OPTIMIZATION OF PRE-TREATMENT FOR ENHANCED SACCHARIFICATION AND BIOETHANOL PRODUCTION FROM WHEAT STRAW USING SSF THROUGH FREE AND IMMOBILIZED *Saccharomyces cerevisiae.*

Project report

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

Master of Technology in Biotechnology

Submitted by

Meenakshi Kanungo (Reg no. 11209231)

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 wheat straw using SSF through free and immobilized *Saccharomyces cerevisiae*" is an authentic record of our own work carried out at school of bioengineering and biosciences, Lovely Professional University, Phagwara Punjab for the partial fulfillment of the award of Master of technology in Biotechnology under the guidance **Mr. Himanshu Singh (**Assistant Professor, Domain of Biotechnology, Lovely Professional University)**.**

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

Place: Meenakshi Kanungo(11209231)

Date:

CERTIFICATE

This is to certify that Meenakshi Kanungo (11209231) have completed her project, entitled "Optimization of pre-treatment for enhanced saccharification and bioethanol production from wheat straw using SSF through free and immobilized *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

ACKNOWLEDGEMENT

I Meenakshi Kanungo 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.

First and foremost, I want to express my thanks to my guide **Mr. Himanshu Singh** (Assistant Professor, Domain of Biotechnology, Lovely Professional University) for his encouraging attitude, affection, inspiration, valuable suggestion and creative guidance for successful completion of this project. I am really thank you sir for giving me an opportunity to work under your guidance.

Meenakshi kanungo

ABBREVIATIONS

s. no.	Abbreviations	Full form
1	*A. Niger*	*Aspergillus niger*
2	AFEX	Ammonium fibre explosion
3	CBP	Consolidated Bio Processing
4	CCD	Central composite design
5	DNS	Dinitrosalicylic acid
6	H_2SO_4	Sulphuric acid
7	FTIR	Fourier transform infrared spectroscopy
8	GC	Gas chromatography
9	LCC	Lignin carbohydrate complexes
10	NaOH	Sodium hydroxide
11	*S. cerevisiae*	*Saccharomyces cerevisiae*
12	SSF	Simultaneous saccharification fermentation
13	SHF	Separate hydrolysis and fermentation
14	*T. viride*	*Trichoderma reesei*
15	*Z. mobilis*	*Zymomonas mobilis*

LIST OF TABLES:

LIST OF FIGURES:

LIST OF GRAPHS:

TABLE OF CONTENT

Abstract

Wheat straw is abundantly found agriculture waste having low commercial low value. Wheat straw is lignocellulosic biomass which can be used for the production of bioethanol. As fuels are depleting source of global world so ethanol can be used for alternative source. Pre-treatment method is used for lignocellulosic biomass for improving the hydrolysis of the wheat straw as it contains high amount of cellulose and removal of lignin and hemicellulose. Cellulose convert into the reducing sugars and then to the ethanol. Acidic and alkaline pretreatment methods used and from both of the method alkaline pretreatment come up with the good results. Free and immobilized *Saccharomyces cerevisiae* was used for the fermentation and was found that pretreated sample fermented with free yeast cell found with the better results for the production of bioethanol. Fourier transforms infrared spectroscopy and gas chromatography methods are used for the estimation of ethanol.

CHAPTER 1

INTRODUCTION

1. Introduction

Bioethanol is an alcohol made by fermentation. Bioethanol is produced by the sugar or from cellulose by the process of fermentation and can also be obtained from the chemical reaction of ethylene with steam. Mainly sugar is used as source for production of ethanol. Source comes from energy crops. The principle fuel used as petroleum alternate is bioethanol. The sources for sugar required to produce ethanol comes from fuel or energy crops .The mainly used crops are also grown for specific energy use these crops are maize, corn, sugarcane, rice and wheat straw crops, waste straw, sorghum plants, sawdust , red canary grass.

Ethanol also called ethyl alcohol with the structural formula CH_3CH_2OH, also known as C_2H_5OH or C_2H_6O. When consumed ethanol can cause alcohol intoxication. Best known as the type of alcohol found in beverages containing alcohol, it is also used in thermometers, as a solvent, and as a fuel. In common, it is simply used as alcohol or spirits. (Shakhashiri 2009).

1.2 Lignocellulose bioethanol

Ethanol is bio-based oxygen enriched fuel. Developing bioethanol as bio fuel, the main requirement is development of lignocellulose bio mass which serves as low cost and enriched in carbon and energy feed biomass. Lignocellulose means to dry matter of plants so abundant raw material is present on earth. Lignocellulose composed of two polymers carbohydrate polymer those are cellulose and hemicellulose and aromatic polymer as lignin. Carbohydrate polymer consists of various sugar monomers and tightly bound to lignin (Saha *et al.* 2005). Different and many advance technologies are there for the bioconversion of lignocellulose to biofuel and choose technologies are based upon the factors of economic and environmental factors (Carroll *et al*., 2009).

Lignocellulose is very recalcitrant so pretreatment is done to simplify the recalcitrant structures. Hydrolysis is performed to hydrolyze polysaccharides example cellulose and hemicellulose into the sugars. Fermentation process is done for converting fermentable sugar into ethanol (Huang *et al*., 2011).

Lignocellulose compounds are taken for agriculture waste, forestry, fruit and household wastes. Hydrolysis of cellulose causes production of glucose and hemicellulose causes production of both hexose and pentose sugar. During the process of hydrolysis production of acetic acid is also there that can also be factor for inhibition in ethanol fermentation process. Lignin is mostly the by-product. Difference between the processing steps of starch and lignocellulose feedstock but hydrolysis stages of lignocellulose is more complicated (Chandel *et al.*, 2007).

1.3 Main processes for bioethanol production

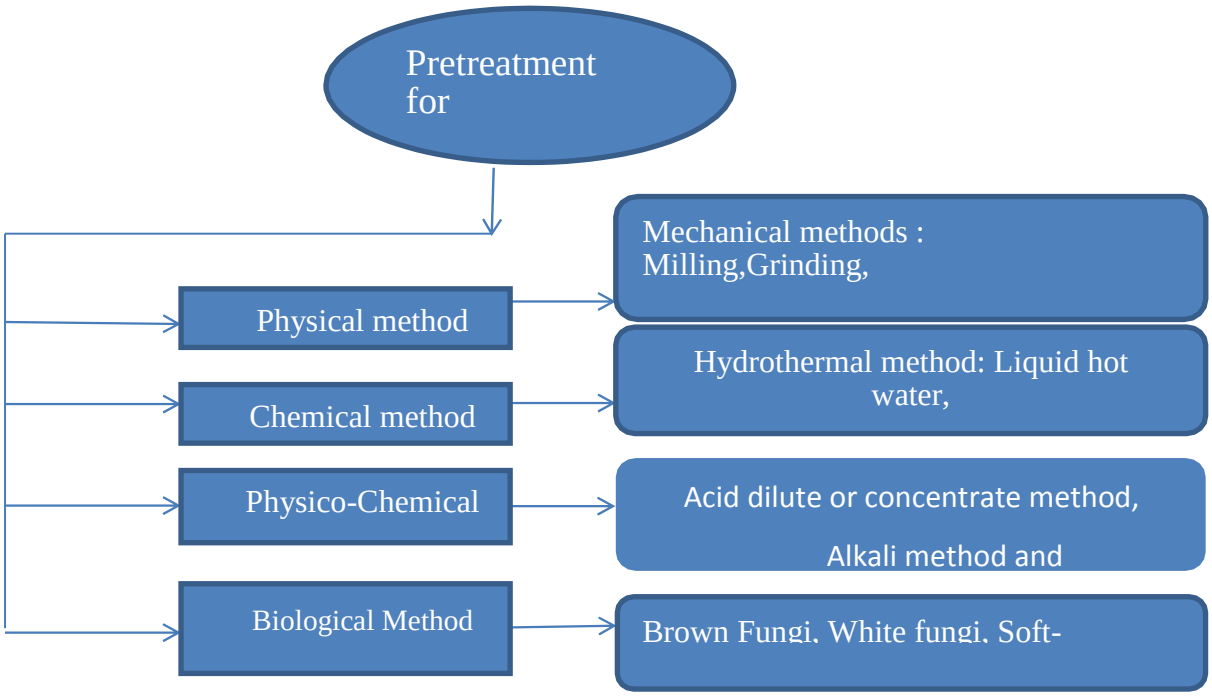


Figure 1. Different pretreatment methods for production of bioethanol from wheat straw.

1.2.1 Pretreatment process

Different methods of pretreatment are physical, physico-chemical, chemical, biological process used according to the cost effective. The main purpose of pretreat is to clear away lignin and hemicellulose, abate cellulose crystallinity and hence the porosity of the feedstock wheat straw. Pretreatment is aimed for no formation of byproducts those act as inhibitor to further process that

is hydrolysis, no loss of carbohydrates, and more formation of sugar (sun *et al.*, 2010).

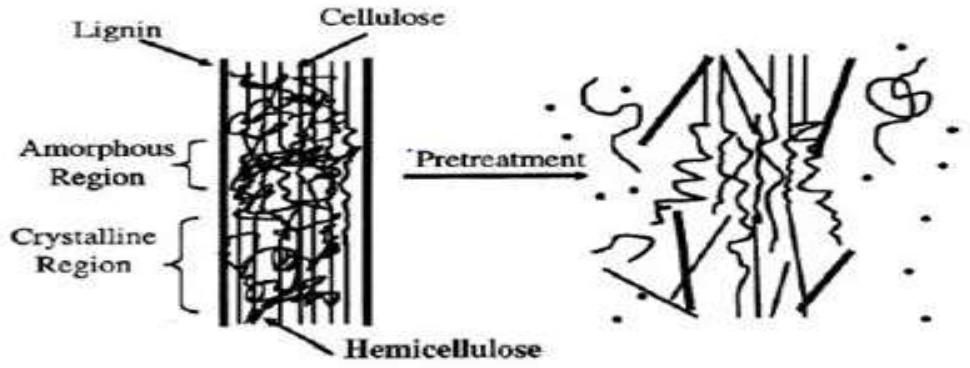


Figure 2 .Cellulose released from lignin structure during pretreatment (Mosier et al. 2005)

1.2.2 Hydrolysis

Hexose and pentose sugars are produced by hemicellulose hydrolysis: mannose, galactose, xylose and arabinose that cannot be fermented with existing strain. During hydrolysis primarily acetic acid is also produced that can inhibit the process of ethanol fermentation. By-products mainly have lignin. As compared to the grains ethanol byproducts have less food value so used as production of ethanol. The difference in process steps between starch and lignocellulosic feedstock is that lignocellulosic biomass requires more complex stages in hydrolysis. Cellulose biomass is biologically converted to ethanol, fuel and chemical through cellulose enzymatic hydrolysis which favors to higher rate of yield (Yang *et al*., 2011).

