CCD	Central composite design
DNS	Dinitrosalicylic acid
H_2SO_4	Sulphuric acid
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
LCC	Lignin carbohydrate complexes
NaOH	Sodium hydroxide
S. cerevisiae	*Saccharomyces cerevisiae*
SSF	simultaneous saccharification and fermentation
SHF	Separate hydrolysis and fermentation
T. viride	*Trichoderma reesei*
Z. mobilis	*Zymomonas mobilis*

LIST OF FIGURES

S.No	Content	Page No.
1.	General composition of lingocellulosic biomass in feedstock	3
2.	Schematic flow sheet for the conversion of biomass to ethanol.	3
3.	Schematic pretreatment of lignocellulosic material.	4
4.	Modified summary of biofule conversion routes	4
5.	Rice Stalks before and after physical pretreatment	23
6.	The sample added to the Mandels medium and then incubated for hydrolysis	26
7.	Calcium alginate bead of immobilized yeast cell	27
8.	Immobilized yeast cells were added to the sample after 3 days of treatment by *Trichoderma reesei*	28
9.	DNS test to know the glucose concentration in the sample	29
10.	Distillation Apparatus	39

LIST OF TABLES

Table No.	Description	Page no.
1.	Composition of Peptone Yeast Dextrose Broth	24
2.	Chemical composition of the fermentation media, Srivastava et al., 2014)	25
3.	Glucose concentration obtained for acidic pretreatment at different concentration for free yeast cell.	32
4.	Glucose concentration obtained for alkaline pretreatment at different concentration for free yeast cell.	32
5.	Glucose Concentration obtained after fermentation for acidic pretreatment at different concentration for free yeast cell	32
6.	Glucose concentration obtained after fermentation for alkaline pretreatment at different concentration for free yeast cell.	32
7.	Glucose concentration obtained for acidic pretreatment at different concentration for immobilized yeast cell.	34
8.	Glucose concentration obtained for alkaline pretreatment at different concentration for immobilize yeast cell.	34
9.	Glucose Concentration obtained after fermentation for acidic pretreatment at different concentration for immobilized yeast cell	34
10.	Glucose concentration obtained after fermentation for alkaline pretreatment at different concentration for immobilized yeast cell	34
11.	Analysis of GC showing the available concentration of ethanol.	42

LIST OF GRAPHS

Graph No.	Description	Page No.
1.	Standard Curve for DNS	31
2.	Comparison of glucose concentration obtained in acidic and basic pretreatments for free yeast cell.	33
3.	Comparison of glucose concentration obtained after fermentation for free yeast cell.	33
4.	Comparison of glucose concentration obtained in acidic and basic pretreatments for immobilized yeast cell.	35
5.	Comparison of glucose concentration obtained after fermentation for immobilized yeast cell.	35
6.	FTIR analysis of control (untreated) sample	36
7.	FTIR analysis for the sample treated with Sulphuric acid	37
8.	FTIR analysis for the sample treated with Sodium Hydroxide	38
9.	GC graph showing the peak for ethanol, the sample was treated with 2% NaOH and free yeast cell	40
10.	GC graph showing the peak for ethanol, the sample was treated with 2% H_2SO_4 and free yeast cell	41
11.	GC graph showing the peak for ethanol, the sample was treated with 2% H_2SO_4 and immobilized yeast cell	41
12.	GC graph showing the peak for ethanol, the sample was treated with 2% NaOH and free yeast cell	42

CONTENT

1. Introduction……..……...……1

1.1 Pretreatment……..……...…..……...…4

1.2 Hydrolysis……..……..…..…..….5

1.3 Fermentation……..……...……..…5

1.4 Determination……..……..……...5

1.5 Immobilization of yeast cell…….....…...5

2. Terminology……..…….……7

3. Review of Literature……...…….....…9

4. Rationale and scope of the study……...……..18

5. Objective of the study……...……20

6. Research Methodology……...…..….22

6.1 Sample Collection…...…….....23

6.2 Pre-treatment……..……...…….....…23

6.2.1 Mechanical Pretreatment…….…….…….....…23

6.2.2 Heat (physical) Pretreatment …..……..……..……...…….23

6.2.3 Acidic pretreatment (H_2SO_4)…….……..…24

6.2.4 Alkaline Pretreatment (NaOH) ……...…….…….…24

6.3 Preparation of Growth Medium…….…….…….……24

6.4 Preparation of Inoculum and Fermentation Procedure……..…….……...24

6.5 Enzymatic Hydrolysis and Fermentation…….……...…...25

6.6 Preparation of rice stalks for the treatment by immobilized yeast cell…..……26

6.6.1 Preparation of immobilized yeast cell……..……...…..26

6.6.2 Calculation of the Bead Diameter……..…27

6.6.3 Inoculation of immobilized yeast cell……..…...…..28

6.7 Analysis Method…….…...…28

6.7.1 Dinitrosalicylic Acid Test……28

6.7.2 FTIR Analysis…….…..29

6.7.3 Gas Chromatography……29

7. Results and Discussion…….……..……..30

7.1 Preparation of Substrate…….....31

7.2 Pretreatment…….....……31

7.2.1 Physical Pretreatment……31

7.2.2 Standard Curve of DNS test……...…..31

7.2.3 Dilute H_2SO_4 solution pretreatment for free yeast cell.....32

7.2.4 Dilute NaOH solution pretreatment for free yeast cell……..…..….32

7.2.5 Dilute H_2SO_4 solution after fermentation for free yeast cell ……32

7.2.6 Dilute NaOH solution after fermentation for free yeast cell …….....32

7.2.7 Dilute H_2SO_4 solution pretreatment for immobilized yeast cell……..…34

7.2.8 Dilute NaOH solution pretreatment for immobilized yeast cell……..……34

7.2.9 Dilute H_2SO_4 solution after fermentation for immobilized yeast cell…..…..34

7.2.10 Dilute NaOH solution after fermentation for immobilized yeast cell..…..34

7.3 FTIR analysis…….36

7.3.1 FTIR result for both acidic and alkaline treated sample……...37

7.4 Filtration and Distillation……...……39

7.5 Gas Chromatography analysis of produced ethanol……..40

7.5.1 Gas Chromatography of NaOH sample treated by free yeast cell……40

7.5.2 Gas Chromatography of H_2SO_4 sample treated by free yeast cell……41

7.5.3 Gas Chromatography of H_2SO_4 sample treated by immobilized yeast cell..41

7.5.4 Gas Chromatography of NaOH sample treated by immobilized yeast cell..42

8. Conclusion and future scope……..……..43

9. Reference……..……..…45

10. Appendix…….……...……..51

ABSTRACT

Bioethanol is considered as one of the most likely next generation fuel for automobiles because it is neutral in carbon content and can be produced from the renewable resources like the lignocellulosic biomass that is obtained from rice stalk which is a major by-product of agriculture and is produced in a large amount in India. There are many technological barriers like pretreatment, hydrolysis and fermentation of the reducible sugars which are needed for efficient conversion of bioethanol from lignocellulosic biomass. The fermentation was performed with free and immobilized *Saccharomyces cerevisiae* (yeast cell) after its hydrolysis by *Trichoderma reesei*. Then the comparison of the two methods was done through FTIR analysis and Gas Chromatography and it was found that the free yeast treatment was better than the immobilized one as more amount of bioethanol was produced.

CHAPTER -1

INTRODUCTION

1. Introduction

Bioethanol has been recognized as the most propitious renewable source of energy, especially as a transport fuel. It is a resource that does not add CO_2 to the atmosphere but on combustion it releases volatile organic compound, nitrogen oxide and carbon monoxide in low concentration [Abo-state *et al*., 2014]. It can be extracted from grains, but it can be unsustainable as the demand is growing for the food supply and farmland. Bioethanol from cellulosic material is more economic than grain derived bioethanol [He *et al*., 2011]. It is produced by the fermentation of cellulosic biomass [Abo-state *et al*., 2014]. Production of ethanol from the cellulosic material has the solution for some of the recent problem of environment, economic and energy that the world is facing today [Srivastava *et al.*, 2014]. The inescapable reduction of petroleum supply from the world and the enlarging greenhouse effect has increased demand of nonpetroleum source of energy. Use of ethanol has reduced carbon dioxide emission from the atmosphere. [Li *et al*., 2009]

Traditional raw materials used for bioethanol production are molasses, sugarcane juice, corn, etc. but they have certain economical barriers. Apart from this rice stalks can also be used as it is one of the largest available biomass feedstock in the world and has about 90% annual global production. It is the staple crop of the world's population with annual global production of about 465.078 MT [Potumarthi *et al*., 2012]. Presence of high sugars in the form of hemicellulose (57-61% weigh), rice stalks is used for producing bioethanol. Rice stalks have a potential of about 0.95-1.2 hm^3 for the production of bioethanol every year [Banerjee *et al*., 2009].

Lignocellulose is a common term that is used for describing the main constituent in many plant with very complex structure as it mainly comprises of cellulose (35-50%), hemicellulose (25-30%), and lignin (25-30%)[Wang *et al*., 2011]. It is resistant towards degradation because of the presence of lignin which negatively affects the conversion step and limits the ethanol production. [Banerjee et al., 2009] [Srivastava *et al*., 2014]

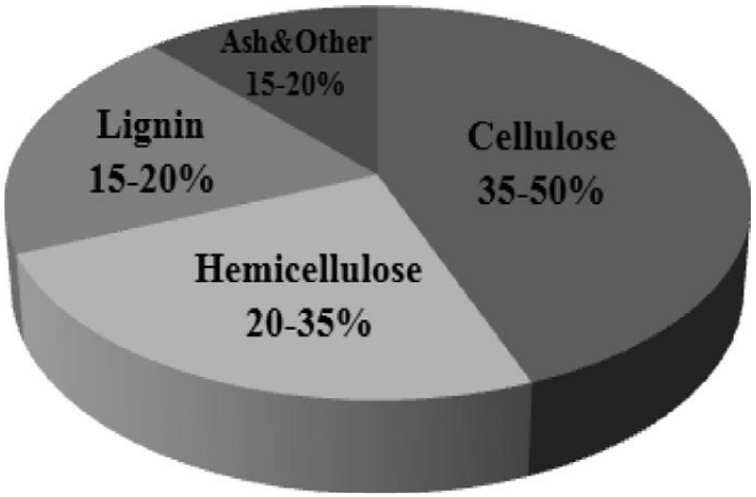


Figure 1: General composition of lingocellulosic biomass in feedstock [Mood et al., 2012]

Naturally lignocelluloses are degraded by many microorganisms [Wang *et al*., 2011]. Cellulose being the major component of the plants cell wall is a glucan polysaccharide which has a large source of energy affording great potential to convert into biofule [Srivastava *et al*., 2014].

