Sequential pretreatment of wheat straw with H_2O_2 and $NaClO_2$ for enhanced bioethanol production

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DEDICATION

I would like to dedicate this thesis to my loving parents, my inspirations in life who taught me how to fight against the struggles of life, my father who supported me through every thick and thin of life, my mother who taught me to be patient and work hard until you achieve your goals, my teachers whom guidance helped me to achieve this status in my life and my friends who were always there for me.

ABSTRACT

Wheat straw is extensively used for the bioethanol production because of its zero-value waste and clean energy source. But still, its recalcitrant structure is a major hurdle to the economical bioethanol production. By keeping in view, this study might focus on the chemical along with biological pretreatments to valorize the wheat straw. Herein, the combination of acidic sodium chlorite ($NaClO_2$) and alkaline hydrogen peroxide (H_2O_2) pretreatment was used in the presence of *bacillus* specie that resulted in the formation of less inhibitory compounds and increased degradation of complex lignin structure. For the bioethanol production, simultaneous saccharification and fermentation (SSF) method was carried out by treating biomass 10g/L at different time concentrations of 24, 48, 72, 96 and 120 hrs and results were analyzed by different characterization techniques e.g., high pressure liquid chromatography (HPLC), scanning electron microscope (SEM). This study also provides the comparative analysis of acidic sodium chlorite, alkaline hydrogen peroxide and their combination using *bacillus* specie for the bioethanol production The maximum bioethanol production rate of 44 g/L and bioethanol yield of 1.26 g/kg was achieved by combined pretreatment of acidic sodium chlorite and alkaline hydrogen peroxide in the presence of *bacillus* specie as compared with other pretreatments methods as mentioned above. The SEM images confirmed the enhanced degradation of lignocellulosic structure by different pretreatment methods resulted in the enhanced production of bioethanol. This study paved the root to further use the combined pretreatment method in different industrial applications.

Keywords: Fermentation, Bioethanol production, Wheat Straw, Sodium chlorite, Hydrogen peroxide, *Bacillus* specie

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LIST OF ABBREVIATIONS

GHG	Greenhouse Gases
SDG	Sustainable Development Goals
XRD	X-Ray Diffraction
GC-MS	Gas Chromatography- Mass Spectrometry
HPLC	High Pressure Liquid Chromatography
FTIR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscope
EDS	Energy-Dispersive X-Ray Spectrometry
$NaClO_2$	Sodium Chlorite
H_2O_2	Hydrogen Peroxide

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Chapter 1

Introduction

1.1 Background

Now-a-days, the outright reliance on non- renewable sources has led the rapid exhaustion of the resources which directly leads to environmental and e1cological concerns. It not only increases the greenhouse gases emissions (e.g., CO_2) but also results in climatic deregulation resulting in melting of ice, polluting air quality and increase in climatic temperature. depicts the energy consumption (million tons) of conventional fossil fuels (coal, oil, and natural gas), nuclear energy, hydro energy, biomass and biofuels, wind and solar energy in the year 2020 and compares it with forecasted energy consumption rate in the year 2030.