1.2.3 Fermentation

After the process of pretreatment and hydrolysis release of sugar, pentose and hexoses to ferment them microorganism for bioethanol production are used. Microorganism is used as per there different capabilities as to hence the production of bioethanol yield and substrate utilization and main is to ferment sugar at high temperature for production of bioethanol. Different microorganism can be used like fungi, Bacteria, yeast, pure and mixed culture (Talebina *et al.*, 2009).

1.3 Properties of bioethanol as fuel

- The main property of bioethanol is having the high octane number (108), wide flammability and combustible limits, and comparably more heat of vaporization from gasoline. All given properties allow the higher compression ratio, spark burn time, thin burn engine, as these moves to more efficient over gasoline in internal burning engine.
- Bioethanol is efficient for the varied biofuel in the' gasoline engine as it has high octane number(108) and cetane number is low and large heat of vaporization disturb self-kindling in the diesel engine . Glow plugs, surface kindling and other are applied to increase self-ignition by using diesel and ethanol blended fuel.
- Popularly blended E85 (85% bioethanol and 15% gasoline) used for light duty vehicles. Ethanol is enriched with oxygen (35%), which lowers hydrocarbon, carbon monoxide, particulate and nitrogen oxide (NO_X) emissions from combustion.
- Bioethanol is oxygenated fuel as it provides sensible antiknock value. As amount of oxygen content of a fuel raises its burning capacity.
- Ethanol has same fuel properties as methanol but ethanol costs more because its production is mostly from biomass bioconversion. Systematically reaction of ethanol is different methanol and ethyl alcohol is fast oxidized in nature. As methanol is easy to recover and recycles because no formation of zoetrope (Balat *et al.*,2008).

1.4 Rate production of Bioethanol

As increase in the world population and industrialization there is high demand for energy. As this resulted in the increment in the cost of crude oil, natural gases (methane, ethane and butane) daily. Thus the development of biofuel like biodiesel, biogas and bioethanol all are the influencing renewable energy among biofuel. The commonly used worldwide renewable fuel is ethanol. Production of bioethanol increased widely all over the world since, in 1970 oil crises. That effect the market as it reduced to billion liters in 1975 and even high in 39 billion liters in 2006, and the expected to attain 100 billion liters in 2015. Although, minimize production costs are essential to make biofuel more combative, mainly when oil costs are below US$ 80 per drum (Tesfaw *et al.*, 2014).

In 2004 ethanol production was calculated to be 40 giga liters. Brazil and US are the leader both consider about 60% of the ethanol production through the sugarcane and corn (Chandel *et al.*, 2007).

1.5 Immobilized yeast cell.

Immobilized yeast cell method has been proposed as an active means for taming ethanol fermentation. Immobilization of cells leads to superior cell masses with resulting increases in rate of reaction and yield. Bioethanol production by immobilized cells has been widely investigated for the last few periods. Immobilized of cell have been performed on a variety of natural and artificial supports. Widely used is method synthetic importer in immobilization methods is founded on cell entrapment in different gels, such as carrageen and Ca-alginate. Limitation of these methods is the absence of mass transmission between product and substrate in the method. Other drawbacks are the volatility of CA alginate in contradiction of the buffer solution and the interruption of gel particles due to CO_2 change during fermentation, which also bounds its usage .(Martini *et al*., 2010)

CHAPTER 2

TERMINOLOGY

2. Terminology

- **Bioethanol:** Ethanol formed from plants such as wheat straw ,rice husk ,sugar cane or maize, used as an substitute to petrol.
- **Chromatography:** A method for the parting of a mixture by transient it in solution or holdup through a medium in which the constituents move at different rates.
- **Distillation:** The action of liberating a liquid by a method of heating and cooling.
- **Fermentation:** The biochemical analysis of a substance by yeast, bacteria or other microorganisms, typically relating foaminess and the giving off of heat.
- **Filtration:** The action or process of filtering something like solid part mixed with liquid and separated out..
- **Hemicellulose:** A hemicellulose is any of several heteropolymers, such as arabinoxylans, present along with cellulose in almost all plant cell walls
- **Hydrolysis:** The chemical breakdown of a compound due to reaction with water.
- **Lignocellulose:** A compound of lignin and cellulose present in the cell walls of woody plants.
- **Pyrolysis:** Decomposition brought about by high temperatures like high temperature.
- **Saccharification:** It is a method of hydrolysis of polysaccharides to soluble sugars is called saccharification.
- **Suspension:** A suspension is a mixed mixture having solid particles that are suitably large for sedimentation

CHAPTER- 3

REVIEW OF LITERATURE

3. Literature survey

Study comprises the advance techniques and challenges during the processes those are pretreatment, hydrolysis, fermentation and distillation. An agricultural waste wheat straw is most abundant and has very less market value. World's21% food varieties depend upon wheat crop so its production should be increased for fulfilling the requirement and human consumption. Wheat straw has a complex mixture of cellulose, hemicellulose and lignin. Pretreatment is done to be make cellulose work for better enzymatic depolymerization. Physical process involves milling, grinding, chipping. Physico-chemical process involves the process hydrothermal i.e. is liquid hot water and steam explosion and chemicals used as H_2SO_4 and AFEX. Chemical process have several acids (dilute, concentrate), alkali (NAOH, lime), oxidizing agents (h_2O_2, WO and ozonolysis). Fungal method as brown white and soft-rot fungi examples as *Aspergillus Niger, pleurotus ostrearus, aspergillus awamori.* Further hydrolysis is performed by using enzymes *Trichoderma reesei* and *Aspergillus niger*. Three main enzymes called endo-glucanase exo-glucanase and β-glucosidase these entire plays vital role in hydrolysis. Optimum temperature (45-55°C) and pH (4-5) is maintained as almost all the enzyme show there activity. Tween 20 non-ionic surfactant as raises the cellulose conversion during hydrolysis. Utilization in industries of lignocelluloses for the production of bioethanol *Saccharomyces cerevisiae* and *Zymomonas mobilis* are microorganism for fermentation offering high yield (90-97%) and increased ethanol tolerance up to Ca. 10 %(w/v). The different technique like separate hydrolysis and fermentation (SHF), saccharification and fermentation (SSF) are performed in different vessels under specific conditions. The future perceptive of the study as *S. cerevisiae* unable to uptake pentose sugar so the metabolize technology is used for the process of fermentation of the mixed sugar. Some yeast and bacteria such as *E.coli, kelbsiella oxytoca, z. mobilis and S. cerevisiae* has shown result and along with commercial scale up (Talebina *et al*., 2009).

According to the literature rice straw is highly available biomass all over the world about 7.31×10^{14} per year .The various methods for pretreatment were carried out such as grinding, milling, pyrolysis, high pressure steam, alkaline hydrolysis, acid hydrolysis, gas treatment, hydrogen peroxide treatment and biological pretreatment. Enzymes help in the conversion of glucose

which is the main for the ethanol production. Several enzymes can be used for the production and anaerobic thermophilic bacteria, like *Clostridium therocellum, Zymomonas, Escherichia coli* and of the filamentous fungi involving *Monilia sp., Neurospora crassa, Neurospora sp., Zygosaccharomyces rouxii, Aspergillus sp, Trichoderma viride, Paecilomyces sp.* Pretreatment of the lignocellulose biomass is done for the production of ethanol from the biomass by using the specific strain of *Saccharomyces cerevisiae* in the submerged fermentation. The sample collected that wheat straw dipped in 3% h_2O_2 +2% NaOH solution 2 hours at room temperature. After the steaming is done at 130 °C for 60 min. Determination of lignin and delignification is expressed by equations:

$$\text{Lignin (\%)} = \frac{\text{lignin weight (g)}}{\text{Substrate weight (g)}} \times 100$$

$$\text{Delignification (\%)} = \frac{L_u - L_t}{L_u} \times 100$$

l_u = lignin (untreated sample)

l_t = lignin (treated sample).

Commercial enzyme with Carboxymethyl cellulose (CM Case) solution was prepared and used for process of hydrolysis. Terminated enzymatic hydrolysis the sample centrifuge at 10,000 rpm for 10min. Calculation of
Saccharification (%).

$$\text{Saccharification(\%)} = \frac{\text{Reducing sugar(g)} \times 0.9}{\text{Cellulose content in pretreated substrate}} \times 100$$

Ethanol production is measured spectrophotometric ally. Fourier transforms infra-red spectroscopy of substrate used to examine the chemical disturbance in treated and untreated sample. ANOVA used for the statistical analysis. They concluded that pretreatment of the sample is important for more amount of sugar as result by method of enzymatic hydrolysis. For conversion of sugar to ethanol *S. Cerevisiae* is used *(*Irfan *et al., 2013).*

According to the study in 2009 production of ethanol rose up to 19,535 millions of gallons.

Immense capacity for conversion sugar and biofuel from lignocellulose feed stock referred as second generation production of bioethanol. For processing the first step that is pretreatment performed with hydrothermal process used hot compressed water as this is useful and efficient because no chemical is required and no toxic aggregate formation occur . Yeast strain *Saccharomyces cerevisiae* and enzymes solution those are β- glucosidase from *Aspergillus niger* and cellulose from *Trichoderma reesei* commercially obtained. Standard procedure was conducted for simultaneous saccharification and fermentation in which flocculating enzyme *saccharomyces cerevisiae* and incubated at various conditions. Concentration of Ethanol and amount of remaining sugar were estimated by HPLC. Ethanol yield calculation and statistical analysis along with experimental design were examined in study.

$$\text{Ethanol yield} = \frac{[EtOH]_f - [EtOH]_0}{0.51f\,[\text{Biomass}] \times 1.111} \times 100\%$$

Where, $[Et\ OH]_f$ final ethanol concentration and $[Et\ OH]_0$ is the initial rate of ethanol concentration and $0.51f$ [Biomass] term is theory based concentration and weight of initial rate of ethanol. Using hydrothermal processing glucan is not that much affected solid aggregate and increased concentration of glucan which is also in relation with hemicellulose that is amorphous and unstable. During SSF yield of ethanol increases and level of glucose get reduced. These results showed that *Saccharomyces cerevisiae* CA11 provides better yield than yeast. ANOVA used for ethanol and carbon dioxide concentration. Temperature plays always crucial role in the SSF process. *Saccharomyces cerevisiae* CA11 has potential for ethanol production. (Ruiz *et al.,* 2011).