There are four main steps for the extraction of bioethanol from the raw material:

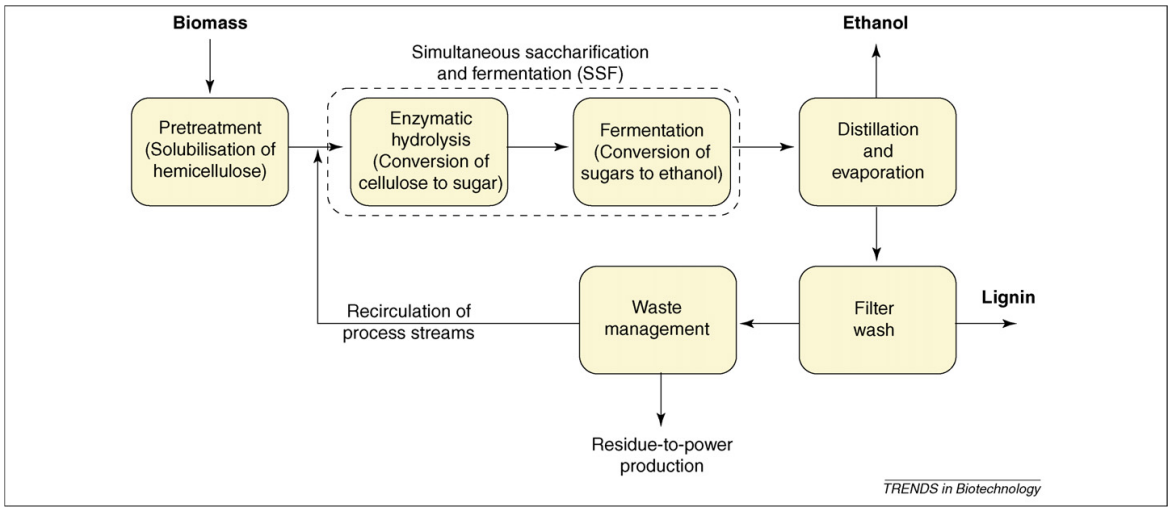


Figure 2: Schematic flow sheet forthe conversion of biomass to ethanol. [Ha¨gerdal, *et al*., 2006]

1.1 Pretreatment-In it the enhancement of cellulose content occur, lignin is removed and pentose and hexose are released.

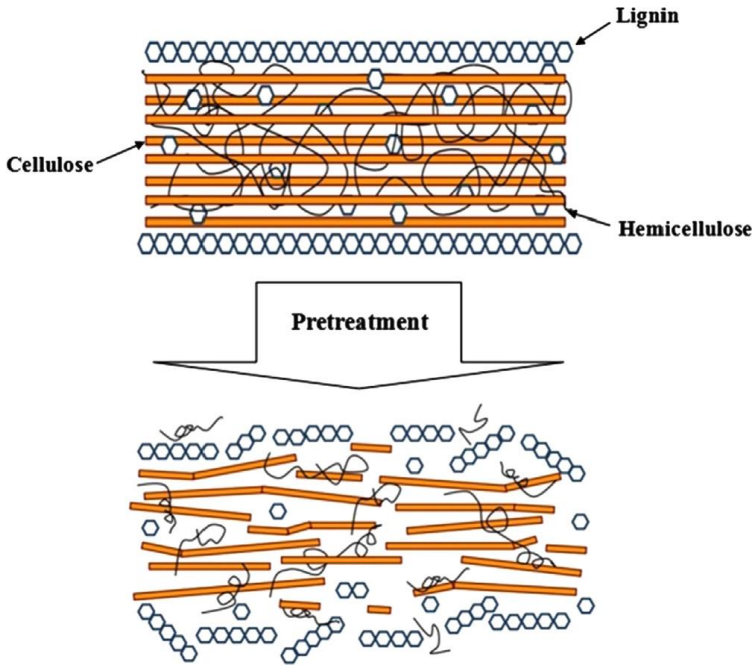


Figure 3: Schematic pretreatment of lignocellulosic material [Mood *et al*., 2012]

Lignocellulose to release monomeric sugars for the production of ethanol needs various treatment mechanisms it is known as pretreatment. The pretreatment mechanism may be physical, chemical, physio-chemical or biological.

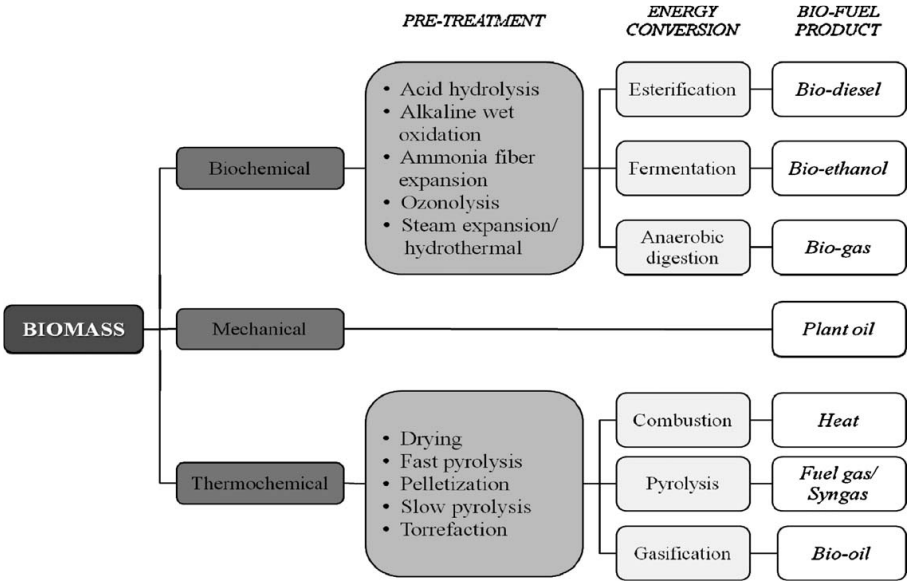


Figure 4: Modified summary of biofule conversion routes [Chew *et al*., 2010]

There are many pretreatment processes like grinding, alkaline or acidic hydrolysis, gas treatment, steam explosion, hot water, biological treatments and recently ionic liquid and cellulose solvent pretreatment. [Banarjee *et al*., 2009]

1.2 Hydrolysis- Produce glucose monomer from cellulose. It is the most important as well as the most expensive step which contribute of about 30% of the cost. Effective treatment aims on lowering the biomass size, reducing hexoses and pentoses loss, increasing the removal of lignin and lowering the cost of the production. The simple and cost effective conversion of lignocellulosic component of rice stalks into biofule is by alkaline hydrolysis. [Srivastava *et al*., 2014]. It mainly involves engage in clearing of the polymers of cellulose and hemicelluloses using certain enzymes. Lignocelluloses can also be degraded by the cooperation of microorganisms such as fungal and bacteria which produce enzyme under aerobic and anaerobic conditions [Wongwilaiwalin *et al*., 2010].

1.3 Fermentation-The cellulosic lignocelluloses fraction can be transformed to ethanol by synchronous saccharification and fermentation (SSF) or discrete enzymatic hydrolysis and fermentation (SHF). SSF mostly advised as its cost is low and has a higher production of ethanol as compared to SHF. A drawback is also suffered by SSF, as there is different temperature of enzyme hydrolysis and fermenting microorganism. The maximum ethanol is produced at 40-50^{0}C but microorganism normally does not tolerate this temperature. The solution to this problem was by using thermo-tolerant microorganism [Karimi *et al*., 2015].

1.4 Determination-The bioethanol produced was determined by the use of biochemical methods or by using gas chromatography techniques [Koshy *et al*., 2014].

1.5 Immobilization Of Yeast Cell

Immobilized cell expertise has been recommended as a useful means for enhancing Bioethanol fermentation. The immobilization of the yeast cells lead to elevated cell densities with subsequent increases in rate of reaction and output. The production of bioethanol by immobilized

cells has been broadly investigated for the last few decades. Cells have been immobilized on a range of natural and synthetic supports. The most generally used synthetic carrier in immobilization methods is based on cell entrapment in gels, such as carrageen and Ca-alginate The main problem of these system is the shortage of mass transfer in between product and the substrate. Other disadvantages are the instability of Calcium alginate against the buffer solution and the disruption of gel particles due to CO2 evolution during fermentation, which also limits its usage [Martini *et al*. 2010]. The most commonly used immobilizing agents are sodium or calcium alginate and agar-agar cubes. Alternatively, new immobilizing agents that are cheap and easy to use have been investigated in several studies. Immobilization of yeast cells has been considered as potential alternative for enhancing ethanol productivity, because immobilizing yeasts reduce risk of contamination, make the separation of cell mass from the bulk liquid easy, retain stability of cell, minimize production costs, enable biocatalyst recycling, reduce fermentation time and protect the cells from inhibitors [A. Tesfaw *et al*., 2014]

CHAPTER-2

TERMINOLOGY

2. Terminology

- **Aerobic:** It is a reaction which requires air, specifically oxygen. This utilizes oxygen as the final electron acceptor in the reactions oxidizing sugars, fats and other carbon sources to CO2.
- **Anaerobic:** Anaerobic normally refers to energy collecting reactions that do not want oxygen, specifically fermentation based processes.
- **Centrifugation:** It is a practice which involves the function of the centrifugal force for the sedimentation of diverse mixtures with a centrifuge.
- **Chromatography:** A method for the separation of a mixture by passing it in solution or suspension through a medium in which the components move at different rates.
- **Fermentation:** The chemical breakdown of a matter by bacteria, yeasts, or other microorganisms, normally involving froth and the giving off of heat.
- **Hydrolysis:** The chemical breakdown of a compound due to reaction with water.
- **Monomeric:** A monomer is a molecule that, as a unit, binds chemically to other molecules to form a polymer.
- **Oxidation:** Used to explain reactions in which an element combines with oxygen
- **Polymers:** A polymer is a large molecule, or macromolecule, tranquil of many recurring subunits.
- **Saccharification:** The hydrolysis of the polysaccharides to form soluble sugars is called saccharification.
- **Suspension:** A suspension is a heterogeneous combination that contains solid particles that are sufficiently large for sedimentation.

CHAPTER-3

REVIEW OF LITRATURE

3. Review of literature

According to Abo State in the milling pretreatment rice stalks was grinded and put into Erlenmeyer flask. Then it was moistened using distilled water. Further it was incubated for about 2 hours and finally it was mixed so that reducing sugar is extracted. The yield obtained of reducing sugar after was milling pretreatment was 1.44 g/L [Abo state *et al*., 2014].

According to Biswas the rice stalks was dried using the hot air oven at 55^0C for about 24 hour and then passed through hammer mill for milling to reduce the size to 1.27 mm. This milled rice Stalks (15% w/v) was then mixed with H_2SO_4 (1% v/v) and treated in autoclave at 121^0C for 1hour. There was about 35% conversion of the cellulose present in the rice stalks to reducible sugar [Biswas *et al*., 2006]. The grinded rice stalks was put into Erlenmeyer flask, moistened and then treated with stem using autoclave at temperature of 121^0C and 1.5 bar pressure for about 20 min. Reducing sugar yield was found to be 6.35 g/L at the end to this process [Abo state *et al*., 2014]. In the pretreatment done by milling and irradiation, grinded rice stalks was given various doses (50 and 70 Mrad) of this γ radiation. Then this radiated rice was put in Erlenmeyer flask and further moistened with distilled water. After incubation of 2 h the sugar is extracted by filtration. 6.62 g/L was found to be the presence of reducing sugar at the end of pretreatment [Abo state *et al*., 2014]. In the combination of milling, irradiation and autoclaving grinded rice stalks was given γ radiation of various frequencies and after moistened with distilled water put into Erlenmeyer flask, then autoclaved at 121^0C and 15 bars for 20 min. After autoclaving the content was extracted. 5.03 g/L was found to be the presence of reducing sugar at the end of pretreatment [Abo state *et al*., 2014].According to the studied conducted by the Ultrasonic wave can also be used to remove the lignin content of the rice stalks. The waves were used for 10, 20, 30, 40, 50, and 60 min at 250 W, 40 KHz. [Yu *et al*., 2009]

In the chemical treatment that was done using sodium chlorite and sodium hydroxide powdered rice stalks was dried and was treated with sodium hydroxide (1-5%) concentration and sodium chlorite (5%) concentration. Further it was washed with deionised water several times then dried by using hot air oven for about 24 hrs at 70^0C. The cake was then processed for microbial saccharification. Maximum yield was obtained with 5% NaOH and 5% NaCl [Srivastava *et al*.,

2007].In the treatment that was done using hydrogen peroxide, rice stalks was grinded and then treated with 2.5 % concentration of alkaline hydrogen peroxide ($NaOH + H_2O_2$) which is at a pH of 4.5 [Krishna *et al*., 2000]. 20 gm of rice stalks was taken after cutting and then suspended 160 ml of 1% NaOH aqueous solution. Then it was kept for boiling for 15 min to 2 h. Further the residue was collected and washed with tap water so that the pH is neutralized and then dried. The cellulose and carbohydrate yield at the end of the treatment was found to be $99\pm$ 0.4% and $80\pm$ 0.6% respectively after 70 min [Zhu *et al*., 2005]. In the study done by Cheng were he used NaOH by first dissolving it in DI water and then incubating at 55^0C and then the solution is mixed with the rice stalks [Cheng *et al*., 2010]

In the phosphoric acid pretreatment 50g of dried material was taken and mixed with 400 ml of concentrated phosphoric acid. It was then incubated in rotary air bath at 120 rpm and temperature of 50^0C for about 1h. After the reaction has occurred the solution was poured in 1.2L of pre-cold acetone and then mixed. Further the mixture is centrifuged at 8000 rpm for 10 min, the supernatant is taken and suspended in 1.2 L of acetone and then centrifugation was done three times. The residue was again washed with distilled water and again centrifuged three times. During the last step the pH was adjusted to 5.0-6.0 with the help of 10M NaOH and then finally the pretreated material was collected [Li *et al.,* 2009].