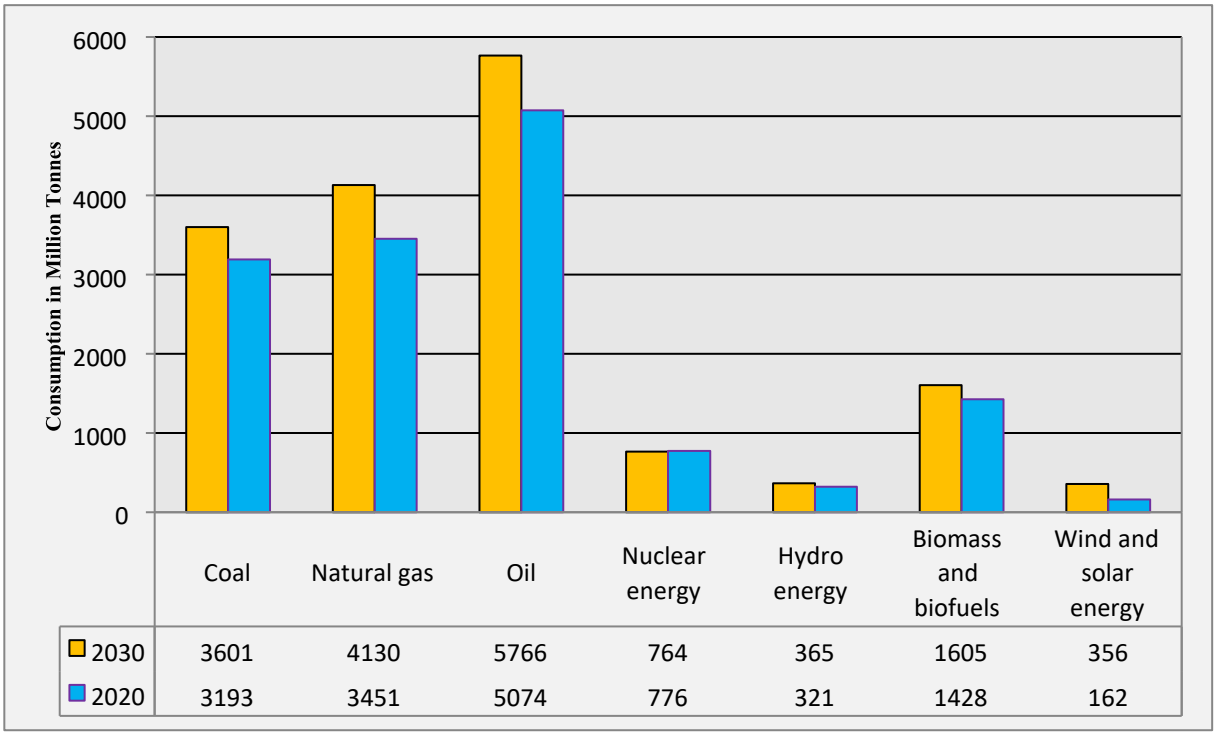

Figure 1-1 2020 and 2030 energy consumption [1]

To reckon with the intense crisis of non-renewable energy coupling with environmental concerns and sustainable development goals, the scientific community is looking for green, renewable, environmental-friendly and sustainable sources of energy. Targeting the use of renewable energy sources results in energy efficiency and conservation, energy optimization, curtailment of fuel prices, reduction of energy elasticity and replaces the import of refined fuels.

Bioethanol is considered as a viable option for clean energy production because of its abundance, low cost, waste management and low environmental impact. Historically, bioethanol gained attention after 'oil crisis in 1970s' and 'Clean Air Act 1990' due to its high cetane number, utilization as an octane booster, high heat of valorization and low environmental impact. It is regarded as clean energy source because of the carbon recyclation through the photosynthesis process during biomass growth. Therefore, 'Henry Ford' called bioethanol as 'fuel of the future'. Brazil and United States contributes to produce more than 80% of the world's bioethanol. Bioethanol production is followed by the utilization of different biomasses e.g., wheat straw, rice husk, corn straw etc.

Figure 1-2 Bioethanol [3]

Biomass is copiously utilized because of its attractive properties like abundance, low cost, and non-reliance with food reserves satisfying SDGs 1 & 2. There are different typed of biomass used for bioethanol production.

Wheat, crop in the emblem of Pakistan, copiously produced manifest the agricultural economy of Pakistan. European Union, Canada, China, India, USA are the leading wheat cultivating countries in the world. Wheat's cultivation holds 3rd position in the cultivation of cereal crops. The left-over stalk

i.e., wheat straw substantially contributes to the carbon dioxide (CO_2) emissions. According to the statistics, the wheat production in 2017 reached 1.04×10^8 t in China (NBS, 2018).

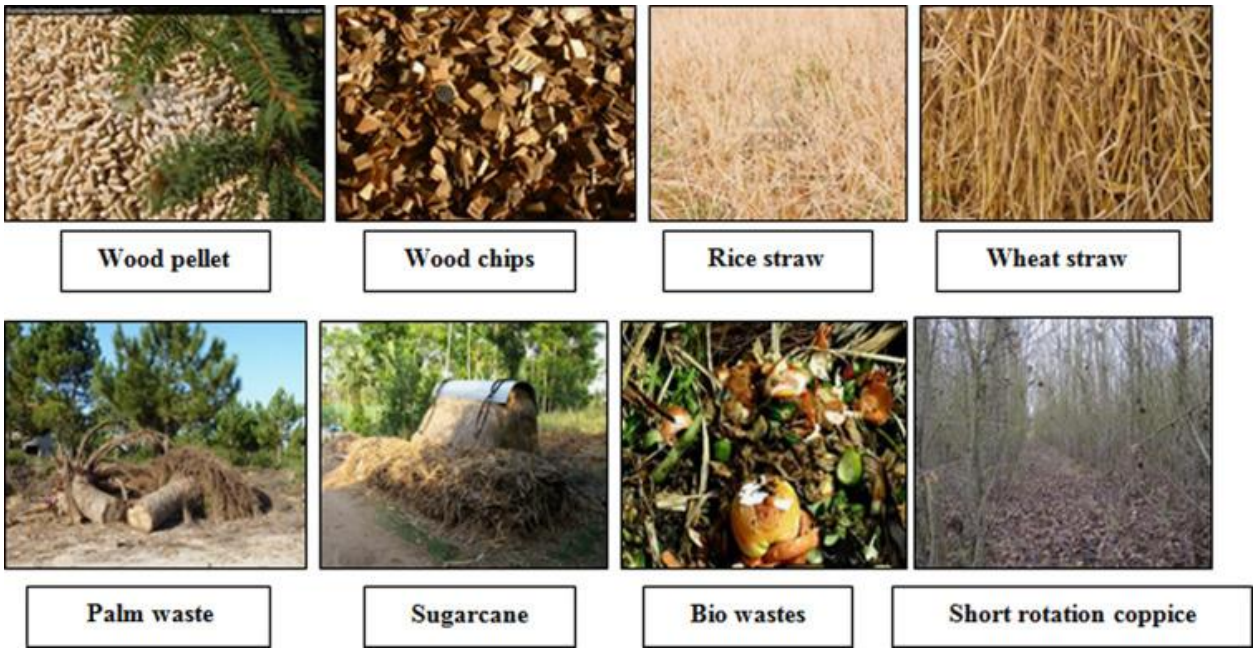

Figure 1-3 Different lignocellulosic biomasses used for bioethanol production [4]