Pretreatment process for ethanol production the other factors like temperature, substrate concentration, enzyme concentration and the final product accumulated depends upon he optimized condition of SSF. For pretreatment sample was treated with 1.0% (w/w) H_2SO_4 for time 12 hour at room temperature. *Saccharomyces cerevisiae* AS2.1 were used and yeast was developed at 30 °C at 100 rpm with rotatory shaking water bath for 24 hours and culture concentration was noted to 2.4×10^8 yeast cells per ml. Concentration of reducing sugar was determined by reagent 3,5-dinitrosalicylic acid(DNS). Total water-soluble hemicellulose content was resulted by phenol-sulfuric acid and ethanol content through gas chromatography.

SSF temperature optimized condition resulted as decrease in temperature 30 °C accordingly decrease in ethanol yield and concentration and also decrease in saccharification rate. Another case of increasing the temperature to 40 °C resulted in decreased limited changes in ethanol concentration. SSF rate dependent upon hydrolysis rate and as saccharification pace of cellulose increased more with increment in enzymatic concentration and as inserting β-glucosidase in effect hydrolyzes prompt cellulose exerting inhibitory resulted in stimulation of cellulose enzyme to glucose and accumulation of sugar was avoided. Evidently 35°C recorded as for favorable reaction temperature and pH for yeast farming and cellulose activity is 4.8 and 5.0-5.5. (Luo *et al.*, 2010).

According to the study Pyrolysis method is used for pretreatment for the feedstock. Temperatures were given more than 300°C and cellulose were quickly decomposed and create gaseous products. By using this method 80-85% bioconversion of cellulose was resulted. Catalyst zinc chloride or sodium carbonate was used but at lower temperature. Other than this steam explosion an auto hydrolysis method is used with initiated temperature 160-120 °C for few minutes and leads to degradation of hemicellulose. 90% enzymatic hydrolysis was attained by steam explosion process. Recent researches evident that rate of lower temperature and longer flat time are additional favorable. One of the major advantages of steam explosion includes consumption of lower rate of energy along with no economic and environmental cost. Ammonia Fiber explosion is other method in which at high temperature and pressure lignocellulosic material are exposed to liquid ammonia helps in improvement in rate of saccharification. Acid hydrolysis, alkaline hydrolysis, oxidative delignification and enzymatic hydrolysis both types of microorganism can be used aerobic as well as anaerobic. For better saccharification mixture of β- glucosidase and *Trichoderma reesei* is used. In process of SSF mostly used microorganism are fungus *Trichoderma reesei* and *yeast saccharomyces cerevisiae* and favorable temperature is 38°C (Sun *et al.*, 2010).

Main hindrance for the bioethanol production is radical of lignocellulosic biomass, impotence of microorganism for fermentation of all presented sugars. Mixed consortium of *Thermomyces lanuginosus* SS-8 (*Humicola lanuginose*) imperfect fungus and *Pichia stipites* known as

xylose fermenting yeast belongs to genus *Schefferomyces* having clear colony with diameter 3 to 5 µm. Simultaneous saccharification and fermentation *Thermomyces lanuginosus* was obtained at 50°C on yeast extract soluble starchy medium. Mixed culture was stored at 4°C and after 15 days both were fortify. Hydrolysis of sample was carried with *Thermomyces lanuginosus* and substrate monosaccharide sugar was treated with HCL. Incubator Shaker at 50°C for 72 h for saccharification and also estimation of hydrolysis. Fermentation by *Pichia stipites* produces pentose sugar which was inoculated with Yeast extract soluble starch and incubated at 28 °C and production rate analyzed after incubation of 72 h. (Shrivastava *et al.*,2014).

Hammer mill method of pretreatment for obtaining less than 0.12 mm particles of wheat straw and used for further processes. Hexose yeast is used for fermenting glucose in the bioethanol production also the inoculation in YM broth at pH 6.0 consisting glucose (10 g/1), peptone (5 g/1), Yeast extract (3 g/1), malt extract (3 g/1) and distilled water and incubated at 30 °C for 48h. Wheat straw is pretreated with per chloric acid with ratio of 1:4 for 20 min at 100 °C and after cooling is filtered and used for hydrolysis. The residual sample is neutralized with KOH. Hydrolysis done with $HClO_4$ for 40 min at 100 °C. HPLC used for measuring by products and sugars formed by hydrolysis. *Saccharomyces cerevisiae* used for the fermentation. Calculation of fermentation yield is done by formula:

$$\text{Fermentation yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100$$

Where, practical yield refers to that produce rate of ethanol and theoretical yield is amount of sugar is consumed. (Ali *et al.,* 2012).

Triticum durum grinded and pretreatment method wheat straw is treated with alkaline peroxide (H_2O_2, pH 11.5 and temperature 121 °C for 6 h). *Fusarium oxysporum* fungi is used for the fermentation Suspension culture with inoculum added to fermenter initially at pH 4.5 and maintained by using H_2SO_4 (0.1) 0r $CaCO_3$ 1 M slurry. 20 rpm is initial stirring rate. For estimation gas chromatography is used (Hossain *et al.,* 2011).

Wheat straw is biodegradable and mostly founded agriculture waste. *Zymomonas mobilis* and

Candida tropicalis mixed culture is used for the fermentation. Acid hydrolysis carried out with help of H_2SO_4 (4%v/v) at temperature 55 °C for 3 hour and after heating is done for 10 min at 150°C. NaOH is used for the alkaline hydrolysis with various concentrations with equal ratio and incubated at two different temperature (35 °C and 55 °C) and result of hydrolysis measured by method dinitrosalicylic acid method. Different enzyme's combination is used for enzymatic hydrolysis as Xylanase, Pectinase and cellulose with standard concentration, incubation, pH, temperature. At 30 °C the inoculation of mixed culture (10% v/v) and samples recorded daily (Patle and Lal. 2007).

Pretreatment is carried out by dilute acid method wheat straw is milled and then treated with H_2SO_4 in autoclave for 1 hour at 121 °C and maintained pH 5.0 with optimum concentration of NAOH the pretreatment is followed twice. Carboxymethyl cellulose and Xylanase activities both enzymes were assayed with their equal amount with acetate buffer at pH 5.0 and with proper solutions of diluted enzyme. After incubation for 30 min at 50 °C, the released sugars estimation is done by dinitrosalicylic acid. Enzymatic saccharification of pretreated wheat straw was done by shaking with slow speed at 45 °C with pH 5.0 with NaOH and additional dose of enzymes. Strain of recombinant E.coli used for the saccharification the production rate is high at the starting stage of fermentation (Saha *et al.*,2005).

In order to increase the enzymatic digestibility pretreatment of wheat straw is performed. Thermal pretreatment of wheat straw is confined in two different streams one is solid fraction having cellulose (Hexose and glucose) and other is liquid mainly hemicellulose (pentose and xylose). By *Saccharomyces cerevisiae hexose* can be easily and effectively can be converted to bioethanol. In the hydrothermal pretreatment wheat straw three serial reactors are used primarly wheat straw sample is soaked at temperature 80 °C for 6 min.in second step that sample of wheat straw is heated for 15 min at 180 °C and final stage again heated for 3 min at 190 °C that resulted in liquid fraction (cellulose and hemicellulose) known as hydrolysate. Saccharification and fermentation is done by S*accharomyces cerevisiae.* HPLC is used for measuring the ethanol production and glucose yield is calculated by:

$$\text{Glucose yield fraction (\%)} = \frac{\text{Free glucose after enzymatic}}{\text{Total glucose fraction}} \times 100$$

By the formulae fraction of glucose yield can be calculated. (Kaparaju *et al.*, 2009)

Alkaline pretreatment is used for the removal of lignin so it become available for the enzymes than they will easily and efficiently allow yeast for glucose to ethanol conversion. Weight loss is evaluated with delignification. Hydrogen peroxide is used at various pHs and after short duration. *Aspergillus Niger* can also use for fermentation. Pre-treatment process effectively removes lignin. High performance liquid chromatography used for monitoring the ethanol production. The results indicate that ethanol can be made from the fruit biomass peel residue. Fermentation process should be optimized if required for the scale up of the bioethanol production (Lalitha *et al.*,2011).

Immobilized and suspended formal *Schefferomyces stipitis* and *Saccharomyces cerevisiae* were castoff to translate pentose and hexose sugars for the production of ethanol. System like batch and continuous, *Schefferomyces stipites* and *S. cerevisiae* co-culture performance was better than *S. cerevisiae*. Continuous production of bioethanol was performed in packed bed type immobilized cell reactor (ICR). In immobilized cell recator, *Schefferomyces stipitis* cells were create to be more subtle to oxygen application and other probable mass transfer confines as compared to *S. cerevisiae*. Usage of co-immobilized *Schefferomyces stipitis* and *S. cerevisiae* caused in concentrated xylose depletion (73.92%) and 41.68 g/L day ethanol was produced at hydraulic retention time of 6 h with wheat straw hydrolysate. At hydraulic retention time of 0.75 h, the peak amount of bioethanol with the values of 356.21 and 235.43 g/L day was formed when artificial medium and wheat straw hydrolysate were used as feeding medium in immobilized cell reactor one-to-one.(Karagoz *et al.*,2014)

CHAPTER-4

RATIONALE AND

SCOPE OF THE STUDY

4. Rationale and scope of study:

Wheat straw was used for the production of the bioethanol. The vital point of using wheat straw for production is that is waste or by product left after the processing of wheat for the different purpose use. Wheat straw is freely available and be used for the production of the bioethanol. In recent era petroleum prices are hiked day by day so it the best alternative that can be used as biofuel. The use of this biofuel is in the chemical industries as solvent, disinfectant and also in lotions.

.

CHAPTER -5

OBJECTIVE OF STUDY

5. Objective of study:

- Pretreatment of wheat straw using different techniques such as acidic and alkaline method.
- DNS test for the analysis of pretreatment of wheat straw.
- Enzymatic hydrolysis and fermentation of wheat straw by use of *Trichoderma reesei* and *Saccharomyces cerevisiae* respectively.
- Use of free and immobilized yeast cell for fermentation of ethanol.
- Distillation of the solution of the sample after fermentation.
- Analysis of bioethanol using FTIR and by Gas chromatography
- To study the effect of immobilized yeast cells i.e. is *Saccharomyces cerevisiae* on the rate of ethanol fermentation as compare to the free yeast cells.

CHAPTER 6

RESEARCH METHODOLOGY

6. Research Methodology

There are main three processes are to be carried out for the production of the bioethanol those are pretreatment, hydrolysis and fermentation.