In the pretreatment that was done using aqueous-ammonia soaking method 10 g of rice stalks was soaked in aqueous-ammonia solution. The solid was then separated from the solution by using filtration cloth and was washed with 2L of distilled water till the pH of the solution reached 6.5-7.0. Further it was dried in vacuum by drying oven at 45^0C for 3 days. In the sulfuric acid treatment the rice stalks was grinded to about 833 μm in size and then 600g was soaked in 41 of 0.5% sulfuric acid solution for about 20h. This mixture was added into 101 reactors, were it was steam heated for 1.5 min till 15bar pressure is achieved. Then this pressure is remained for 10 min. Further the solution is cooled within 3 min to achieve 2 bar pressure, the material is the collected and washed up to five times with tap water and finally filtered [Karimi *et al*. 2006].

During the sodium hydroxide treatment the rice stalks was grinded and in the hot-air oven it was dried at 70^0C. This dried rice stalks were treated by 1% sodium hydroxide (NaOH) at solid-to-liquid ratio of 10% (w/v). The residue is collected by the process of filtration and then washed with the help of distilled water so that the pH can be neutralized [Sandhu *et al*. 2013]. According to pretreatment performed by Wei powdered rice stalks was taken and first treated with dilute sulfuric acid with a concentration of (0.25~1.5% v/v) at a temperature of (100~160^0C) for about 10 min to 1 h. then the slurry was filtered and the filter cake was collected and suspended in a solution of dilute sodium hydroxide and placed in autoclave with a pressure range from 0.0 to 1.0 Mpa at 120~160^0C. For the neutralization the residue was washed with tap water. The reducing sugar that was obtained after the pretreatment was 46% [Wei *et al*. 2009].

Wet Air Oxidation Reactor of 1.8 L volume was taken in the wet air oxidation treatment method and 30 g or dried rice stalks mixed with 500 g of distilled water and 1 g of Na_2CO_3 was added in the WHO reactor. The suspension was then mixed and sealed so that there is no leakage. Pressure of the air (at 0.5 and 1.0 MPa, corresponding to 0.05 and 0.11mol of O_2 respectively) was applied and then the solution was heated. During heating the temperature was kept $\pm$ 5^0C and a constant stirring of 100 rpm. The suspension was then left for the reaction to occur and finally the pretreated slurry was cooled and filtrated, giving a cake that is rice in cellulose and hemicelluloses [Banerjee *et al*. 2009].

In the PCS (Peptone Cellulose Solution) pretreatment method 5g of the sample was taken in a 200 ml of flask which contain 100 ml of PCS medium (0.1% of yeast extract, 0.5% of peptone, 0.2% of $CaCO_3$, 0.5% of NaCl, 0.5% of filter paper and pH 7.0) that was autoclaved. This culture was the incubated at 50^0C under the optimum conditions. Once the paper strip was degraded and rice stalks had become soft, 5 ml of culture was transferred into fresh enrichment medium. This process was then repeated for 10 times. The remaining culture as stored and kept in cold [Wang *et al*., 2011].

Domestic microwave oven was used with the microwave frequency was 2450 MHz 20 g of rice stalks was grinded and then suspended in 160 ml of 1% NaOH aqueous solution in a beaker and

then it was placed at the center of rotating circular glass plate inside the microwave oven and the microwave treatment was given for 15 min to 2 h. Yield of about 99 $\pm$ 0.6% and 75 $\pm$ 1.2% for cellulose and carbohydrate respectively was found [Zhu *et al*., 2005].The rice Stalks according to Saha was milled and 15.0 % w/v was taken along with 1.5 % w/v of lime and slurry was made. The slurry was further autoclaved at 121^0C for 1 h. The pH of the pretreated rice stalks was then adjusted to 5.0 using concentrated solution of HCl. The reducing sugar obtained was increased with increased doses of lime and found that at 100mg lime g-1 the yield was 126 $\pm$ 1 mg [Saha *et al.*, 2008]. In AFEX (Ammonia Fiber Explosion) pretreatment liquid ammonia was given to 1-2 kg of biomass at a temperature of 90^0C [Chundawat SP, Venkatesh B, Dale BE, 2007] [Thomsen MC, Belinda A (2007)]. 75% of the glucose was released after 24 h of hydrolysis [Sun Y, Cheng J., 2002]. Ethanol has a yield of about 83% at the end of the pretreatment [Jeoh T, Agblevor FA (2001)].

The biological pretreatment was carried out with fugal, *Trichoderma reesei* [Srivastava *et al*., 2007]. Different fungal strain spores such as F66, F94 or F98 (F94 and F98 were strains of *Trichoderma viride* and *Aspergillus terreus* respectively) can also be used by inoculating them in the grinded rice stalks and incubating them for 7 days at a temperature of 30^0C [Abo state et al., 2014]. Studies that have been conducted show that white-rot fungi are the most effective microorganism used for the pretreatment of lignocellulose that is present in agricultural waste [Akin *et al*., 1995]. Many fungi produce hydrogen peroxide with the help of certain enzymes like glyoxal oxidase, ary- alcohol oxidase and pyranose-2 oxidase which degrade the lignin content of the rice Stalks [Martinez *et al*., 2009].

Either the enzymatic or chemical hydrolysis can be carried out for the cellulose or hemicellulose content of rice stalks. For the chemical hydrolysis dilute-sulfuric acid was used for the conversion of lignocellulose in rice stalks into corresponding sugar. In this dilute acid hydrolysis at lower temperature hemicelluloses was depolymerised but if higher temperature was applied further hydrolysis occur of the formed monosaccharide. Therefore the hydrolysis process was carried out in two steps, in the step first milder conditions were applied in which fraction of hemicellulose are hydrolyzed and in the second step, enzymatic or dilute acid hydrolysis was carried out a comparatively higher temperature in which cellulose content is

hydrolyzed [Karimi *et al*., 2006]. Hydrolysis can be performed with the help of certain enzymes such as Acremonium cellulase and Optimash BG which is a supplement used to increase β-xylosidase activity. In this treatment pretreated rice stalks was taken and mixed with 1.0 ml enzyme and 50 mM acetate buffer which is at the pH 5.0 and then incubated at 45^0C for about 72 h. At the end sugar yield was calculated using the equation:

Sugar yield (%) = [weight of monomeric sugar after enzymatic hydrolysis (g) / weight of maximum monomeric sugars after hydrolysis of rice stalks using sulfuric acid per gram (g)] × 100 [Hideno *et al*., 2008]

Cellulose enzyme is commonly used for the hydrolysis of cellulose to obtain glucose but the rate of conversion is very slow and this saccharification was not found to be cost-effective. An alternative method that can be used is to first convert cellulose into value-added cellulose acetate. This conversion will reduce the overall cost of the production of bioethanol. It was performed on the pretreated rice stalks by taking 2 gm of sample and acetic acid (0.5 gm), acetic anhydride (5.0 gm), methylene chloride (30 ml) and sulfuric acid (0.04 g). Then the mixture was heated at 80^0C along with stirring on magnetic stirrer and the temperature measured and maintained by Thermo-o-watch. The mixture was cooled after 4 hour and then it was sieved. Then 60 ml of chloroform was added to the collected filtrate, further it was stirred constantly for 30 min at 38^0C and then sieved again. The filtrate is collected and again filtered using Whatman filter paper, the filtrate is dried and cellulose acetate was obtained as a film inside the flask. To remove this film ethyl alcohol is used and then dried at 80^0C in vacuum oven [Biswas *et al*., 2006].

According to research done by Krishna SSF (Simultaneous Saccharification and Fermentation) method can be used to minimize the inhibition of product and increase the ethanol yield and also eliminate the use of two separate reactors for fermentation and then saccharification. This also reduces the cost of production of ethanol. The only drawback is that it requires different temperature for saccharification and fermentation i.e. 50^0C and 35^0C respectively. In the SSF reaction the mixture used contains the substrate that is pretreated by alkaline peroxide, the

cellulose, yeast and SSF basal medium. The reaction is carried out in a flask which is kept on the shaking orbital at a pH of 5.1. This method increased the yield up to 10% [Krishna *et al*., 2000]. After the hydrolysis of the pretreated rice stalks *Saccharomyces cerevisiae* was added for the reduction of sugar to ethanol. SDDL broth (which is a mixture of glucose 20.0 gL^{-1} and yeast extract 5.0 gL^{-1}) was used for the growth of microorganisms at a temperature of 30^0C for about 48 hrs [Srivastava *et al*., 2014]. The cellulose fermentation can also be done where 1% of *C. thermocellum* was inoculated followed by the strain X514 or 39E when sugar has accumulated. If needed yeast extract 0.6% (w/v) can be added to the defined medium [He *et al*., 20011]. *Saccharomyces cerevisiae* is common used for the fermentation of the hexoses sugar to ethanol. Zygomycetes which is a filamentous fungus is recently being used for the ethanol fermentation. *Mucor indicus* and *Rhizopus oryzae* are the class of fungi that are capable of producing hexoses and pentoses. The yield of ethanol by *M. indicus* was 0.45 gg^{-1} and from *R. oryzae* was 0.83 gL^1h^{-1} [Abedinifar *et al*., 2009].

Xylose which is one of the most abundant pentose sugars that is present after the hydrolysis of the pretreated rice stalks. Therefore Xylose-fermenting microorganisms are commonly found among the bacteria, filamentous fungi and yeast. Anaerobic bacteria are able to ferment the pentose sugar but it gets inhibited at low sugar and concentration of ethanol. *Pichia stipitis* CBS 6054 which is naturally occurring yeast is able to ferment the xylose sugar to ethanol and has reasonable yield. These yeast strains are inhibited by compounds. These inhibitions are tolerated by the filamentous fungi but are slow. For the fermentation of the pentose sugar Escherichia coli and Klebsiella oxytoca are generated, it can ferment almost all sugar that is derived from lignocellulose [Hagerdal *et al*., 2006]. Number of species of yeast like *Candida shehaatae*, *Pachysolen tannophilus* and *Pichia stipitis* are efficient in the production of bioethanol from the fermentation of the xylose sugar. Out of all the species *P. stipitis* was found to produce very high yield of ethanol but has high sensitivity towards organic acids that are in lignocellulosic hydrolysate therefore the hydrolysate was neutralized, by first heating it to 50^0C and then holding at that temperature for 15 min. Then it was followed by the addition of calcium hydroxide (lime) which raises the pH of filtrate to 10.0. Agitation is then carried out for 30 min. and then the sludge is separated using filtration and the pH of the filtrate was adjusted to 5.0. The

culture of *P. stipitis* was then inoculated and then fermentation was carried out at a temperature of 30^0C [Huang *et al*., 2009].