Wheat straw, an herbaceous crop and bio-composite mainly consists of cellulose, hemicellulose and lignin which can be easily transported. WS serves as the leading lignocellulosic biomass used for the bioenergy in the 21st century. It is mainly composed of xylan chains substituted with arabinose units. Full removal of wheat residue leads to soil erosion. To overcome this, a fraction of wheat straw is left there, depending on the existing soil fertility, weather, crop rotation, slope of the land and tillage practices. The lignin component present in the cell wall makes the biodegradability of wheat straw difficult to achieve. Therefore, different pretreatment techniques can be applied to release the cellulose sugars in the presence of different microorganism i.e., yeast, bacteria, fungi.

Bioethanol production can be achieved by passing through different stages. First stage is the pretreatment of biomass. In this step, the recalcitrant structure of wheat straw is unraveled to break down the cellulose, hemicellulose, and lignin complex bond to achieve the fermentable sugars. The next step is hydrolysis and fermentation. This can be achieved by different approaches depending on the conditions and by analyzing the structure of biomass. For example, separate hydrolysis and fermentation (SHF), simultaneous hydrolysis and fermentation (SSF). Next, we have to distillate the fermentation broth made from the previous step. Distillation is done at 78.2°C i.e., the boiling point

of ethanol. Finally, the distillated product is dehydrated to remove moisture content to achieve the desired product.

Pretreatment of biomass is a crucial step in removing the recalcitrant structure of lignocellulosic biomass. In fermentation, the reducing sugars obtained from hydrolysis are utilized in fermentation in the presence of bacteria yeast or fungi for bioethanol production. Fermentation can be performed by various ways:

It is the commonly used process because of it shows flexible nature towards the selection of hydrolysis process [15]. In SHF, before introducing microorganisms for the fermentation, the hydrolysis process is first completed and the reducing sugars are recovered via filtration or centrifugation [16].

In the SSF process, an enzyme/ organism such as yeast are introduced into the reactor for the saccharification and fermentation that occur simultaneously under same operating conditions.[17]. Since both the processes occur at the same time, therefore, this process is cost effective. But the problem lies in the operating conditions that is difficult to achieve. SSF is reported to be a higher yielding process than SHF [18], [19].

Fermentation takes place in the cytoplasm with little or no oxygen present and the presence of abundant fermentable sugars. Yeast has internal glycogen reserves that it uses for reproduction. In the fermentation phase, the yeast stops the replication process and begin to convert the simple sugars into ethanol, carbon dioxide, and other waste products that give the beer specific flavors. In the beer fermentation process, the yeast found in diploid state undergoes asexual budding [20]. The glycolytic enzymes represent about 65 ~ of the total soluble protein that could favour the existence of protein-protein complexes. That's why glycolytic pathway is considered for yeast [21].

Bacillus sp. are gram-positive, rod-shaped endospore-formers and are facultative anaerobes or aerobes [22]. Genetically, *Bacillus* species are characterized by a very high diversity with a % G + C content ranging from 35% to 46%, [23]. From an industrial viewpoint, the members of genus *bacillus* are recognized as important industrial bacteria. The *bacillus* genus is used in different sectors, including food, beverage industries. Special industrial attention on this genus is due their rapid growth rates, which produce short fermentation cycles [22], [24].

1.2 Problem Statement

Bioethanol production is obtained by the utilization of cellulose sugars. This can be achieved by the fermentation of cellulose, hemicellulose and lignin component of biomass. Different studies showed the pretreatment techniques of biomass using *saccharomyces cerevisiae*. But the formation of

inhibitory compounds results in lower bioethanol production. Therefore, combined pretreatment method is used in this study using *saccharomyces cerevisiae* and *bacillus* specie. By looking into the pros and cons, we will be able to see the influence and shows the comparison between different pretreatment methods used in this study.

1.3 Research Objectives

✓ **Pretreatment**

To investigate the effect H_2O_2 and $NaClO_2$ on the production of bioethanol.

✓ **Wheat Straw**

To characterize the fermented residues of wheat straw

✓ **Bioethanol**

To study and characterize the bioethanol of pretreated wheat biomass

1.4 Thesis Framework

Chapter 1

- Provides an overview about world energy consumption, followed by the discussion on bioethanol fuel.
- Various bioethanol production methods using wheat straw as a biomass have been discussed in detail. The objectives of this research and the scope of the research was also discussed.

Chapter 2

- The comprehensive literature review was presented in this chapter.
- Different pretreatment techniques, bioethanol production using different species, bioethanol yield and fermentation process were discussed in detail.