Wheat straw was collected from nearby plantations of the region.

6.1 Pretreatment: The main objective is to remove lignin of wheat straw.

6.1.1 Mechanical method: Used for the size reduction of the sample through the grinding. The biomass composition was analyzed before pretreatment.

Figure 3 After applying physical method on wheat straw.

6.1.2 Physical (heat) method: 5g dry wheat straw and 95ml distilled water was mixed in 250 ml flask. Physical method of pretreatment performed in autoclave at temperature121 °C and 15 psi for 30 minutes. Wheat straw was filtered and afterwards solid residue of sample was air dried and stored for further proceedings.

6.1.3 Acidic pretreatment (H_2SO_4): As different concentration of H_2SO_4 1 %(v/v), 1.5 %(v/v) and 2 %(v/v) was used for acidic pretreatment of wheat straw. For all the concentrations 5% solution of wheat straw was prepared and afterward all three solutions were autoclaved at 121 °C and 15 psi for 30 minutes. Then solid residues was separated from liquid and washed until pH of 7 was obtained. Pretreated sample was dried and stored for further use.

6.1.4 Alkaline Pretreatment (NaOH): 1 %(w/v), 1.5 %(w/v), 2 %(w/v) concentrations of NaOH were used for alkaline pretreatment of wheat husk. 5% solution was prepared using wheat straw for all concentration, then both the solutions was autoclaved at 121 °C and 15 psi for 30 minutes. Then solid residues was separated from liquid and washed until pH of 7 was obtained. Pretreated sample was dried and stored for further use.

6.2 Preparation of growth Medium:

Trichoderma reesei growth broth that is Peptone yeast dextrose broth was prepared and pH of the broth was set at 4 and for *Saccharomyces cerevisiae growth* broth set at pH 5.5 then autoclave at 121°C and 15 psi for 30 minutes.

Sr. No.	Chemical	Amount
1	Peptone	2gm
2	Yeast extract	1gm
3	Dextrose	1gm
4	Distilled water	100ml

Table 1. Composition of Peptone yeast dextrose broth.

6.3 Preparation of Inoculum and Fermentation Procedure.

Cotton plugged conical flask (250ml) was taken with 100ml of broth so to inoculate *Trichoderma reesei*. The ph. of this media is 4. Now the autoclaved media was inoculated with the strain of Trichoderma reesei. These flasks were kept on incubation at 25-26°C for 3-7 days, can be kept in either rotary shaker or in incubator without shaking.

Trichoderma reesei was inoculated in Peptone Yeast Dextrose broth, incubation was given for 24 hours at 28°C. After both the yeast and fungus were found well grown, then enzymatic hydrolysis along with fermentation was setup in co-culture manner.

6.4 Enzymatic Hydrolysis and fermentation: Defined Mandel's medium was prepared for the hydrolysis 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)

For enzymatic hydrolysis, the pH of this media is adjusted 5.5 to 6.0 before autoclaving and then 5gm of chemically treated straw was added to 100 ml of fermentation media. After sterilization of media it was inoculated with the fungus culture with these flasks. Incubation of 3-6 days was given at 28°C temperature. After 6 days the culture media was allowed to undergo the fermentation by inoculating it with the grown yeast at temperature of 28°C. Again the fermentation was performed for next 6 days in between the concentration of glucose was determined by the DNA test.

Figure 4. Fermentation media added to the pretreated wheat straw with NaOH and inoculated with enzyme.

Figure 5. Fermentation media added to the pretreated wheat straw with H_2SO_4 and inoculated with enzyme.

6.5 Preparation of wheat straw sample for treatment with immobilized yeast cells.

For SSF through immobilized *Saccharomyces cervisae* all the pretreatment methods such as mechanical method, physical method, acidic method, and alkaline pretreatment were performed. Then, *Trichoderma reesei* was inoculated with sample in fermentation media. The samples were kept at 28°C for 6 days after this fermentation immobilized yeast cells were inoculated.

6.5.1 Preparation of immobilized yeast cells.

1. 2% calcium chloride ($CaCl_2$) solution prepared and kept at 4°C for chilling.

2. 2gm sodium alginate was dissolved in hot water with constant stirring on magnetic stirrer.

3. 2gm free yeast cells were added to sodium alginate slurry with stirring for uniform dispersion.

4. Drop wise added the slurry solution with yeast biomass into 2% chilled calcium chloride solution and stored at 4 °C.

Figure 6: Immobilization of *Saccharomyces cerevisiae* for inoculation.

6.5.2 Calculate the diameter of bead

200 beads were taken in a measuring cylinder, drop wise added distilled water with pipette till the beads upper surface get merged into water. The volume of the water noted at the same time called 'void volume'.

Then the total volume was noted (i.e. volume of bead + distilled water). Then the volume of water was minsused from the total volume so we can get the total volume of the 200 beads. Then divided the total volume of the bead by 200 to get volume of an individual bead. With the help of formula for volume of a sphere, the radius of the bead was calculated and then

diameter was obtained by multiplying the radius by 2 because d=2r.

$$V=4/3\times\pi\times R^3$$

Where,

V=volume of bead, R=Radius of bead

6.5.3 Inoculation of immobilized Yeast cell

After preparation of immobilized yeast cell (*Saccharomyces cervisae).* 30 beads *were* inoculated in the fermentation sample of wheat straw that was inoculated with *Trichoderma reesei* after 6 days. The sample was kept in the incubated at 28°C for 6 days. After the duration the distillations of the samples were performed.

Figure 7. Inoculation of immobilized *Saccharomyces cerevisiae* after 6 Days.

6.6 Analysis method:

6.6.1 Dinitrosalicylic acid test: Estimation of reducing sugar content miller method was performed known as DNS test. Fresh DNS reagent was prepared in brown bottle because of its sensitivity to light. Dilutions for different concentration for standard graph were made using stock solution of glucose having pH 4. Dilutions were made each of 10ml with 0.2, 0.4, 0.6 0.8 and 1ml then volume was made up to 2ml with distilled water. In each tube 1ml of DNS reagent was added and then kept in water bath at 90 °C for 5 minutes then after cooled at room temperature and 9 ml of distilled water added for 10ml. for making standard curve OD of all the dilutions was observed at 540nm in the spectrophotometer. For the sample 3ml of DNS

reagent was added to 3ml of sample. The test tubes were covered and kept in the water bath at 90 °C for 5 minutes then red brown color appears. After that 1 ml of 40% potassium sodium tartrate (Rochelle salt) was added to stabilize the color. Then the test tube was allowed to cool down at room temperature then OD was taken 540 nm in the spectrophotometer.

6.6.2 FTIR Analysis: To analysis the amount of glucose or cellulose present in the pretreated sample FTIR spectroscopy was done. By the results in the graph it was confirmed the destruction of lignin and cellulose presence.

6.6.3 Gas chromatography: According to the graphs of the FTIR the sample was sent to the analysis for the ethanol content at Herbal health research consortium (HHRC), Amritsar.

CHAPTER-7

RESULT AND DISCUSSION

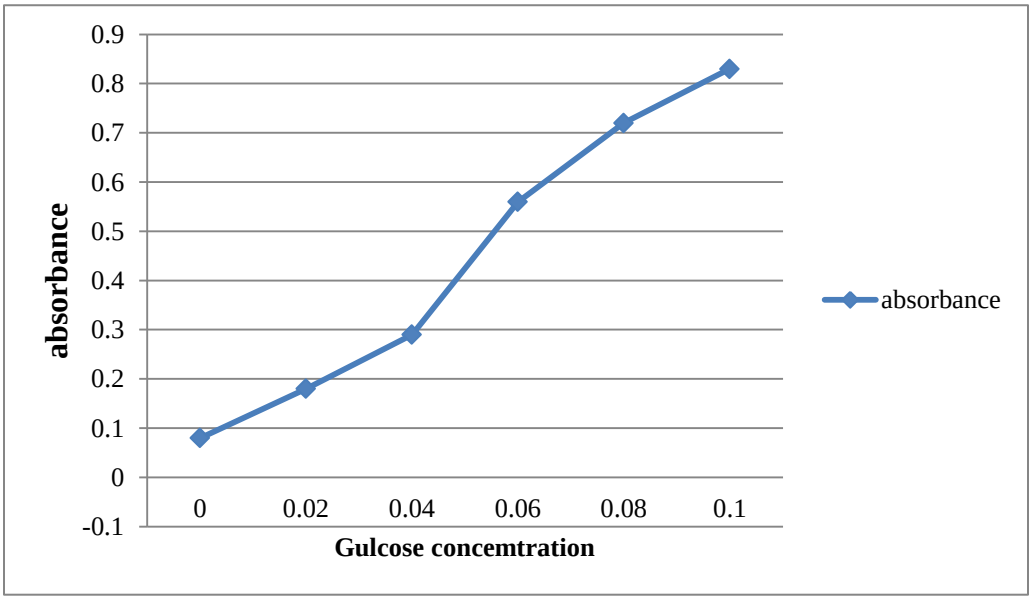
7. Result and Discussion

7.1 Preparation of substrate: Wheat husk was collected from the local milling center. For the further process the size reduction of wheat straw is done by grinding.