The analytical methods that can be used while measuring the amount of carbohydrate content that is present in the raw material is Anthrone method. To determine the amount of reducing sugar that is present in the pretreated rice stalks DNS (dinitrosalicylic acid) method [Srivastava et al., 2007], phenol-sulfuric acid method [He *et al*., 2011] and HPLC (High particular liquid chromatography) with refractive index detector can be used [Hui *et al*., 2009]. In the DNS method 3, 5-Dinitrosalicylic acid is taken which is an aromatic compound; it reacts with the reducing sugar to produce 3-amino-5-nitrosalicylic acid which absorbs light at 540 nm. In this method presence of the free carbonyl group (C=O) [Koshy *et al*., 2014].

Soluble proteins can be analyzed by the Lowry method. To estimate the amount of pentose sugar present in the solution can be done by using fuming Ferric chloride (Iron solution) [Srivastava *et al.*, 2007]or the glucose and xylose in the mixture can be determined after acetone and hydrolysis is removed by 4% sulfuric acid at a temperature of 121^0C for about 1h [Hui *et al*., 2009]. To measure the amount of ethanol produced after the fermentation process, spectophotometric method can be used [Srivastava *et al*., 2007]. Commonly bioethanol is estimated by gas chromatography or chemical oxidation method, this estimation was found to be good for the quantification of ethanol [Krishna *et al*., 2000]. Ethanol can also be analyzed on Aminex HPX-87 H column at 60^0C with 0.6 ml/min eluent of 5mM sulfuric acid [Karimi *et al.*, 2005].

Gas chromatography or more commonly gas-liquid chromatography encompasses the vaporization of the sample and then injecting it in a chromatographic column. The sample is then transported through column. Liquid stationary phase is present in the column which absorbed on inert solid. Difference in the partition coefficient of the stationary liquid phase and the mobile gas phase analyze the ethanol present in the solution [Koshy *et al*., 2014].

Potassium dichromate method is a biochemical method that can be used for the quantitative estimation of the ethanol. The principal of this chemical oxidation method is complete oxidation of ethanol with the help of dicarmonate along with the presence of sulfuric acid, this leads to the formation of acetic acid. The stoichiometry reaction is as follows:

$$2Cr_2O_7 + 3C_2H_5OH + 16H \text{ ---------> } 4Cr + 3CH_3COOH + 11H2O$$

This is commonly used as potassium dichromate is available easily and is stable in air [Koshy *et al.*, 2014].

Martini used the pre-cultured commercial baker yeast from Korea. The pre-culture medium for yeast has the composition as (in g/L): glucose-15; sucrose-15; yeast extract-3; peptone-5; malt extract-3. The cells were cultured in this medium in a controlled environment shaker at 30^{o}C for 20 hours in order to obtain high cell density. At the end of incubation period, cells were centrifuged aseptically and re-suspended in fresh pre-culture medium to be used as inoculum. The composition of the fermentation mediums (in g/L) was: glucose, 50; MgSO4, 1; KH2PO3, 1. Then it was adjusted to pH 5.5 using citrate buffer solution and autoclaved at 121^{o}C for 15 minutes before used the *Saccharomyces cerevisiae* cells were immobilized in the bioreactor, having size of 3 cm in diameter and 30 cm of height. The carriers were aseptically transferred to the sterilized equipment. The carrier volume was about 25-50% of the working volume of 170 ml. After the rice stalks were washed, they were sieved to remove other contaminants. The average size of the rice stalks was between 5-7 mm. There is no size reduction in this work. The cleaned rice stalks were then dried. Rice stalks as much as 2.5 and 5 g were autoclaved at 121^{o}C for 15 min and then mixed with 50 ml pre-culture inoculums in which the concentration was 2 x 108 cells/ml in flask. After 24 hours, the liquid from immobilizing reactor was discharged, filled with another 50 ml of fresh pre-culture medium, and kept for the next 24 hours before it was used for fermenting sugar [Martini *et al*. 2010].

CHAPTER-4

RATIONALE AND SCOPE OF THE STUDY

4. Rationale and scope of the study

For the production of bio-fuel also known as bioethanol rice stalk was used. There are various advantages of using rice stalk and the most important one is that it is the waste or by product left after the processing of rice for the regular use in food. Therefore, rice stalk being of no use and freely available was used for the production of bioethanol. In addition due to increase in petroleum prices and depleting fossil fuel it serve as the best alternative in the future as it is eco-friendly and more efficient. It can also be utilized in chemical industries as a disinfectant, lotions and also solvent in the future.

CHAPTER-5

OBJECTIVE OF THE STUDY

5. Objective of the study

- Pre-treatment of rice stalks using different techniques such as acidic and alkaline and there comparison.
- DNS test for the determination of glucose content in the sample.
- Hydrolysis and fermentation of rice stalks using *T. reesei* and *S. cerevisiae.*
- Fermentation using free and immobilized *S. cerevisiae* and comparison between them.
- Distillation of the sample for the extraction of ethanol after fermentation.
- Depiction of obtained bioethanol by FTIR (Fourier Transform Infrared) spectroscopy and Gas Chromatography.
- Study the amount bioethanol produced through free and immobilized *S. cerevisiae.*

CHAPTER-6

RESEARCH METHODOLOGY

6. Research Methodology

6.1 Sample Collection

The raw material i.e. rice stalks was collected from the nearby field. Further it was gone through many physical and chemical treatments for the production of bioethanol.

6.2 Pretreatment

Various approaches for the pretreatment to remove the lignin are used for the rice stalks have been discussed in this report.

6.2.1 Mechanical Pretreatment

Raw rice stalks was taken and was grinded with the help of grinder to reduce the size of the rice stalks.

Figure 5: Rice Stalks before and after physical pretreatment

6.2.2 Heat (physical) pretreatment

Grinded rice stalks 5 gram was fixed with 95 ml distilled water in 250 ml conical flask. The flask was autoclaved at 121^0C, 15 psi for 30 minutes and then the sample was filtered, the solid residue was air dried and stored for further use.

6.2.3 Acidic Pretreatment (H_2SO_4)

Concentrated solution of sulphuric acid 1%, 1.5% and 2% were used for the acidic pre treatment of rice stalks. Then 5% solution was prepared using rice stalks for all the concentrations and then the solution was autoclaved at 121^0C at 15 psi for 45 minutes. After autoclaving the solid residual was separated and dried.

6.2.4 Alkaline Pretreatment (NaOH)

For the alkaline pretreatment of the rice stalks 1% (w/v), 1.5% (w/v) and 2 %(w/v) concentration of sodium hydroxide was prepared. Then 5% solution of the grinded rice stalks was prepared for all the concentration. Further the solutions were autoclaved at a temperature of 121^0C, 15 psi for 15-45 min. After autoclaving the solution the solid residue was separated from the liquid and washed in tap water until pH 7 is obtained. The pretreated stalks was dried and stored.

6.3 Preparation of Growth Medium

Trichoderma reesei growth medium was prepared by mixing Peptone Yeast Dextrose Broth. The pH was set to 4 and for *S. cerevisiae* yeast-malt broth was prepared at pH 5.5. The two medium was sterilized by autoclaving them at 121^0C, 15psi for 15-45 min.

Sr.No.	CHEMICALS	AMOUNT(gm)
1	Peptone	2
2	Yeast	1
3	Dextrose	2
4	Distilled water	100ml

Table 1: Composition of Peptone Yeast Dextrose Broth

6.4 Preparation of Inoculums and Fermentation Procedure

The conical flask (250ml) was cotton plugged with 100ml of broth so as to inoculate *T. reesei* having pH 4. The media was autoclaved and then stain of *T. reesei* was inoculated in the broth. The flask was further incubated at 25-26 degrees for 6 to 7 days.

S. cerevisiae was inoculated in the prepared Peptone Yeast Dextrose Broth and then incubation was given for 24 hours at 28 degrees. After the growth of yeast and fungus, then enzymatic hydrolysis with fermentation was set up.

6.5 Enzymatic Hydrolysis and Fermentation

For hydrolysis defined Mandels medium was prepared which was further followed by fermentation. The composition of media is as follows:

S. NO.	CHEMICAL	AMOUNT(gms)
1	Urea	0.3
2	Bactopeptone	1.0
3	Yeast extract	0.25
4	Diazanium Sulfate($(NH_4)_2SO_4$)	1.4
5	Potassium Dihydrogen Phosphate(KH_2PO_4)	2.0
6	Calcium Chloride Dihydrate ($CaCL_2.2H_2O$)	0.4
7	Magnesium Sulfate Heptahydrate($MgSO_4.7H_2O$)	0.15
	Trace elements	
8	Iron(II) Sulfate Heptahydrate($FeSO_4.7H_2O$)	0.15
9	Manganese(II)Sulfate Monohydrate($MnSO_4.H_2O$)	1.6
10	Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$)	1.4
11	Cobalt(II) Chloride ($CoCL_2$)	2.0

Table 2: Chemical composition of the fermentation media, Srivastava *et al*., 2014)

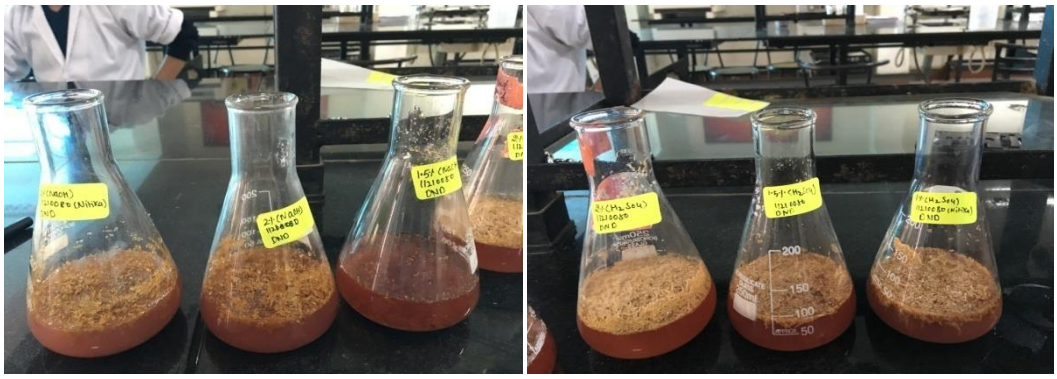


Figure 6: The sample added to the Mandels medium and then incubated for hydrolysis.

For caring out enzymatic hydrolysis the pH of the media was adjusted 5.5 to 6.0 and then the media was sterilized. 5 gm of chemically treated stalks was added in 100ml of fermentation media, after the sterilization of the media it was inoculated with fungus culture, to these flasks incubation of 3 to 6 days was given at a temperature of 28^0C [Srivastava *et al.*, 2014]. After 3 days the culture medium was allowed to undergo fermentation by inoculating it with grown yeast at a temperature of 28^0C. Again the fermentation was performed for next 6 days; in between the concentration of glucose was determined by DNS test.

6.6 Preparation of rice stalks for the treatment by immobilized yeast cell

For the treatment of rice stalks by immobilized yeast cell, 5 gm of the sample was taken and given pre-treatment with alkaline and acidic method at different concentration, same as the pre-treatment was prepared for the fee yeast cell. After the pre-treatment *Trichoderma reesei* was inoculated in the pre-treated sample 3 days before the inoculation of immobilized yeast cell.