Chapter 3

- Raw materials and brief methodology was discussed in this chapter
- Principles of different characterization techniques was also described in this chapter

Chapter 4	• This chapter covered the complete flow of research. The discussion started from the designing of the MEC, followed by its construction and configuration. • At the end of this chapter, the startup of MEC followed by analysis and calculatons was discussed.
Chapter 5	• All the results obatined using lab-sclae MEC was plotted in this chapter. • Discussion on the parameters and reasons that could be responsible for these results have been ellaborated.
Chapter 6	• This chapter concludes the complete research methodology, obatined objectives, the results obtained by this research and a thorough discussion on obtained results. • Recommendations for upcoming researchers have also suggested.

Summary

Instead of presence of extensive natural resources, Pakistan is dealing crucially energy needs. Pakistan's woes have been further inflamed by its excessive reliance power plants, using furnace oil and importing crude oil ads up to another financial burden. Keeping in view the worldwide prospective where Brazil is using 100% in cars in last 25 years, Pakistan is lagging in technological advancement unremitting options to overcome this catastrophic situation using biofuels because of favorable climatic conditions. As Pakistan is an agricultural country, wheat is produced annually resulting in the production of wheat straw. This waste of today has an ability to turn into fuel of tomorrow. Bioethanol not only reduces energy import bills but also helps in earning foreign exchange. The fermentation technology is quite mature but, in another side, it has challenges with different climate and parametric conditions. From last few years, bacteria took some attention in the fermentation technology although bacteria have not been commercially used. Bacteria have some advantages over yeast in many factors, but the screening and usage is quite challengeable due to its harsh behavior, it genetically modifies due to externalities. Nowadays, scientists are taking more attention in the bacteria to make commercially viable for fermentation process. In this study, the leading parameters have been discussed and analyzed the bioethanol production from yeast and bacteria.

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Chapter 2

Literature Review

2.1 Bioethanol production

Bioethanol is produced after passing through different stages i.e.,

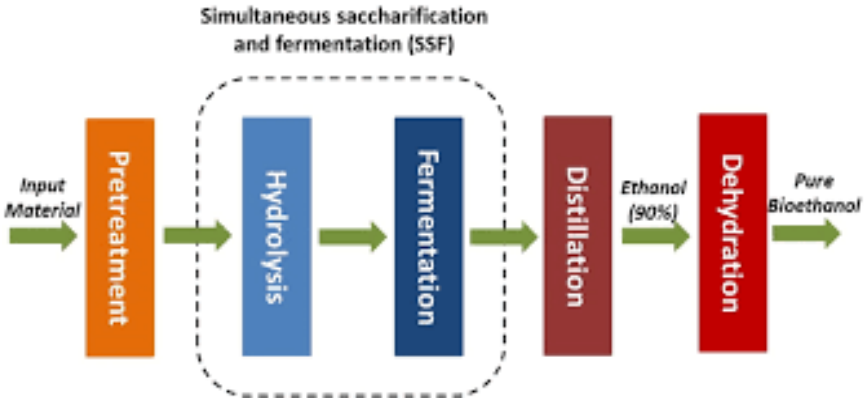

Figure 2-1 Stages of bioethanol production [1]

2.2 Pretreatment

In light of the process of ethanol production, when using lignocellulose substrate, it is essential to treat the substrate with a set of conditions that partially alter the structure of the substrate for maximum exposure of the cellulose content and reduction in lignin, which acts a barrier to the cellulose micro-fibrils [1], [2]. This has been termed as pretreatment. Various forms of pretreatment have been studied by scientists all over to decipher which conditions suit a particular substrate to achieve efficient removal of lignin from the substrate to expose cellulose.

Pretreatment as simple as steam explosion has been used and deemed sufficient in some cases, using a combination of steam and pressure to delignify the substrate [3]. Quoted the effect of treatment of rice husk under pressures generated by steam ranging 2.55 to 4.02 MPa and along with temperatures ranging from 225 to 251 °C and steaming times from 0.5 to 10 mins [4], [5]. The study revealed a pressure of 3.53 MPa for a short period of 2 mins had a profound influence on saccharification efficiency [6]. Though the amount of sugars released increased with steaming time e.g. 11 g/L at 10

mins, the saccharification efficiency declined for any steaming times above 2 mins. Microwaves have also been found to efficiently break the silica coated waxy surface and partially remove lignin and hemicellulose in herbaceous substrates [7]. According to the study, microwave intensity[4], [8], [6]. Irradiation time and substrate concentration were found significant for pretreatment, 30.3% respectively used the standard autoclave conditions of 121 °C for 15 mins for pretreatment of crushed corn stover which was then used as substrate for simultaneous saccharification and fermentation (SSF) using *A. Niger* and *S. cerevisiae*, achieving a final ethanol yield of 10.08% [9].