7.2 Pretreatment:

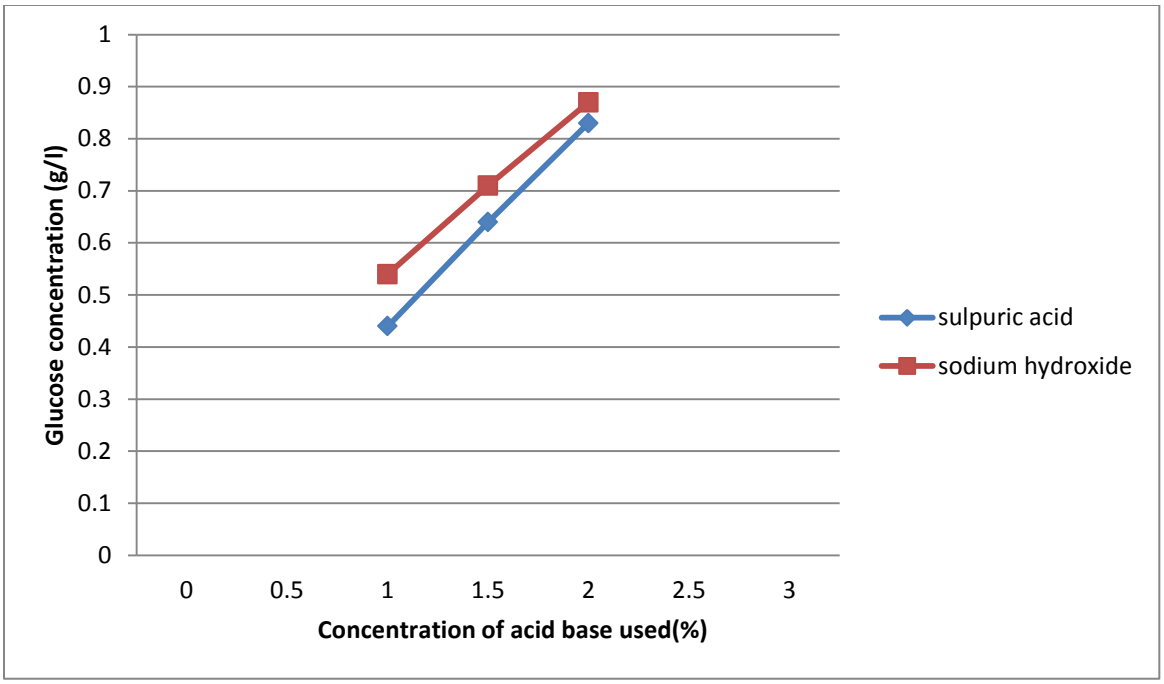
7.2.1 Physical pretreatment: After grinding, heat pretreatment was given to the sample. Autoclaving was done at 121 °C, 15 psi for 30 min.

7.2.2 Standard curve of DNS test:

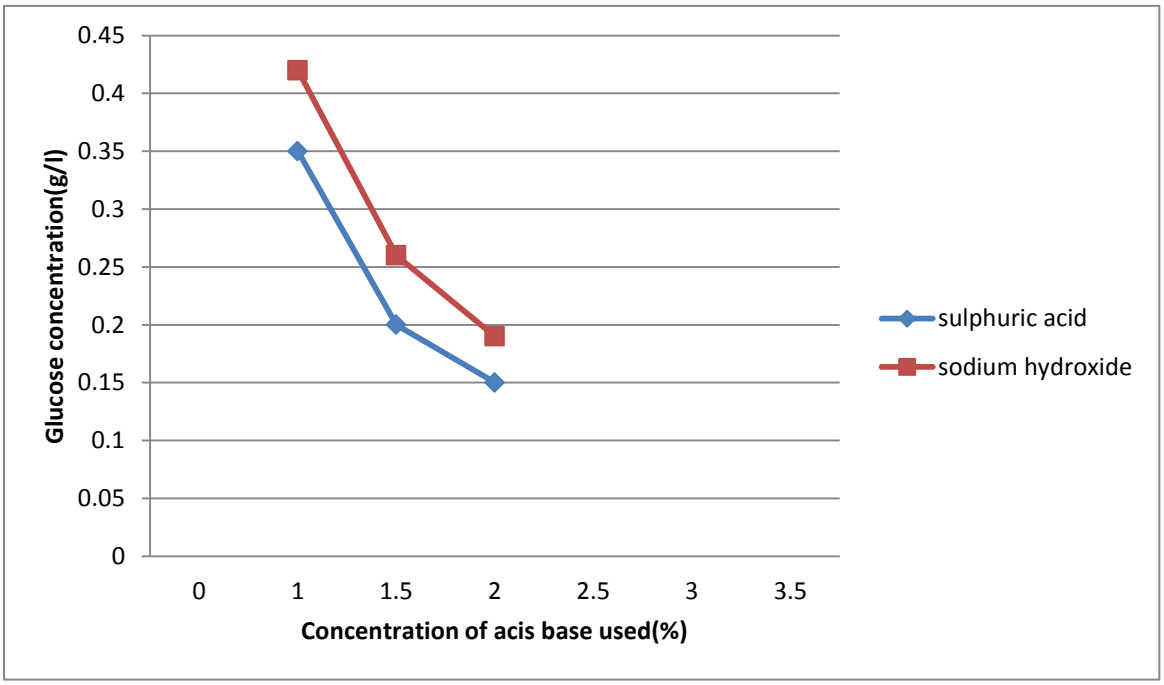


Graph 1: Glucose standard curve.

Figure 8 DNS reagent with sample for estimation of glucose.



Graph 2: Comparison of glucose concentration between acidic and alkaline pretreatment.



Graph 3: Comparison of glucose concentration obtained after fermentation.

7.2.3 Dilute H_2SO_4 solution pretreatment for free yeast cell:

S.NO	Concentration(g/l)	Concentration of Glucose (g/l)
1	1%	0.44
2	1.5%	0.64
3	2%	0.83

Table 3: Glucose concentration obtained for Acidic pretreatment (H_2SO_4) at different concentration.

7.2.4 Dilute NaOH Solution pretreatment for free yeast cell :

S.no	Concentration (g/l)	Concentration of glucose (g/l)
1	1%	0.54
2	1.5%	0.71
3	2%	0.87

Table 4: Glucose concentration obtained for Alkaline pretreatment (NaOH) at different concentration.

7.2.5 Dilute H_2SO_4 solution pretreatment after fermentation with free yeast cell.

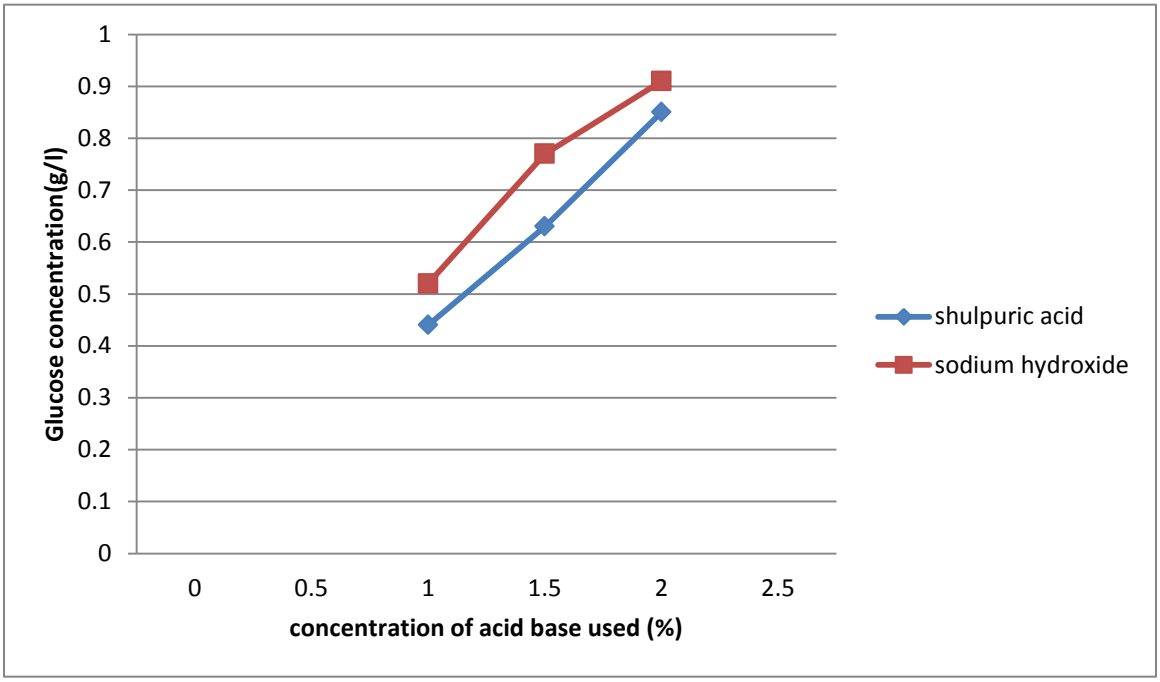
S.no	Concentration (g/l)	Concentration of glucose (g/l)
1	1%	0.35
2	1.5%	0.2
3	2%	0.15

Table 5: Glucose concentration obtained for Acidic pretreatment (H_2SO_4) at different concentration after fermentation.

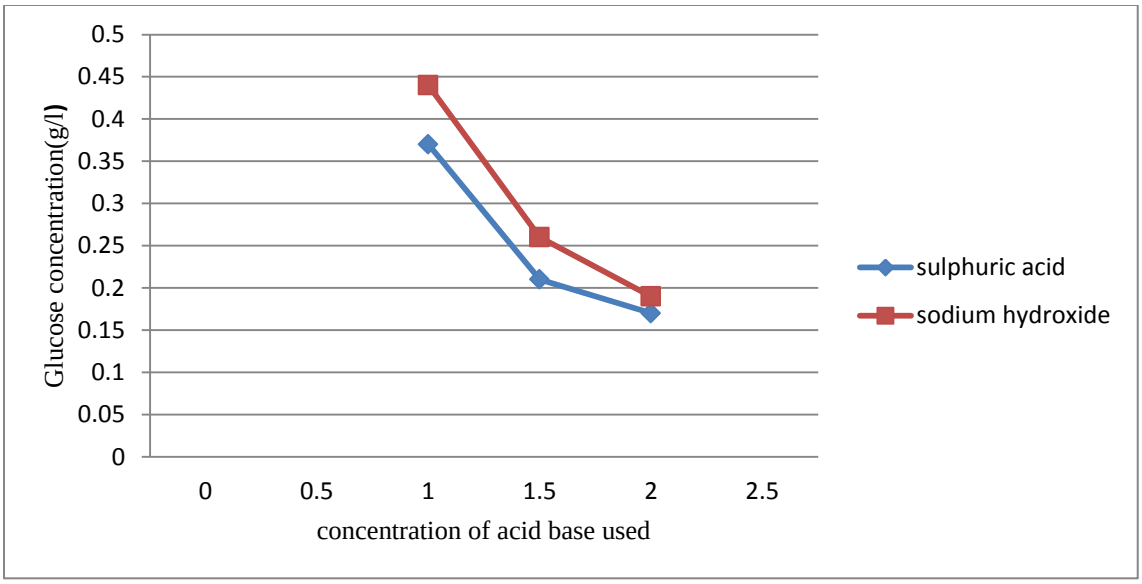
7.2.6 Dilute NaOH solution pretreatment after fermentation with free yeast cell.

S.no	Concentration (g/l)	Concentration of glucose (g/l)
1	1%	0.42
2	1.5%	0.26
	2%	0.19

Table 6: Glucose concentration obtained for alkaline pretreatment (NaOH)) at different concentration after fermentation.



Graph 4. Comparison of glucose concentration between acidic and alkaline pretreatment for immobilized cell.



Graph 5: Comparison of glucose concentration after fermentation with immobilized cell.