6.6.1 Preparation of immobilized yeast cell

1. 2% $CaCl_2$ was prepared and kept at 4^oC for chilling.
2. Sodium alginate was taken at a concentration of 2gm and it was dissolved in hot water through constant stirring.
3. The free yeast cell (2gm) was added to sodium alginate slurry; it was stirred for even distribution.

4. With the help of dropper sodium alginate added with yeast cell was dropped into the chilled calcium chloride solution and was then stored at 4^{o}C.

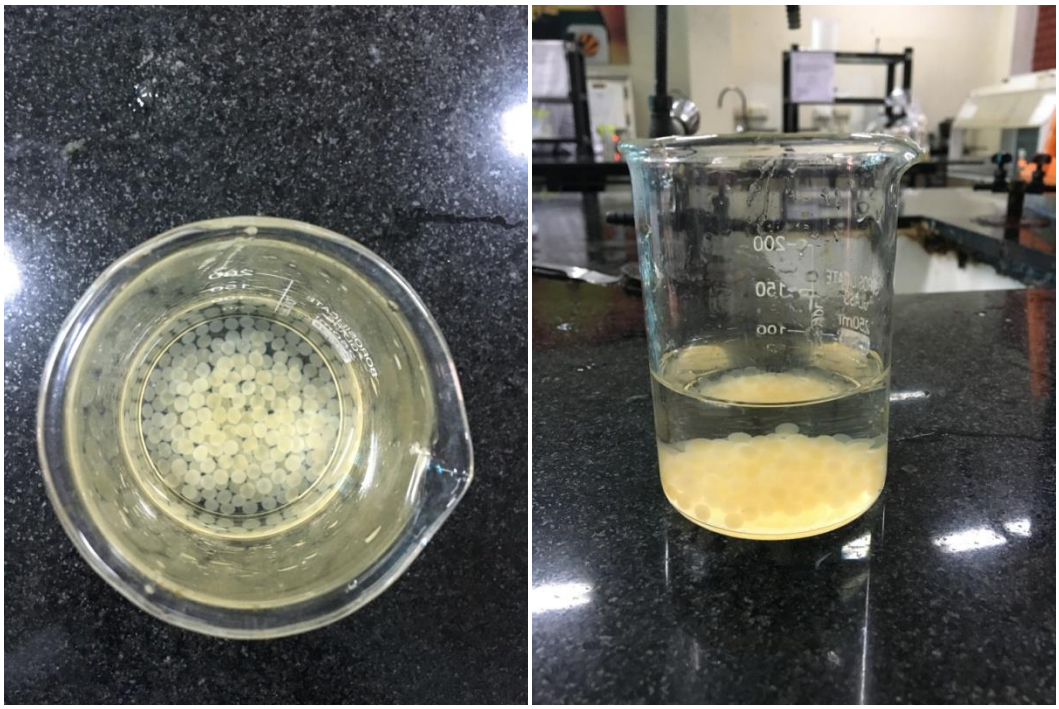


Figure 7: Calcium alginate bead of immobilized yeast cell.

6.6.2 Calculation of the Bead Diameter

In a measuring cylinder 200 beads were taken and drop wise distilled water was added to it through a pipette until the upper surface of the bead is merged into water. The volume of the water added was noted and is known as the 'Void Volume'. Then the total volume was calculated as:

Total Volume = Volume of bead + Volume of distilled water

To calculate the total volume of 200 beads:

Volume of 200 beads = Volume of water – Total volume

Then for calculating the volume of individual bead:

Volume of 1 bead = Volume of 200 bead / 200

With the help of the formula for volume of the sphere:

$$\text{Volume of the sphere} = \frac{4}{3}\pi r^3$$

Where: $\pi = 22/7$ and r = radius of the bead

The radius of the bead was calculated and then diameter was obtained by multiplying the radius by 2 as D = 2

6.6.3 Inoculation of immobilised yeast cell

After 3 days of inoculation of *Trichoderma reesei,* immobilised *S. cerevisiae* beads 30 in count was added to the every flask and kept for the 6 days of incubation. After the incubation DNS test was performed to estimate the amount of glucose preset in the sample. Then filtration and distillation was performed.

Figure 8: Immobilized yeast cells were added to the sample after 6 days of treatment by *Trichoderma reesei*.

6.7 Analysis Method

6.7.1 Dinitrosalicylic Acid Test

For the estimation of the glucose content in the solution DNS test was performed. A fresh DNS reagent was performed in the brown bottle because it is light sensitive. Further standard glucose curve was prepared by making the dilution of stock solution of glucose with the range or 0.2, 0.4, 0.6, 0.8 and 1ml. The volume in each test tube was brought up to 2ml with the help of distilled water. Further 1ml of freshly prepared DNS reagent was added to each tube and then was placed in the boiling water for 5min. After boiling tubes were allowed to cool at the room temperature and 9ml of distilled water was added to each tube and mixed well. For the preparation of standard graph 1ml of solution was taken from each tube and its OD was taken at 540 nm in the spectrophotometer. Then for the estimation of reducing sugar 3ml of DNS reagent was added to

3ml of the sample. The test tubes was capped and placed in the boiling water bath at 90^0C for 5 min to develop red brown color. After taking it out of the water bath 1ml of 40% Potassium Sodium Tartrate (Rochelle salt) was added to stabilize the color. The tubes were then allowed to cool at the room temperature then its OD was taken at 540 nm in the spectrophotometer.

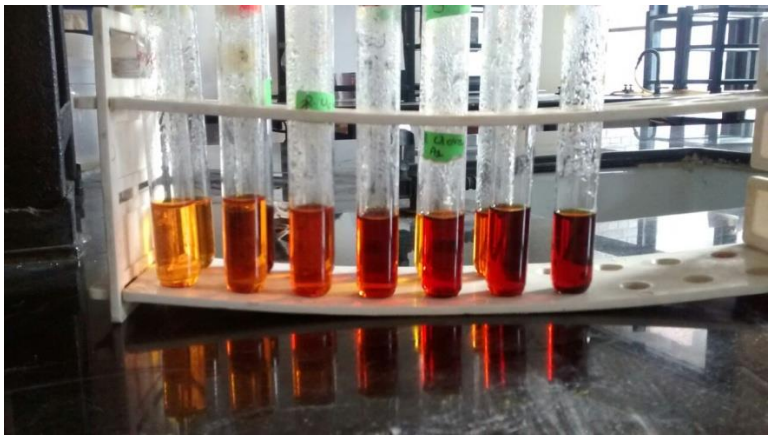


Figure 9: DNS test to know the glucose concentration in the sample

6.7.2 FTIR Analysis

To estimate the presence of cellulose or glucose in the pretreated samples FTIR spectroscopy was performed, which further confirms the presence of cellulose and removal of lignin.

6.7.3 Gas Chromatography

For those samples for which peak showing the presence of ethanol was found good in FTIR analysis gas chromatography was performed. In order to identify the concentration of ethanol in the sample volatile substance were analyzed and separated [Koshy *et al.*, 2014].

CHAPTER-7

RESULT AND DISCUSSION

7. Results and Discussion

7.1 Preparation of substrate:

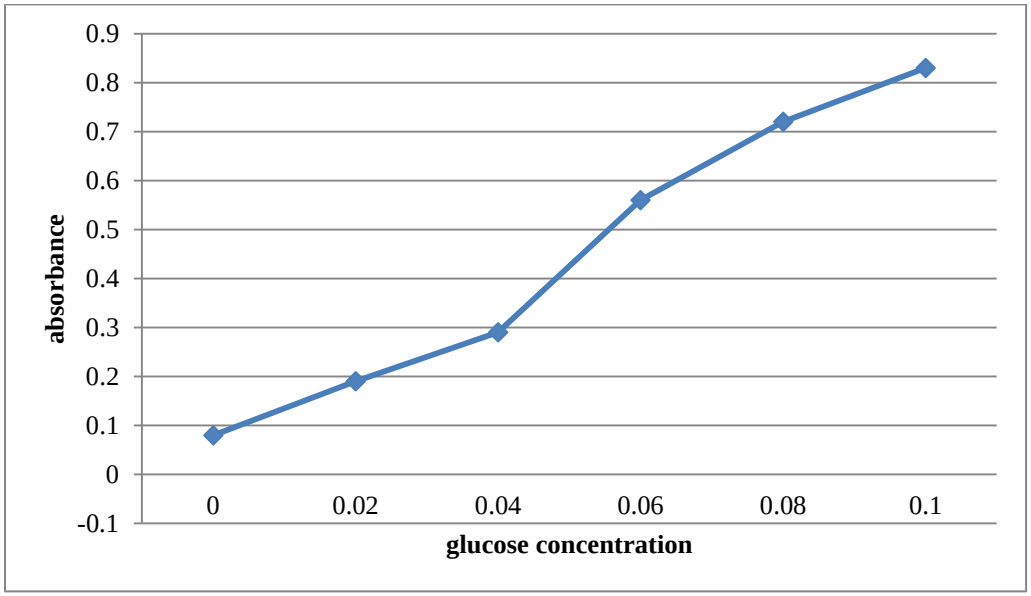
The Rice stalks were collected from nearby milling centre. It was further washed and dried for 24 hour. This was followed by grinding it into small pieces.

7.2 Pretreatment:

7.2.1 Physical Pretreatment:

After grinding the rice stalks it was given heat treatment in the form of autoclaving at 121^0C, 15 psi for 30 min.

7.2.2 Standard Curve of DNS test:



Graph 1: Standard Curve for DNS

7.2.3 Dilute- H_2SO_4 Solution Pretreatment for free yeast cell:

Concentration	Glucose Obtained(g/l)
1% H_2SO_4	0.24
1.5% H_2SO_4	0.26
2% H_2SO_4	0.62

Table 3: Glucose concentration obtained for acidic pretreatment at different concentration for free yeast cell

7.2.4 Dilute-NaOH Solution Pretreatment for free yeast cell:

Concentration	Glucose obtained(g/l)
1% NaOH	0.33
1.5% NaOH	0.41
2% NaOH	0.71

Table 4: Glucose concentration obtained for alkaline pretreatment at different concentration for free yeast cell.