A simpler pretreatment of corn cobs was done by Ado et al., (2009) by refluxing crushed corn cobs with 0.2M NaOH 101br 1% and 10% of treated corn cobs resulted in 4.17% and 6.17% ethanol respectively used hot water treatment for rice straw that was further directly degraded by a modified diploid *S. cerevisiae* giving a yield of 7.5 g/L of ethanol. Employed a different approach with a two-step pretreatment involving a stage of 1.21% sulfuric acid at 142 °C for 11.6 mins from a previous study [10]. The second stage involved soaking of rice straw with a solid: liquid ratio of 1:12 in 20.63% aqueous ammonia at 42.75 °C for 72 hrs with agitation at 250 rpm, which gave about 50% delignification [11]. The resulting ethanol yield came up to 83% of the theoretical maximum. Investigated the effect of popping pretreatment on sugarcane bagasse at 220 °C and 1.96 MPa [6]. Fermentation of the released sugars from the popped substrate via saccharification produced 0.172 g/L ethanol, making up to 80.3% of the theoretical maximum [12]. A formidable change in the process was studied by for Cocksfoot grass in six experiments, three having oxygen within the pretreatment vessel at different pressures and others having 0.2% sulfuric acid [13]. All experiments were maintained at variable parameters for 15 mins [14]. It revealed that lower severity conditions i.e., 160 °C, 15 mins, 89 psi oxygen, gave the highest ethanol yield of 17.98 g/L which was 90% of theoretical maximum.

Considered olive tree pruning's for ethanol production by using 1% (w/w) of sulfuric acid at 180 °C for 10 mins. Fermentation of the treated hydrolyzate gave 0.44 g ethanol/g sugar. Investigated release of xylose with reaction times from dilute sulfuric acid of different concentrations on sugarcane bagasse at 121 °C and found optimal concentration of 19.35 g/L xylose released by 0.24 mol/L of acid in 30 mins [15]. A deviant approach was tested by comparing any inhibitory effects from use of washed and unwashed 0.5% H_2SO_4 pretreated sugarcane bagasse slurry for saccharification. Pretreated sugarcane bagasse at 170 °C was found to produce more efficient saccharification [16]. The unwashed slurry resulted in an ethanol yield of 77.3% in contrast to washed pretreated sugarcane

bagasse had a glucose recovery of 74.4%. Alkaline pretreatment also holds many promises for ethanol production. They set up factorial experiments using sugarcane bagasse treated with hydrated lime at 95 °C and NaOH at 55 °C. Their study indicated an overall increase in delignification in the range 13.1-21.7% for lime pretreatments and 8.3-23.1% for NaOH. Alkaline loading and reaction time were found significant in case of delignification, whereas alkaline loading proved important for the subsequent saccharification step. Higher temperature was also found to contribute to the removal of lignin.

Some studies point out mild treatment with H_2O_2 in comparison to NaOH may be more efficient in lignin removal compared the influence of H_2O_2 and NaOH on saccharification rates for sugarcane bagasse [3], [17]. Saccharification ratio of H_2O_2 pretreated sugarcane bagasse (82.23%) was higher compared to NaOH pretreated sugarcane bagasse (70.38%) when pretreatment was performed at 50 °C at 35% H_2O_2 and 30 °C for 2.0% NaOH for 24 hrs [18]. Further analysis revealed that selectivity for lignin removal decreased beyond 2.0% H_2O_2. Pretreatment at lower temperature was elaborated using sodium bicarbonate, acidified sodium chlorite separately and simultaneously, followed by autoclaving at 122 °C for 20 mins [19]. Using both chemicals together showed potential to remarkably reduce lignin present in rice straw (up to 80%) than when used separately [20]. This method falls short for substrates with higher lignin content, for which it has to be repeated multiple times, but fermentation of the treated substrate revealed a greater ethanol yield of 0.28 g/L implying an increase in efficiency of fermentation as compared to untreated sugarcane bagasse. Substrates with higher lignin content such as softwood chips need relatively harsh conditions than herbaceous substrates, as depicted immersing softwood chips in 2% HCl and 0.5% (v/v) $FeCl_2$ at 170 °C for 30 mins [21]. Efforts in biological pretreatment have been done, with a possibility of combining them with chemical pretreatments to overall reduce costs and need for use of excess chemical.

In this regard has done formidable work to test two cellulolytic fungi *Cerioporiopsis pannocinta* (brown rot) and *Phanerochaete chrysosporium* (white rot) for pretreatment of a high cellulose and hemicellulose energy [22]. The combined biological pretreatment of *C. pannocinta* and *P. chrysosporium* yielded maximum ethanol but still less compared to dilute acid pretreatment of 3% sulfuric acid. A combined treatment of white rot with brown rot followed by 1% acid pretreatment produced an ethanol content of 2876 mg/L which supports the amalgamation of both treatments. Another instance of using white rot fungi in pretreatment was brought about. The study revealed that *termites hirsuta* had immense potential of delignifying paddy straw with innate lignin degrading

enzymes and celluloses. Subsequent saccharification of *T.hirsuta* treated straw with accelerate 1500 gave more sugars and saccharification efficiency reached 52.69% within 72 hrs [23], [24].