7.2.7 Dilute H_2SO_4 solution pretreatment of immobilized yeast cell:

S.NO	Concentration(g/l)	Concentration of Glucose (g/l)
1	1%	0.43
2	1.5%	0.63
3	2%	0.91

Table 7: Glucose concentration obtained for Acidic pretreatment (H_2SO_4) at different concentration of immobilized yeast cell.

7.2.8 Dilute NaOH Solution pretreatment immobilized yeast cell :

S.no	Concentration (g/l)	Concentration of glucose (g/l)
1	1%	0.52
2	1.5%	0.77
3	2%	0.82

Table 8: Glucose concentration obtained for Alkaline pretreatment (NaOH) at different concentration of immobilized yeast cell.

7.2.9 Dilute H_2SO_4 solution pretreatment after fermentation of immobilized yeast cell.

S.no	Concentration (g/l)	Concentration of glucose (g/l)
1	1%	0.37
2	1.5%	0.21
3	2%	0.17

Table 9: Glucose concentration obtained for Acidic pretreatment (H_2SO_4) at different concentration after fermentation of immobilized yeast cell.

7.2.10 Dilute NaOH solution pretreatment after fermentation of immobilized yeast cell.

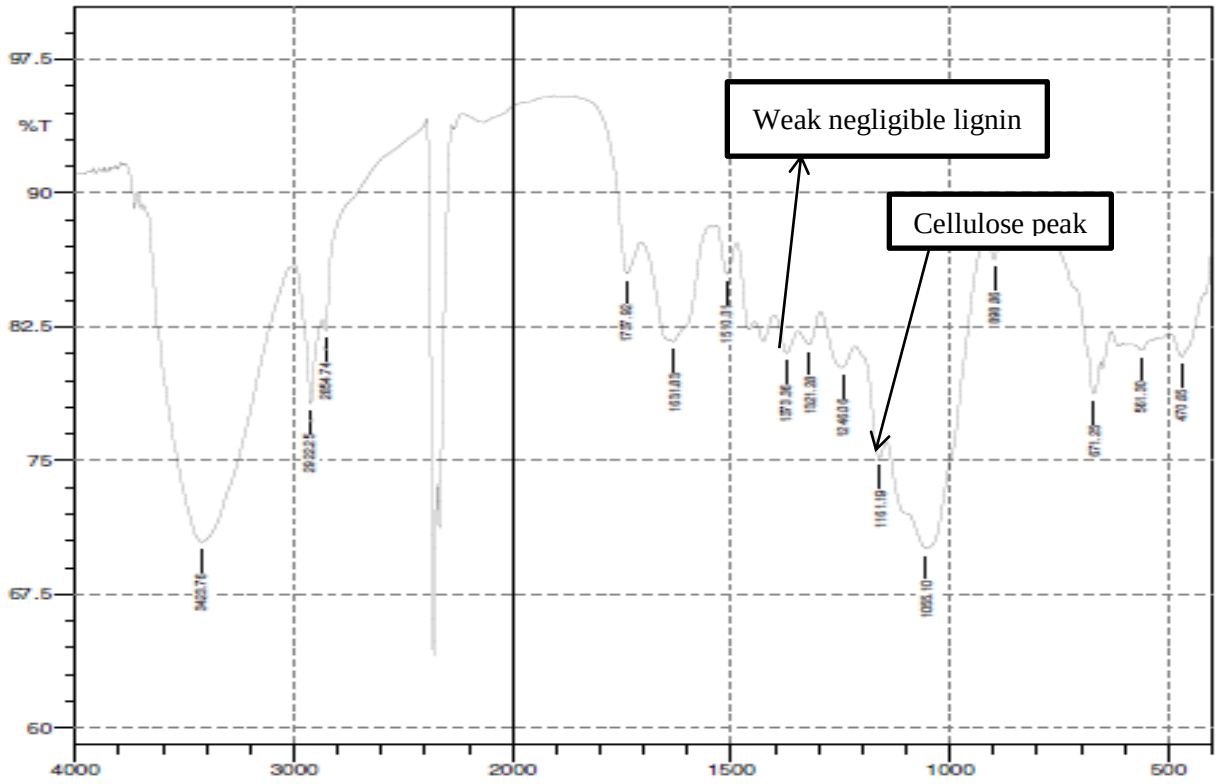
S.no	Concentration (g/l)	Concentration of glucose (g/l)
1	1%	0.44
2	1.5%	0.26
3	2%	0.19

Table 10: Glucose concentration obtained for alkaline pretreatment (NaOH)) at different concentration after fermentation of immobilized yeast cell.

7.3 FTIR analysis:

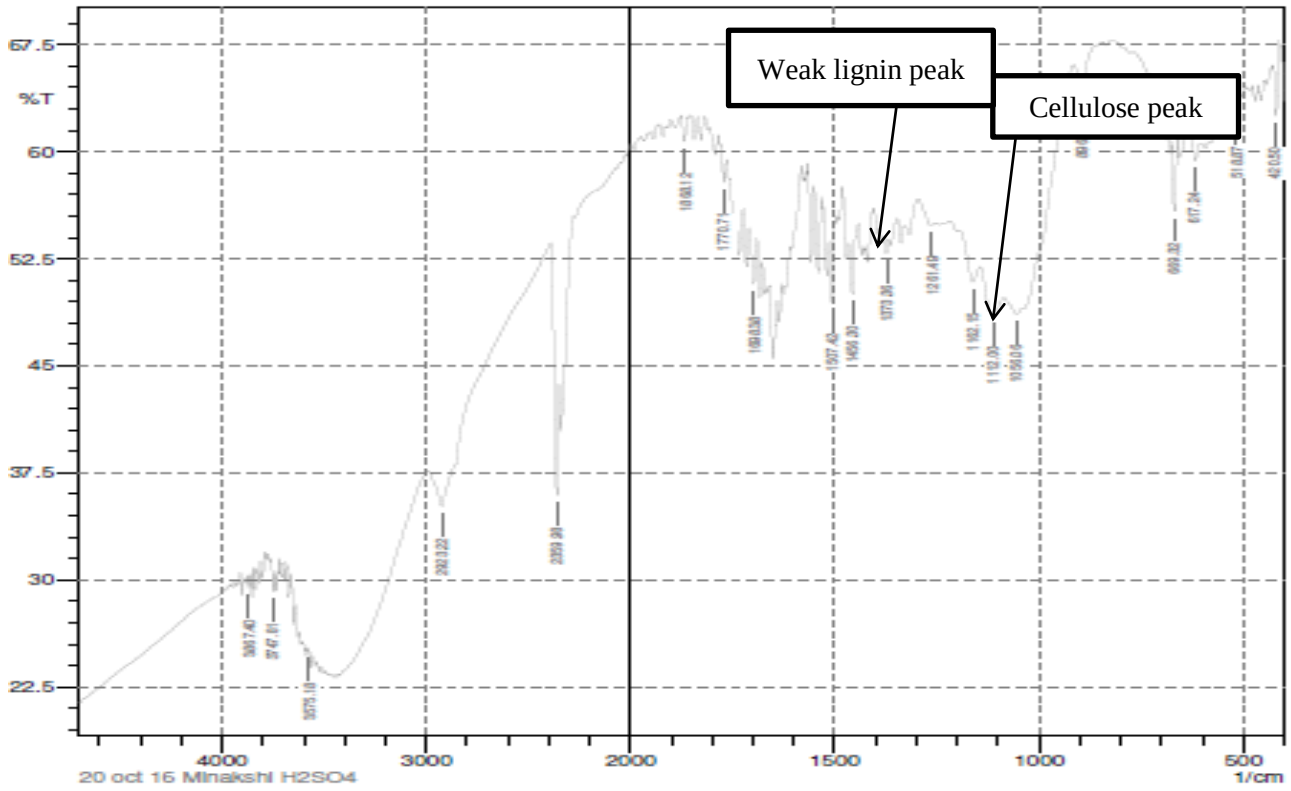
For checking of presence of cellulose or glucose content the FTIR spectroscopy was performed for the pretreated sample. The result shows the cellulosic content and removal of the lignin.

7.3.1 FTIR results:



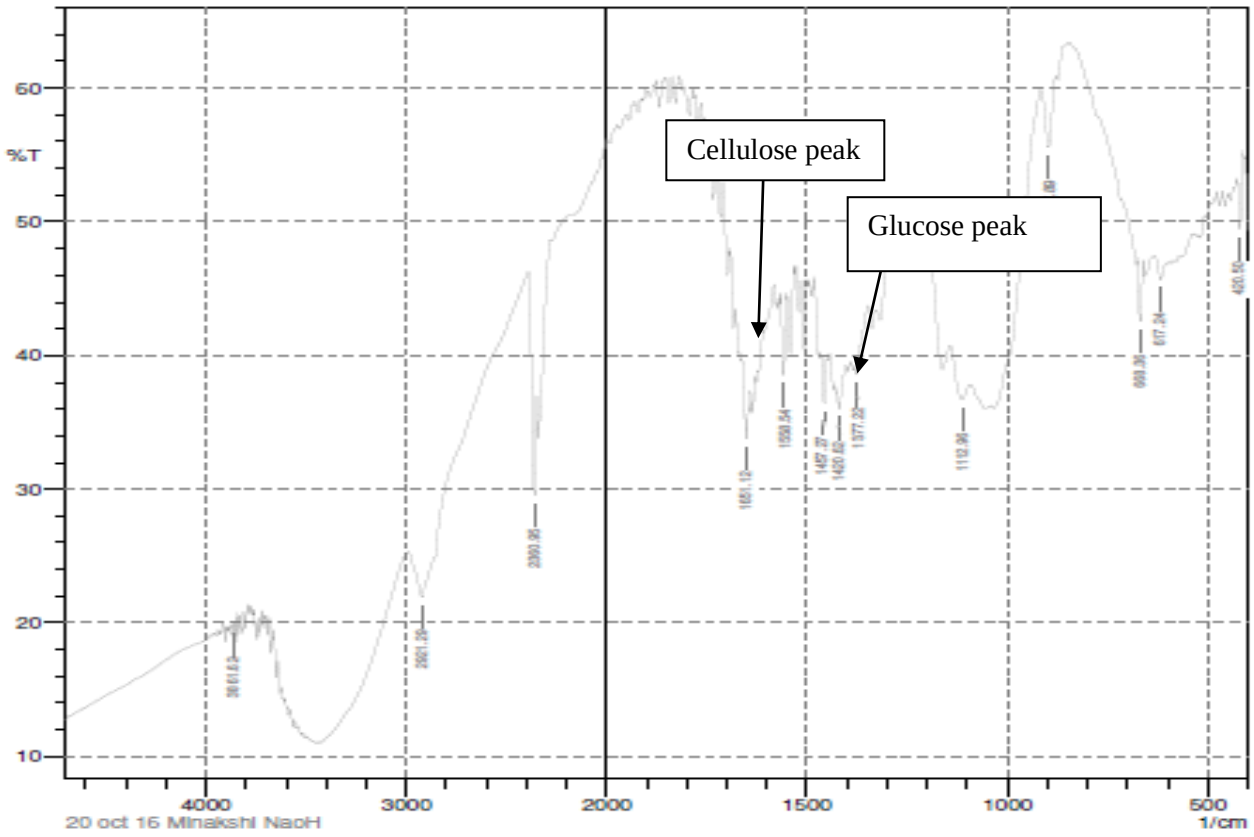
Graph 6: FTIR results for the untreated wheat straw sample.