7.2.5 Dilute H_2SO_4 solution after fermentation for free yeast cell:

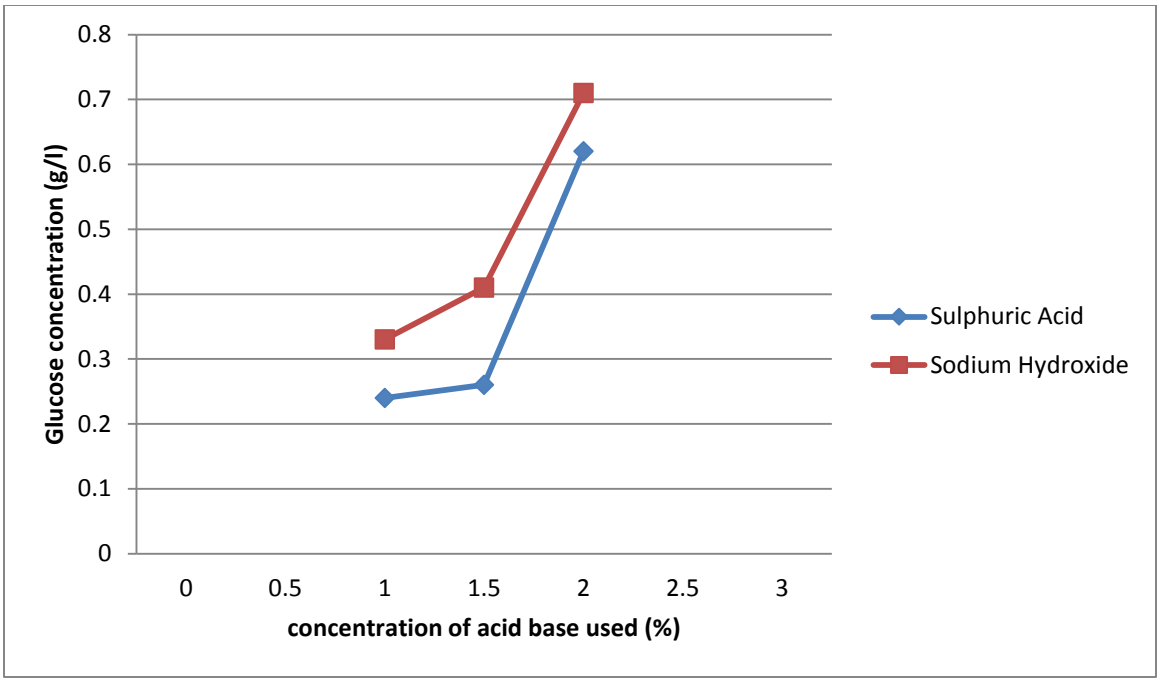
Concentration	Glucose obtained(g/l)
1% H_2SO_4	0.15
1.5% H_2SO_4	0.12
2% H_2SO_4	0.02

Table 5: Glucose Concentration obtained after fermentation for free yeast cell

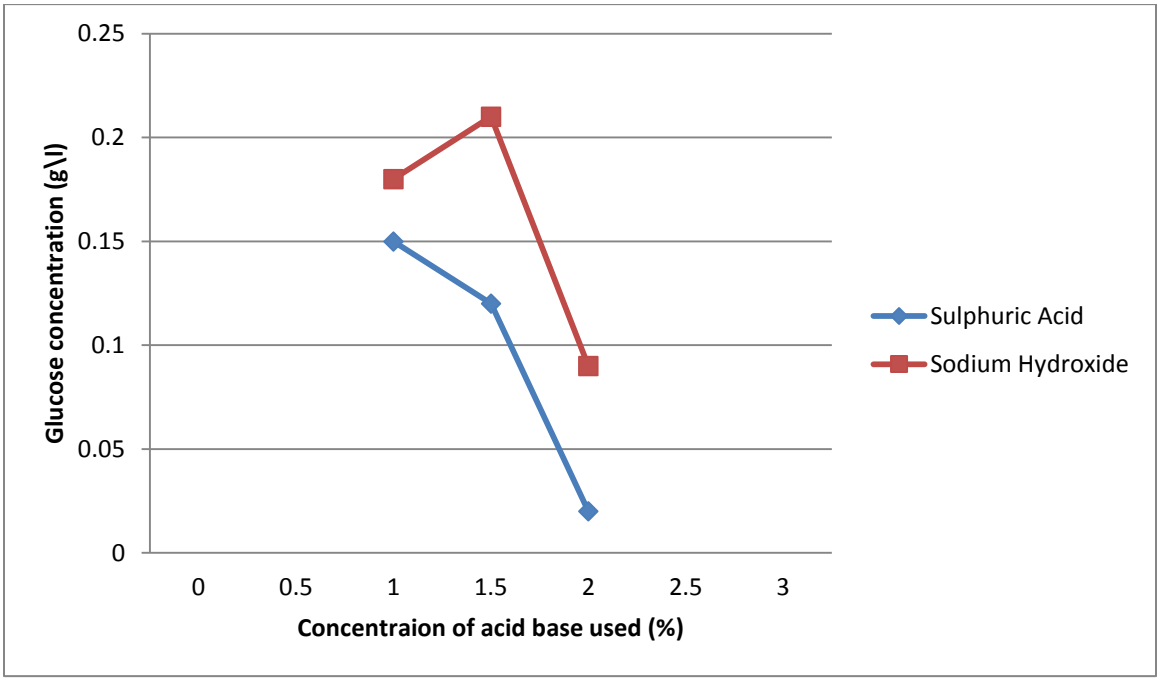
7.2.6 Dilute NaOH solution after fermentation for free yeast cell

Concentration	Glucose obtained(g/l)
1% NaOH	0.18
1.5% NaOH	0.21
2% NaOH	0.09

Table 6: Glucose concentration obtained after fermentation for free yeast cell



Graph 2: Comparison of glucose concentration obtained in acidic and basic pretreatments for free yeast cell.



Graph 3: Comparison of Glucose concentration obtained after fermentation for free yeast cell.

7.2.7 Dilute- H_2SO_4 Solution Pretreatment for immobilized yeast cell:

Concentration	Glucose Obtained(g/l)
1% H_2SO_4	0.25
1.5% H_2SO_4	0.27
2% H_2SO_4	0.64

Table 7: Glucose concentration obtained for acidic pretreatment at different concentration for immobilized yeast cell

7.2.8 Dilute-NaOH Solution Pretreatment for immobilized yeast cell:

Concentration	Glucose obtained(g/l)
1% NaOH	0.36
1.5% NaOH	0.45
2% NaOH	0.76

Table 8: Glucose concentration obtained for alkaline pretreatment at different concentration for immobilized yeast cell.

7.2.9Dilute H_2SO_4 solution after fermentation for immobilized yeast cell:

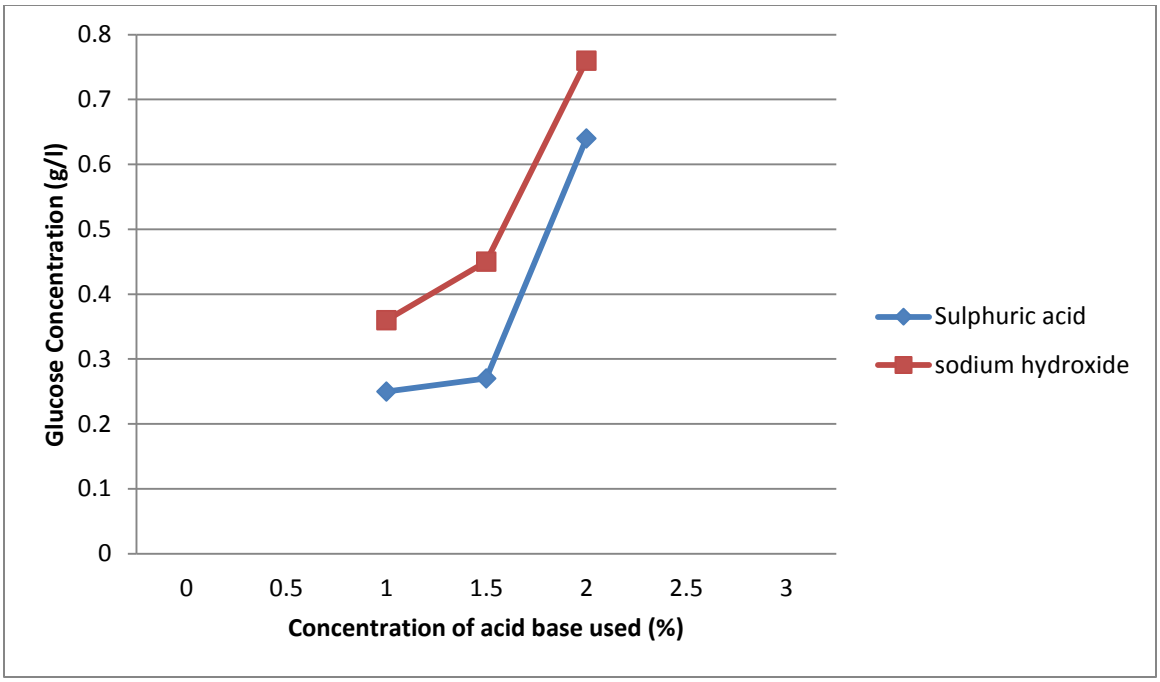
Concentration	Glucose obtained(g/l)
1% H_2SO_4	0.19
1.5% H_2SO_4	0.14
2% H_2SO_4	0.08

Table 9: Glucose Concentration obtained after fermentation for immobilized yeast cell

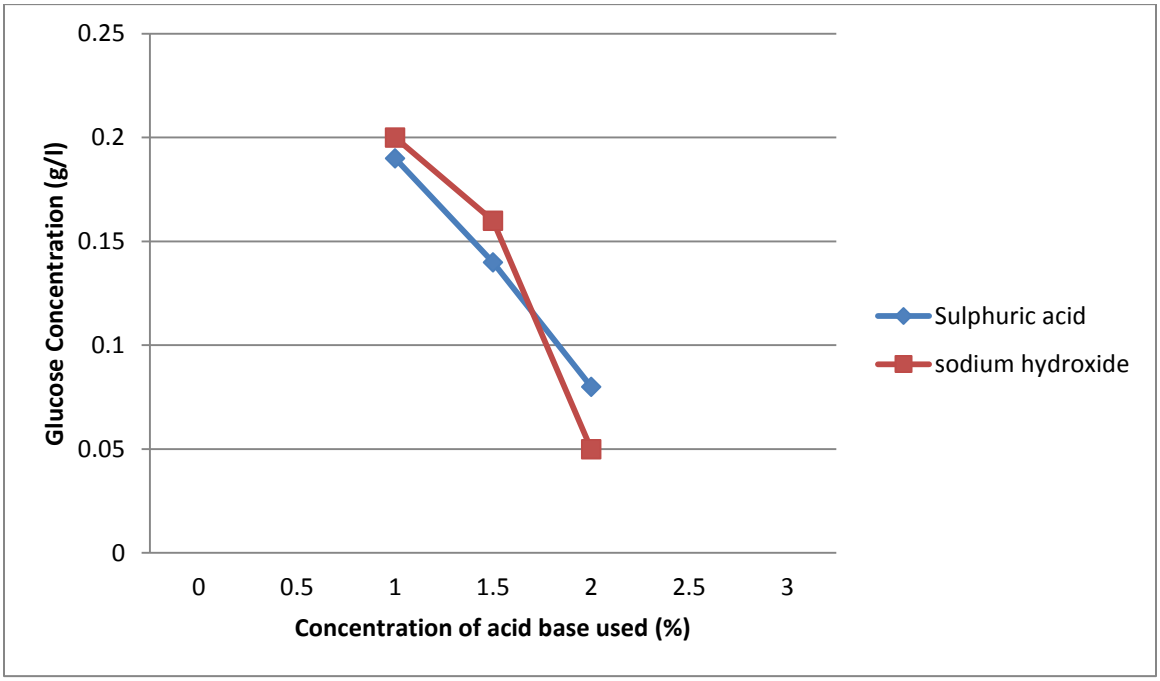
7.2.10 Dilute NaOH solution after fermentation for immobilized yeast cell

Concentration	Glucose obtained(g/l)
1% NaOH	0.2
1.5% NaOH	0.16
2% NaOH	0.05

Table 10: Glucose concentration obtained after fermentation for immobilized yeast cell