Table 2-1 Comparison of different pretreatment methods

Pretreatment Methods	**Main Effects**	**Advantages**	**Disadvantages**	**References**
Ammonia Fiber Explosion (AFEX)	Helps in the removal of hemicellulose and lignin to some extent	Low concentration of inhibitor formation	Expensive due to ammonia	[2]
Ammonia recycled percolation (ARP)	Helps in the removal of lignin	Selective delignification	High energy consumption	[3]
Alkali	Increase in surface area	High digestibility	Long residence time	[4]
Diluted acid	Helps in hydrolyzing hemiocelluloses	Less corrosion problems	High temperature results in the formation of degraded products	[5]
Wet oxidation	Lignin removal	Low formation of inhibitors	High cost of oxygen and alkali catalyst	[6], [7]

Supercritical fluid technology	Increase in accessible surface area	No inhibitor formation	Very high pressure requirements	[8]
Steam explosion	Causes lignin transformation	Higher yield of glucose	Deficiency in disruption of the lignin-carbohydrate matrix	[9]
Ozonolysis	Lignin content reduction	Mild operational conditions	Excessive amount of ozone is required	[10], [11]
Mechanical	Crystallinity of cellulose decreases	No formation of inhibitors	Increase in energy and power consumption	[12]
Biological pre-treatment	Degrades lignin and hemicellulose	Low energy consumption	Low rate of hydrolysis	[13]
Organosolv	Hydrolyses lignin and hemicelluloses	Pure lignin recovery	Recyclation and drainage of solvents	[10]
Ionic liquids	Removes lignin	Green solvents	Not applicable for industrial applications	[14]

2.3 Saccharification

The preceding fermentation simultaneously the saccharification step. Different approaches have been studied to maximize sugar yields i.e., glucose from the pretreated substrate. Scientists have utilized

either of the following methods to achieve maximum saccharification of their substrates: Enzymatic saccharification using commercial enzymes; biological saccharification using cellulose secreting fungal or bacterial strains; SSF or SHF. SSF using a thermos-tolerant *Kluyveromyces marxianus* CECT 10875, which had the ability to survive at temperatures above 40 °C, on woody substrates (poplar and eucalyptus) and soft substrates (wheat straw, brassica craniate and sorghum, sugar bagasse) [6], [25]. The substrates were pretreated by steam explosion to elevate overall cellulose content and increase access for subsequent enzyme action.

A commercial cellulose was used at 42 °C, substrate concentration of 10% and 15 FPU of enzyme. The results revealed that glucose yields could reach 50-72% of maximum theoretical yields within 72-82 hrs and ethanol yields, depending on the substrate, ranged from 16-19 g/L using fermentation media. Recombinant *saccharomyces cerevisiae* has been used in SSF comparing digestibility of three different substrates: sugarcane bagasse pretreated cell lignin, crystalline cellulose and carboxymethyl cellulose. A commercial cellulose from *Trichoderma reesei* was used in a prehydrolysis step at 47 °C for 12 hrs at 200 rpm [26]. Then the temperature was lowered to 37°C for addition of recombinant *S. cerevisiae* harboring β-glycosidase gene from *humicola grisea* at 2 g/L concentration for 96 hrs. Out of the three substrates produced highest levels of glucose (69.79 g/L) as compared to (59.54 g/L), both also producing cellobiose. At the end of SSF, no traces of cellobiose were left, suggesting the effectiveness of β-glucosidase activity as key for promoting complete conversion of sugars to ethanol [27].

Use of commercial celluloses supplemented with other hydrolases, have been known to boost glucose recovery from lignocellulosic substrates. Employing a purified enzyme from a hyper-xylanase found an increase of up to 69.5% of sugars released from rice straw pretreated with steam after 72 hrs of saccharification. The application of xylenes with celluloses and β-glucosidase yielded a 12.4% increase in glucose as well as total sugars. A method of vacuum cycling had been developed while implementing SSF on rice straw. A thermos-tolerant strain of *Candida* acid thermophile ATCC 20381 was used along with culture filtrates of hyper cellulose producing *Tricoderma reesei* mutant D 1/6 and β-glycosidase producing *Aspergillus wentii* P 2804 culture filtrates. It was reported that within 14 hrs., 58% of cellulose content had been utilized and due to the vacuum cycling, saccharification remained unhindered, with regular feeding of citrate buffer and 1.5% NaOH pretreated rice straw between cycles. This method promoted maximum cellulose conversion to sugars.

The use of thermotolerant *C. acidothermophilum* made the SSF implantable with its ability to ferment

at reactor temperature of 40°C. Processed soybean intermediates have also been sought as a potential substrate for SSF trials used commercial enzymes i.e., Novozymes cellulose. Glucosidase and pectinase as an enzyme cocktail with soybean meal (SM), soybean hulls (SH), white flakes (WF) and ground soybean (SB) in SSF trials run at 50°C with agitation (250 rpm) and substrate loading at 10%. Maximum enzyme efficiency was recorded with soybean hulls giving higher soluble carbohydrates (58.23 g/L) in contrast to soybean meal showing higher enzymatic action releasing more soluble carbohydrates (47.24 g/L) than white flakes (37.92 g/L). Whole soybeans recorded the lowest soluble carbohydrates (14.90 g/L) due to lack of any pretreatment as compared to the processed intermediates. This study gave hints that fiber content of the substrate may have a role in efficient enzyme action [28]. Trilization of crude and partially purified enzyme extracts from fungi has also been considered as a method for saccharification have used partially purified cellulose for saccharification on H_2SO_4 pretreated rice straw. The saccharification efficiency was brought up to 41.8% with 110 mg reducing sugars/g pretreated rice straw after 72 hrs at 45 °C and agitation of 100 rpm in a water bath-shaker. Investigated the combined use of a cellulose complex cellulose' produced from recombinant *Clostridium thermocellum* S14 and Glucosidase from *Thermos anaerobacter brockii* to achieve higher saccharification yields. Formation of cellobiose units strongly affected the action cellulose, which can be eliminated by treatment with β-glucosidase.