According to Salmen et al., (2012) 1506 cm^{-1} is the IR range for the aromatic ring which is the characteristic of the lignin. Untreated wheat straw range was founded at 1510.31 cm^{-1} for lignin and for cellulose the range fall around 1161.19 $cm^{-1.}$



Graph 7: FTIR result after pretreatment of wheat straw with H_2SO_4.

In case of pretreated sample with the H_2SO_4 wheat husk has the weak or negligible peak for lignin was observed whereas peaks of IR range for cellulose were seen in all three different concentration of sulphuric acid used for the pretreatment of wheat straw. In case of 2% H_2SO_4 peaks were obtained at 1162.15 for the cellulose as similar to paper Bodirlau *et al.*,2007.



Graph 8. FTIR result after pretreatment of wheat straw with NaOH.

Analysis was done for the sample treated with NaOH wheat straw sample to obtain cellulosic and glucose peak where no lignin was observed. Range for the cellulose peak falls in 1457.27 cm^{-1}.glucose peak range is 1112.98 cm^{-1}.the findings match with the Bodirlau *et al.*, 2007.

7.4 Filtration and distillation

Fermentation was done by filtration and distillation was done at 78.37 degrees, this is the boiling temperature for the ethanol, so at this temperature ethanol gets separated out from the solution.

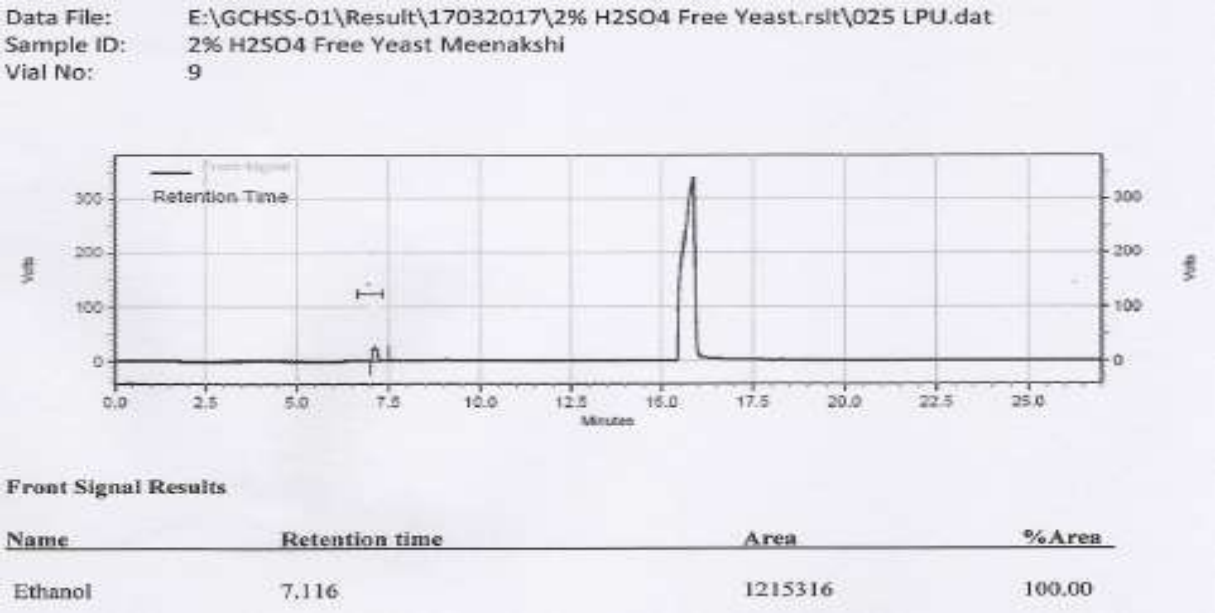
Figure 9 Distillation apparatus.

7.5 Gas chromatography result:

For the ethanol analysis gas chromatography was performed. The sample was sent to the HHRC lab Amritsar for the analysis. (Hossain *et al.*, 2011).

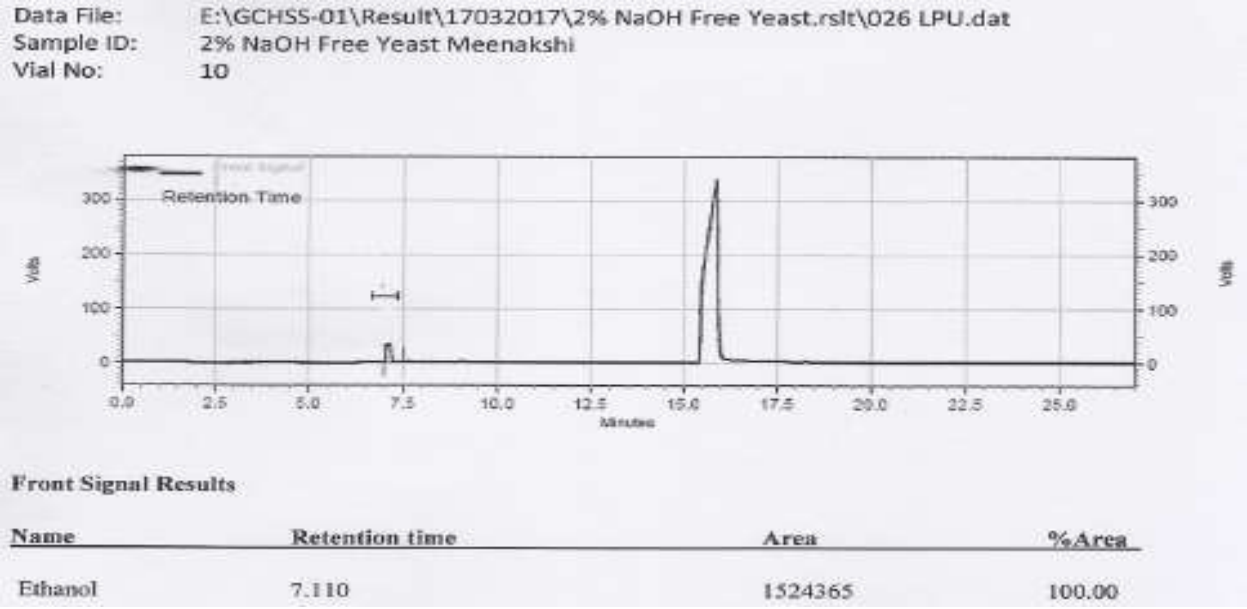
7.5.1 Gas chromatography result of sample treated with free Yeast cell.

GC results sample treated with 2% $H_2SO_4$and inoculated with free yeast cell.



Graph9. Gas chromatography graph showing the peak for ethanol, in the sample treated with 2% H_2SO_4 sample free yeast cell.

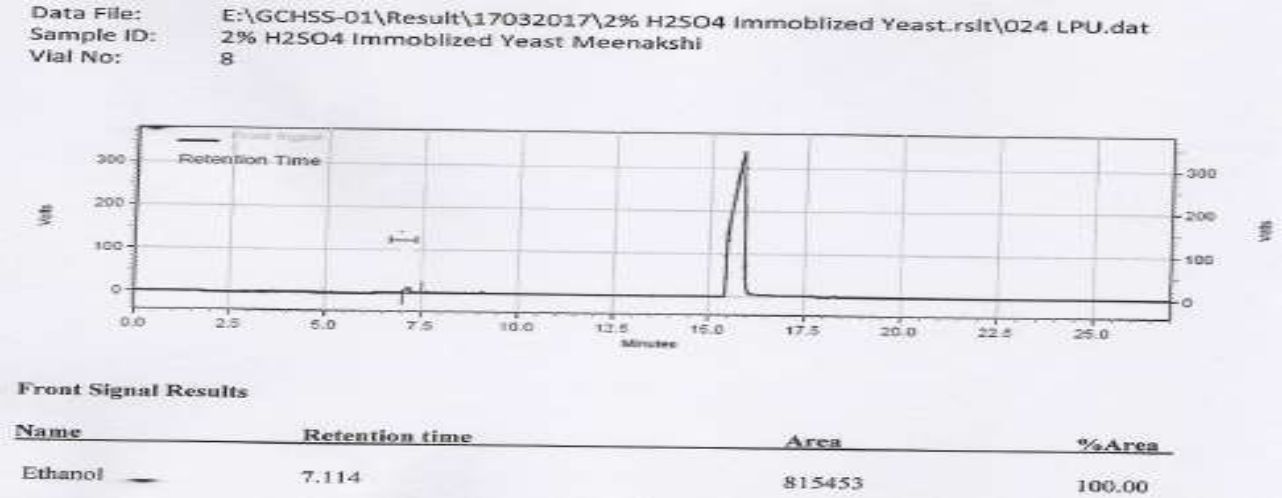
GC result for sample treated with 2% NaOH with free yeast cell.



Graph10. Gas chromatography graph showing the peak for ethanol, in the sample treated with 2% NaOH sample free yeast cell.