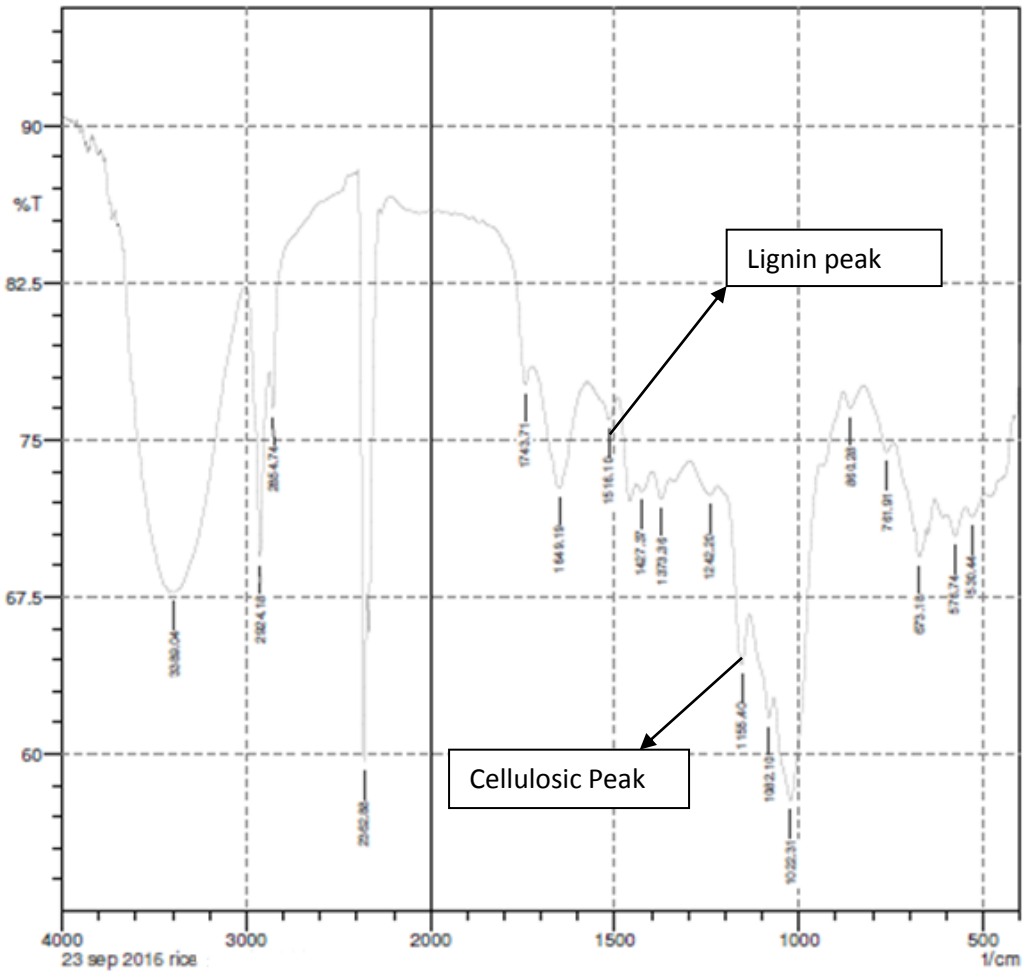


Graph 4: Comparison of glucose concentration obtained in acidic and basic pretreatments for immobilized yeast cell.



Graph 5: Comparison of Glucose concentration obtained after fermentation for immobilized yeast cell.

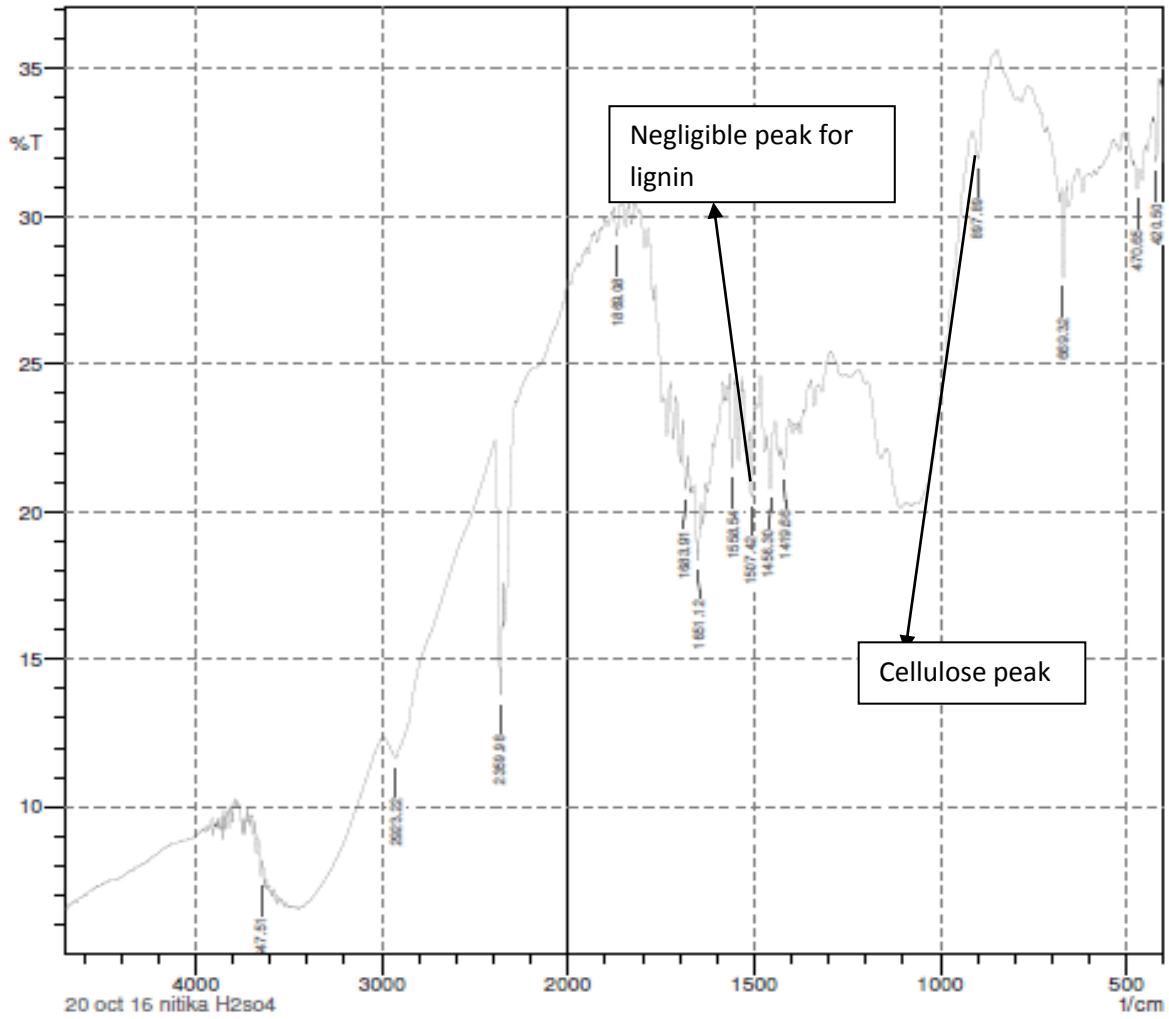
7.3 FTIR Analysis



Graph 6: FTIR analysis of control (untreated) sample

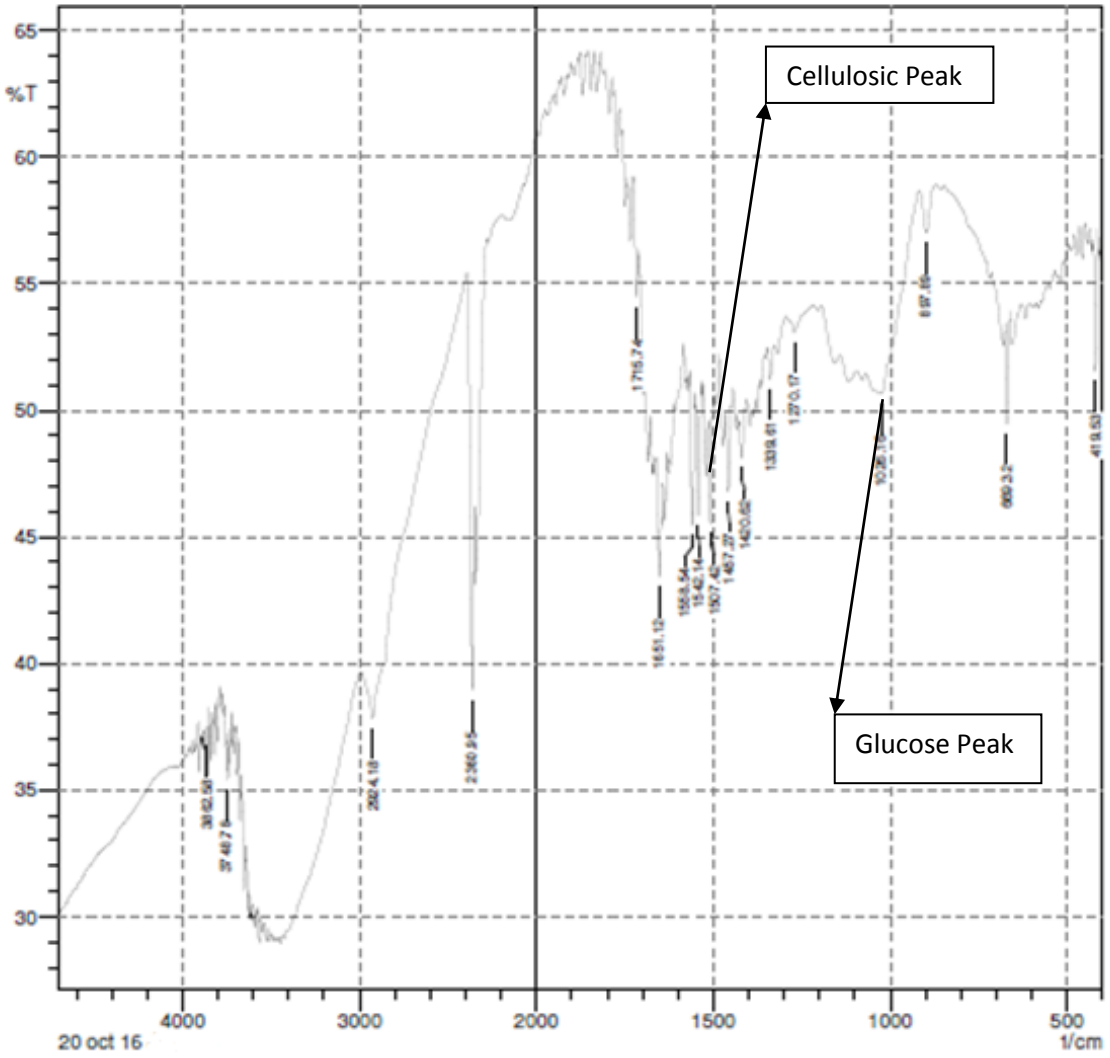
As stated by Salmen et al. (2012) the IR range of the aromatic ring also known as the characteristic of lignin was known to be 1506 cm^{-1}. In the untreated sample of rice stalks 1516.99 cm-1 and 1554.75 cm-1 result was obtained. The IR spectrum of lignin shows a wide range of band between 3500-3100cm-1it was stated by Bykov *et al*. (2008).

7.3.1 FTIR results for both acidic and alkaline treated sample



Graph 7: FTIR analysis for the sample treated with Sulphuric acid

In the sample which was pretreated with acid negligible peak of lignin as obtained for all the concentration of the sulphuric acid. In 2% H_2SO_4 the cellulose peak was obtained in the range of 1570.54 cm-1 it was similar to the study of nikonenko *et al*, (2002).



Graph 8: FTIR analysis for the sample treated with Sodium Hydroxide

The analysis that was done for the sample treated with Sodium Hydroxide to obtain the glucose and cellulose peak. It was found that glucose peak found in the range 1026.16 cm-1 and cellulosic peak was found in the range 1457.27 cm-1. This range was similar to the study of Adina *et al*, (2010).