On addition of 2 mg protein/g glycan cellulose and 10 units of β-glucosidase to ammonia pretreated rice straw, the team achieved saccharification yields of up to 91%. It was later speculated that fermentation of the scarified liquor by Saccharomyces cerevisiae had the potential to produce theoretical ethanol yields of 95%. A bold effort at direct saccharification of rice straw. They have developed and tested a solid acid catalyst for reaction parameters of time, temperature, substrate particle size and sugars and organic acids liberated. Pentose and hexose sugars, majorly xylose and glucose, were released as well as by-products such as formic, levullinic and acetic acid during saccharification. It was found that sugar and organic acid yields increased with increase in temperature from 110 to 150 °C whereas organic acids accumulated over time from 1 to 6 hrs.

Furthermore, particle size and substrate to water ratio also affected the final yield. With a 10% rice straw loading at 150 °C, a yield of 148.7 g sugar/ kg rice straw was obtained in 3 hrs. With most of the sugars coming from hemicelluloses. Effects of treatment of different substrates with cellulose secreting fungi have been studied by several scientists, including. Their study focused an evaluation of cellulolytic ability of 3 fungi: *trichoderma reesei* (MTC-4870), *Aspergillus awamori* (MTC-6652)

and *Phanerochaete chrysosporium* (MTC-787) on 3 substrates: wheat straw, rice husk and rice straw. The substrate concentration was kept at 6 g/150 mL. In comparison to individual treatments, combined treatment with all 3 rungs on rice straw gave highest values for cellulose activity and production.

Maximum ethanol yield of 9.5 g/L was produced by fermentation of rice straw in contrast to the other two substrates had done similar work regarding cellulose secreting fungi. Their study revolves around evaluation of fungal species in situ SSF on cellulosic material. Initially three major woods rot molds, namely brown rot (*Gloephyllum Traebum*), white rot (*Phanerochaete chrysosporium*) and soft rot (*Trichoderma reesei*) were grown on filter paper and then the scarified filter paper was fermented using *saccharomyces cerevisiae*. The study revealed that *T.reesei* the most efficient of the three molds, giving 5.13 g ethanol/100 g of filter paper with *P.chrysosporium* lagging behind at 1.79 g ethanol/100 g filter paper and lastly none from *G. traebum*. The study suggested the potential of using enzymatic saccharification of biomass via *T. reesei* and *P. chrysosporium* in SSF could be economical in production of bioethanol studied the effect of bacterial celluloses on alkaline pretreated substrates. Celluloses from *bacillus* specie in crude form were employed for saccharification of 2% NaOH pretreated wheat straw, bagasse. After 20 hrs. at 50 °C, saccharification rates of 33, 26 and 16.9% were obtained at 4, 6 and 10% wheat straw concentration. It was noted that substrate concentration affected hydrolysis rates, which decreased as the former was increased. This trend was also seen in case of rice straw and bagasse, as substrate concentration approached 10%.

However, this study showed enzyme concentration to have a positive effect on hydrolysis rates as increase in enzyme concentration increased percentage saccharification. Other bacterial sources have also been exploited for cellulose production for saccharification. Isolated and cultured *Lysini bacillus* sugarcane bagasse [29]. Purified enzyme from the bacterium were prepared and conditions such as what rate load, enzyme load, incubation time and tween-80 concentrations were timed which gave maximum saccharification efficiency of 69.5% at 1.84% (w/v), 57.4 hrs. and 0.76 mM respectively, suggesting use of surfactant may as well boost enzymatic action on cellulose in the substrate [30].

2.4 Fermentation

After pretreatment and saccharification have been successfully implemented, the final product yielding step is fermentation [31]. The scope has widened since the past years for choice of microbe, now utilizing bacteria, besides fungi and yeasts for fermentation. Different types of fermentation have been used for ethanol production: solid state fermentation and mostly submerged fermentation [32].

The process of fermentation has been either coupled with saccharification in SSF or performed separate in SHF. Instance of the fed-batch process in bioethanol production featured cultivation of both saccharifying and cellulolytic yeast strains thereby implementing SSF, was presented. Jiang and colleagues cultured *clostridium thermocellum* strain LQR1 with *thermoaneraebacter pseudethanolicus* strain X514 in semi continuous bioreactors and found out the effect of pH control was significant when the initial concentration of cellulose at 80 g/L resulted in an overall production of 474 mM ethanol within 72 hrs.

Of anaerobic fermentation at pH maintained around 6.5 to 6.8. The study supported the advantages cyclic fed-batch operations to for ethanol production from lignocellulose has implementation of the continuous model for fermentation of sorghum and cassava hydrolysate from scarification [33]. The study confirmed the addition of baker's yeast as inoculum at regular intervals was necessary to maintain ethanol production levels.