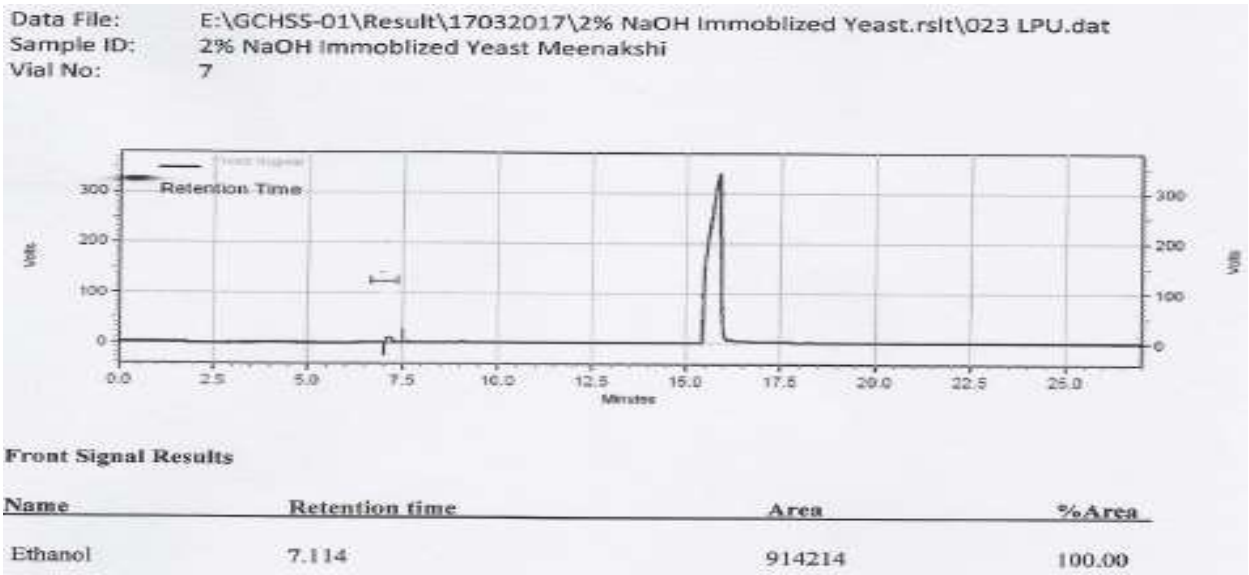
7.5.2 Gas chromatography result of sample treated with immobilized Yeast cell.

GC results sample treated with 2% H_2SO_4 and inoculated with immobilized yeast cell.



Graph11. Gas chromatography graph showing the peak for ethanol, in the sample treated with 2% H_2SO_4 sample immobilized yeast cell.

GC result for sample treated with 2% NaOH with immobilized yeast cell.



Graph12. Gas chromatography graph showing the peak for ethanol, in the sample treated with 2% NaOH sample immobilized yeast cell.

7.6 Ethanol concentration

Sample	Area of test	Vol of sample	%Ethanol(v/v)	Purity of standard
2%NaOH Immobilized yeast cell	914214	100	0.167716654	99.5
2%h2so4 Immobilized yeast cell	815453	100	0.149598506	99.5
2%h2so4 free yeast cell	1215316	100	0.222955165	99.5
2%NaOH free yeast cell	1524365	100	0.279651589	99.5

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

According to the results obtained after Gas chromatography, it was found that highest amount of ethanol was produced in the wheat straw sample which was treated with 2% NaOH tested with free yeast cell, which was found as 0.27965(v/v). For other sample like 2% h2so4 free yeast cell found was 0.222955165(v/v). Among both sample treated with the immobilized yeast cell 2% NaOH Immobilized yeast cell was found 0.167716654(v/v).

CHAPTER 8

CONCULSION AND FUTURE SCOPE

Biofuel substitute bioethanol has achieved reputation in recent time due to number of profit such as reduces emanation of pollutants, it is economically feasible and reduces the dependency nonrenewable resources. The project aims for the successful pretreatment of wheat straw and bioethanol production using SSF through free and immobilized *saccharomyces cerevisiae.* For production of bioethanol different pre-treatment methods has been described. Wheat straw is lignocellulosic biomass consisting of lignin, cellulose and hemicellulose. During the production of bioethanol lignin contribute as the inhibitor because lignin belongs to complex organic polymer and chemically, lignin referred as polymer of phenolic so pre-treatment process is performed so that further process can be performed easily.

The process as physical, chemical, biological pre-treatment is used. Cellulose and hemicellulose were saccharified for fermentation which leads to the production of bioethanol. For pretreatment alkaline method (NaOH) was more efficient than acidic method .Co-culture technique with free yeast cell and immobilized yeast cell among both the treatment free yeast cell give highest results by gas chromatography was 2% NaOH that was treated with the free yeast cell 0.2796(v/v) .

CHAPTER-9

REFERENCES

References:

Ali Ismail, W ., Braim, R. Rasul, Ketuly, K. Aziz, Awang bujag, D. Siti Shamsiah, Arifin, Zainudin "Production of biocellulosic Ethanol from Wheat straw" *cta Polytechnic*,2012, 52,3.

Alvira, P., E. Tomas-pejo, M. Ballesteros, M.J. Negro., Pretreatment technologies for an efficient bioethanol production processbased on enzymatic hydrolysis: A review, *Bio source Technology* 2010,101,4851-4861.

Balat Mustafa, Havva Balat, ahide Öz, "Progress in bioethanol processing" *Progress in energy and Combustion Science, 2008, 32, 551-573.*

Bodirlau R, Teaca CA " FTIR and thermal analysis of lignocellulosic fillers treated with organic anhydrides"*Romanian journal of physics .2007 45(2),93-104.*

Carroll., Andrew and Chris Somerville (2012 "cellulosic Biofuels", *Annual Review Plant Biology 2009.60:165-82.*

Chandel., Anuj Kumar, Chan ES, Ravinder Rudravaram, M. Lakshmi Narasu, L.Venkateswar Rao and Pogaku Ravindra, "Economic and environmental impact of bioethanol production technologies: an appraisal. *Biotechnology and molecular biology Review, 2007,2(1), 014-032.*

Hossain., Masud Sk , N nantharaman, Manas Das " Bioethanol fermentation from untreated and pretreated lignocellulosic wheat straw using fungi *Fusarium oxysporum"* 2012,19,63-70.

Hendricks, A.T.W.M, G. Zeeman., Pretreatment to enhance the digestibility of lignocellulosic biomas., 2009 ,100, 10-18.

Huang., Renliang, Rongxin Su, Wei Qi, Zhimin HE, "Bioconversion of Lignocellulose into Bioethanol: Process Intensification and Mechanism Research" *Bioenerg. Res.(2011),4, 225-245*.

Irfan., Muhammad, Muhammad Nadeem, Quratualain Syed, "Ethanol production from agricultural wastes using *Saccharomyces cervisae, Brazilian Journal of Microbiology 2014,45, 457-465.*

Kaparaju., Prasad, Maria Serrano,Anne Belinda Thomen,Prawit kongjan,Irini Angelidaki "Bioethanol,bihydrogen and biogas production from wheat straw in a biorefinery concept" 2009,100, 2562-2568.

Lalitha, G., and Rajeshwari Sivaraj.,"Use of fruits biomass peel residue for ethanol production"., *International Journal of Pharma* and *BioScienc*es.,2011 Vol 2, 2.

Lee., Jeewon "Biloical conversion of lignocellulosic biomass to ethanol" 1997,56,1-24.

Luo. Peng, Zhong Liu, "Bioethanol Production Based on Simultaneous Saccharification and Fermentation of Wheat Straw" *IEEE computer society DOI 10.1109/CESCE.2010.191.*

*Martini edita,*Dian Andriani, Sriramulu GobiKrishnan, Kyeong Eop Kang , Surn-Teh Bark, Changshin Sunwoo, Bambang Prasetya , and Don-Hee Park "IMMOBILIZATION OF Saccharomyces cerevisiae IN RICE HULLS FOR ETHANOL PRODUCTION" *MAKARA, TEKNOLOGI, VOL. 14, NO. 2, NOVEMBER 2010: 61-64*

Mosier. Nathan, Charles Wyman,Bruce Dale, Richard Elander,Y.Y. Lee"Features of promising technologies for pretreatment of lignocellulosic biomass",*Bioresource technology 2005,96,673-683.*

Patle.,Sonali, Banwari lal, "Ethanol production from hydrolysed agricultural wastes using mixed culture of *Zymomonas mobilis* and *Candida tropicalis*" *2007 ,29,1839-1843.*

Saha., Badal ., Loren B. Iten, Michael . otta, Y. Victor Wu,"Dilute acid pretreatment, enzymaticsaccharificationandfermentationofwheatstrawtoethanol",*ProcessBiochemistry 2005,40,3693-3700.*

Salmen L, Olsson AM, Stevanic JS, Simonovic J and Radotic K "Structural organization of the wood polymers in the wood fibre structure " 2012",*Bioresource,71,521-532*

Shakhashrir Professor, " hemical of the week" *General chemistry Revised date: 5 Feb 2009.*

Shrivastava., Samriti, Krishna Gopal tekriwal, mit Kharkwal and jit Varma " Bioethanol production by simultaneous saccharification anfd fermentation using microbial consortium" *2014, 3, 505-511.*

Sun Ye, Jiayang heng, "Hydrolysis of lignocellulosic materials for ethanol production: a review", *Bioresource technology, 2011, 83, 1-11.*

Ruiz, Hector A., Daaniel P. Silva, Denise S. Ruzene, Luis F. Lima, Antonio A. Vicente, Jose A. Teixeira, "Bioethanol production from hydrothermal pretreatment wheat straw by a flocculating *Saccharomyces cerevisiae* strain- Effect of process conditions. *Fuel,2015,95,528-536.*

Talebina, Farid , Dimitar Karakashev, Irini ngelidaki,"Production of bioethanol from wheat straw: An overview on pretreatment hydrolysis and fermentation*",Bioresource technology 2010,101, 4744-4753.*

Taherzadeh., Mohammad J. and Keikhosro Karimi "Pretreatment of lignocellulosic Wastes to improve Ethanol and Biogas Production: Review" *International journal of Molecular Sciences*

ISSN 1422-0067

Tesfaw, Asmamaw. and Fassil ssefa, " urrent Trends in Bioethanol Production by Saccharomuces cerevisiae: Substrate, Inhibitor Reduction, Growth variables, Coculture, abd Immobilization". *Hindawi publishing corporation ,2014, Article ID532852.*

Tutt , M., T. Kikas and J. Olt., Influence of different pretreatment methods on bioethanol production from wheat straw, Agronomy Research, 2012 ,1, 269-276.

Wang, Lei., Jade Littlewood, Richard J. Murphy., Environment sustainability of bioethanol production from wheat straw in the UK., ScienceDirect,2013, 28,715-725.

Yang Bin, Ziyu Dai, Shai-you Ding and harles E Wyman, "Enymatic hydrolysis of cellulosic biomass. *Biofuels 2011 2(14), 421-450.*

CHAPTER 10

APPENDIX

1. Composition of Peptone yeast dextrose broth.

Sr. No.	Chemical	Amount
1	Peptone	2gm
2	Yeast extract	1gm
3	Dextrose	1gm
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