7.4 Filtration and Distillation

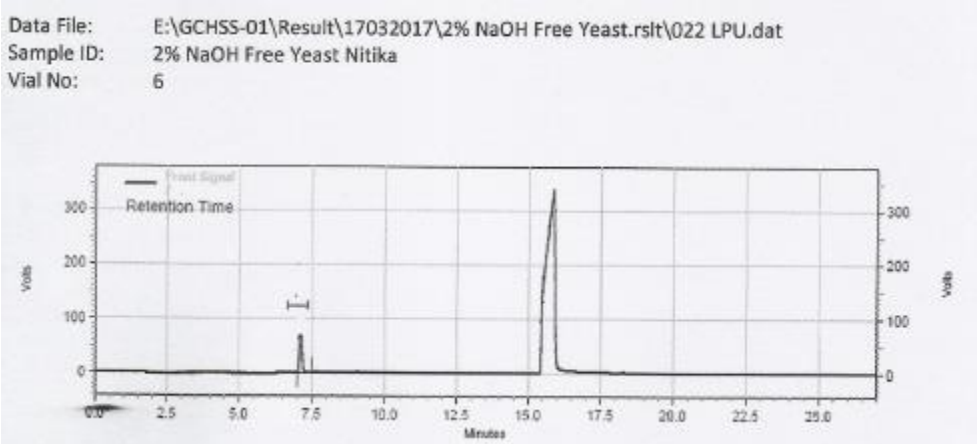
After the process of fermentation it was followed by filtration and distillation. This process was done at a temperature of 78.37 degrees because it is the boiling and evaporation temperature for ethanol, due to this temperature ethanol got separated from the rest of the solution.

Figure 10: Distillation Apparatus

7.5 Gas Chromatography analysis of produced ethanol

To know the concentration of ethanol and to obtaining the result of Gas Chromatography the samples was send to HHRC (Herbal Health Research Consortium) Amritsar and following results were obtained.

7.5.1 Gas chromatography of NaOH sample treated by free yeast cell

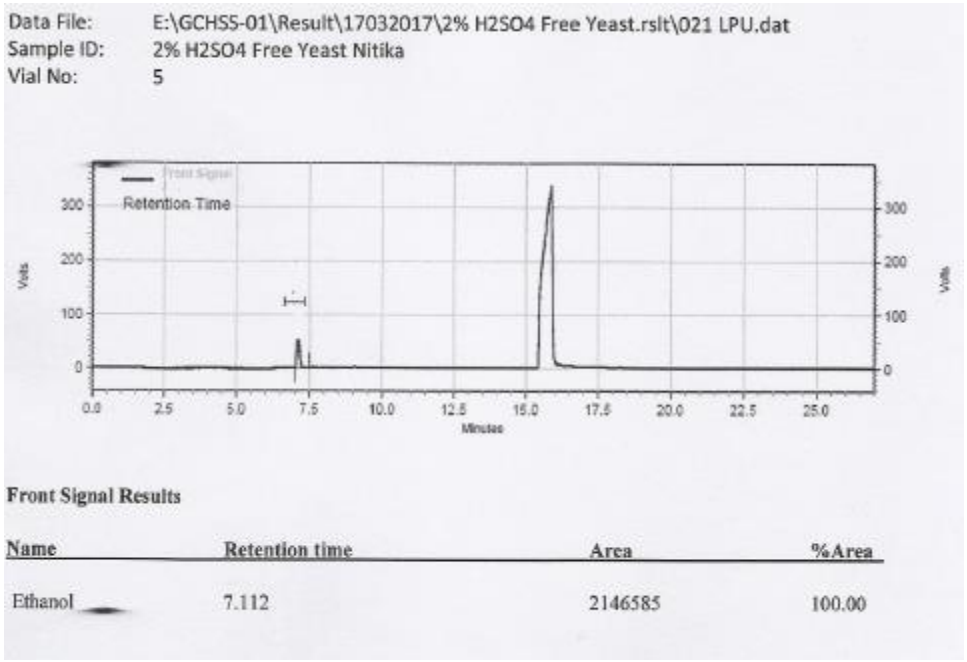


Front Signal Results

Name	Retention time	Area	%Area
Ethanol	7.110	3145658	100.00

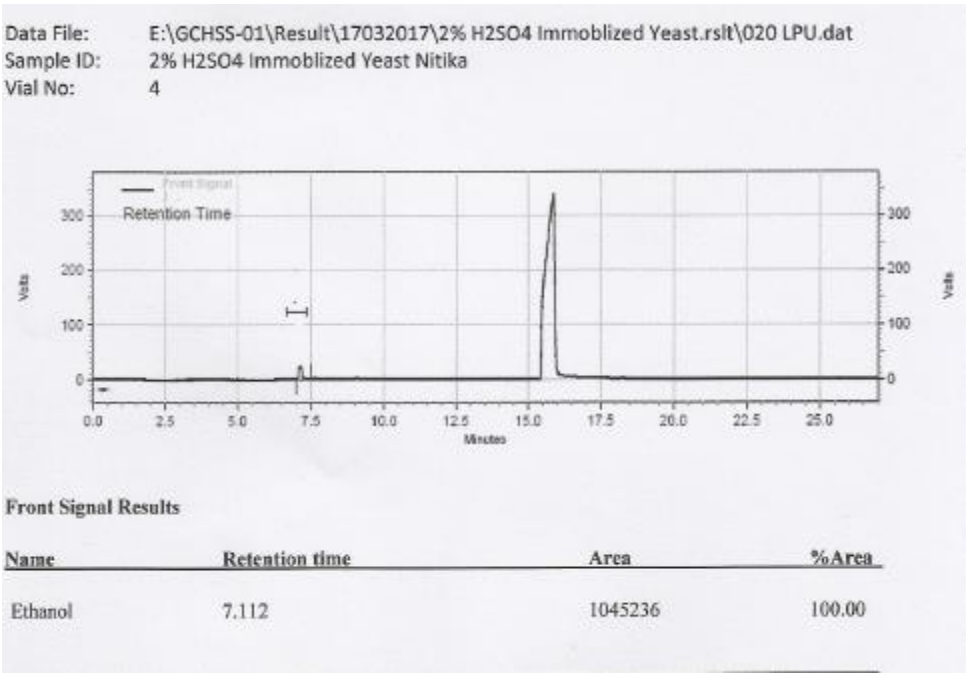
Graph 9: GC graph showing the peak for ethanol, the sample was treated with 2 % NaOH and free yeast cell.

7.5.2 Gas Chromatography of H_2SO_4 sample treated by free yeast cell



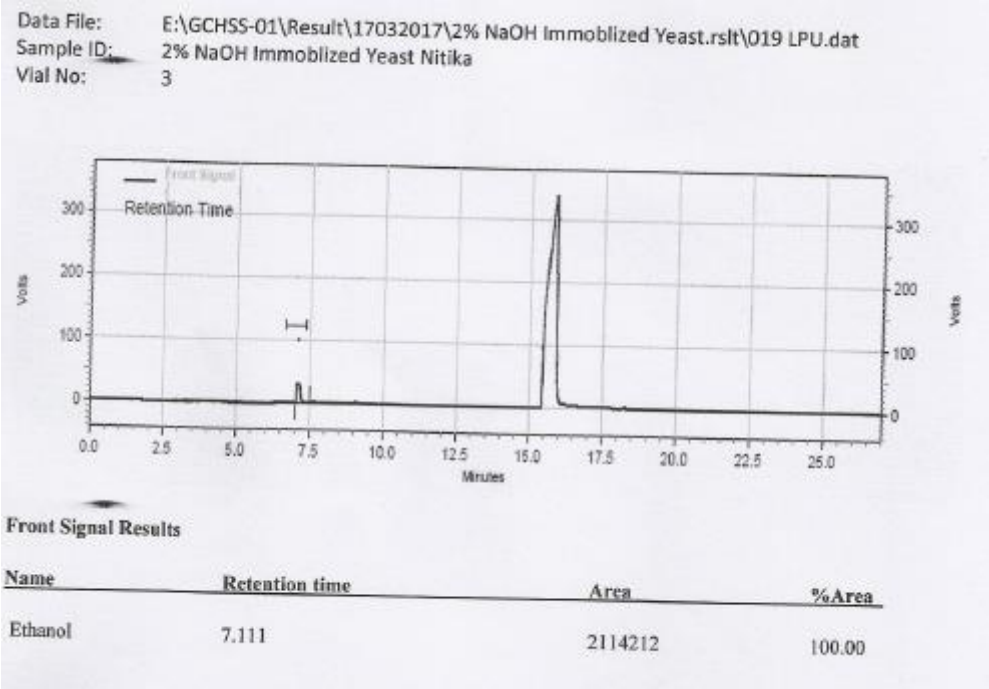
Graph 10: GC graph showing the peak for ethanol, in sample treated with 2% H_2SO_4.

7.5.3 Gas Chromatography of H_2SO_4 sample treated by immobilized yeast cell



Graph 11 : GC graph showing the peak for ethanol, in sample treated with 2% NaOH

7.5.4 Gas chromatography of NaOH sample treated by immobilized yeast cell



Graph 12: GC graph showing the peak for ethanol, in the sample treated with 2% NaOH

	Area of test	Vol of sample	% Ethanol(v/v)
2% NaOH Immobilized	2114212	100	0.387861663
2% H_2SO_4 Immobilized	1045236	100	0.191753227
2% H_2SO_4 free	2146585	100	0.393800635
2% NaOH free	3145658	100	0.577085053

Table 11: Analysis of GC showing the available concentration of ethanol.

After the Gas Chromatography it was found that in 2% NaOH treated with free yeast cells had the highest amount of ethanol i.e. 0.57% and ethanol obtained by the 2% H_2SO_4 was 0.39% , 2% NaOH immobilized yeast cell was 0.38% , and 2% H_2SO_4 immobilized yeast cell was 0.19%.

CHAPTER-8

CONCLUSION AND FUTURE SCOPE

Bioethanol serves as the best alternative for the renewable energy that can be extracted from agricultural waste. The project was aimed for optimizing the pretreatment method and enhanced bioethanol production from rice stalks using SSF through free and immobilised *Saccharomyces cerevisiae.* Cellulose, hemicellulose and lignin are the major structural component of the substrate. There are many hindrances that occur during the extraction process, this is due to the complex structure of the lignocellulose content of the biomass. Various pretreatment processes are used for the removal of this lignin content and the exposure of the glucose so that it can be hydrolyzed and fertilized to yield bioethanol. Cellulose and hemicellulose were saccharified for fermentation which leads to the production of bioethanol that was characterized by FTIR and Gas Chromatography.

When the pretreatment was done using alkaline (NaOH) and acidic (H_2SO_4) it was found that NaOH was better than H_2SO_4. And when the fermentation was done using free and immobilized *S. cerevisiae,* free *S. cerevisiae* treatment was found give higher amount of bioethanol than the immobilized one. From the results obtained by Gas Chromatography it was concluded that 2% NaOH sample which is treated with free *S. cerevisiae* has the highest amount of bioethanol produced i.e. 0.57%, this was more than other treatments done.

CHAPTER-9

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CHAPTER-10

APPENDIX

1. Composition of Peptone Yeast Dextrose Broth

Sr.No.	CHEMICALS	AMOUNT(gm)
1	Peptone	2
2	Yeast	1
3	Dextrose	2
4	Distilled water	100ml

2. Chemical composition of the fermentation media, Srivastava *et al*., 2014)

S.NO.	CHEMICAL	AMOUNT(gms)
1	Urea	0.3
2	Bactopeptone	1.0
3	Yeast extract	0.25
4	Diazanium Sulfate($(NH_4)_2SO_4$)	1.4
5	Potassium Dihydrogen Phosphate(KH_2PO_4)	2.0
6	Calcium Chloride Dihydrate ($CaCL_2.2H_2O$)	0.4
7	Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	0.15
	Trace elements	
8	Iron(II) Sulfate Heptahydrate($FeSO_4.7H_2O$)	0.15
9	Manganese(II) Sulfate Monohydrate ($MnSO_4.H_2O$)	1.6
10	Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$)	1.4
11	Cobalt(II) Chloride ($CoCL_2$)	2.0