Another fact supported the addition of inorganic salts and peptone to the fermentation mix would sufficiently improve fermentation in sorghum hydrolysate but not for cassava at 7.32% (without salts and peptone) and 10.98% (with salts and peptone) respectively. In light of the process of ethanol production, when using lignocellulosic substrate, it is essential to treat the substrate with a set of conditions that partially alter the structure of the substrate for maximum exposure of the cellulose content and reduction in lignin which acts a barrier to the cellulose micro-fibrils. This has been termed as pretreatment various forms of pretreatment have been studied by scientists all over to decipher which conditions suit a particular substrate to achieve efficient removal of lignin from the substrate to expose cellulose.

Pretreatment as simple as steam explosion has been used and deemed sufficient in some cases, using a combination of steam and pressure to delignify the substrate Substrates with higher lignin content such as softwood chips need relatively harsh conditions than herbaceous substrates, as depicted immersing softwood chips in 2% HCl and 0.5% (v/v) $FeCl_2$ at 170 °C for 30 min. quoted the effect of treatment of sugarcane bagasse under pressures generated by steam ranging 2.55 to 4.02 MPa and along with temperatures ranging from 225 to 251 °C and steaming times from 0.5 to 10 mins [34]. The study revealed a pressure of 3.53 MPa for a short period of 2 mins had a profound influence on saccharification efficiency [33]. Though the number of sugars released increased with steaming time e.g. 11 g/L at 10 mins, the saccharification efficiency declined for any steaming times above 2 min used the standard autoclave conditions of 121 °C for 15 mins for pretreatment of crushed corn Stover

which was then used as substrate for simultaneous saccharification and fermentation achieving a final ethanol yield of 10.08%.

A simpler pretreatment of corn cobs was done by refluxing crushed corn cobs with 0.2M NaOH for 2 hrs, 1% and 10% of treated corn cobs resulted in 4.17% and 6 170% resulted in 4.17% and 6.17% ethanol respectively employed a different approach with a two-step pretreatment involving a in situ stage of 1.21% sulfuric acid at 142 °C for 11.6 mins from a previous study, which gave about 50% delignification [35]. The resulting ethanol yield came up to 83% of the theoretical maximum. Use of commercial cellulases supplemented with other hydrolases, have been known to boost glucose recovery from lignocellulosic substrates [2].

2.5 *Bacillus subtilis* experimentation conditions

Different strains of *bacillus subtilis* are streaked down in either media or semi defined media [36]. The fermentation medium was then agitated at 120 rpm. Temperature should be maintained steady at 30° C and so is pH=5 [36]. In order to maintain anaerobic conditions into fermentation broth, nitrogen gas is being purged. However, once the cell growth reached the exponential phase, the nitrogen flow was stopped and culture was kept anaerobic by the CO_2 evolved in the fermentation broth [3].

2.6 *Saccharomyces Cerevisiae* use for fermentation

Saccharomyces cerevisiae is regarded as an industrial working horse for ethanol production because of its capability to produce ethanol in high titre using hexose sugars and increased ethanol tolerance capacity [3], [27], [37]. The traits that are considered for an industrial strain for fermenting lignocellulosic hydrolysate are high ethanol production, efficient utilization of hexoses and pentoses, fast fermentation rates, sugars and fermentation inhibitors, high tolerance to ethanol, [38].

Summary

Alcoholic fermentation is the process in which one mole of sucrose is converted to give two moles of ethanol and two moles of carbon dioxide, producing two moles of ATP in the process. This process takes place in anaerobic conditions. The presence of oxygen results in the complete oxidation of pyruvate to carbon dioxide and water. This process is called cellular respiration. However, some yeasts such as the *saccharomyces cerevisiae* yeast undergoes fermentation through cellular respiration. The yeast will produce ethanol under the desired nutrition important for the respiration. Significant advances have been made towards the technology of ethanol fermentation. Compared with *saccharomyces cerevisiae*, the ethanol yield and productivity of *bacillus subtilis* are higher, because less biomass is produced, and through entner-doudoroff pathway glucose with high metabolic rate is produced.

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Chapter 3

Review on Experimentation and Characterization Methods

3.1 Preparation of culture media

Culture media is either in the solid form (agar) or liquid media (broth) that is prepared by the supplementation of nutrients required for the growth of microorganisms. It is prepared on the basis of microorganisms used [1].

3.1.1 Autoclave sterilization

For IL of liquid, autoclave sterilization is used with the operating parameters of 15 minutes at 15 pounds of pressure and 121 °C temperature. These operating conditions are called the standard autoclaving conditions. A time of 15 minutes is recommended to sterilize the tubes in which carbohydrate media is present used for fermentation studies [2]. Oversterilization or prolonged heating results in the breakdown of lactose in lactose-containing media that will change its composition. On prolonged sterilization, agar media are apt to show a precipitate [3].

3.1.2 Media pouring

After sterilization, media is poured in the petri plates in the laminar flow hood near and stay it until the media solidifies. This whole experiment is performed near bunsen burner to avoid any external contamination [4].

Figure 3-1 Media Pouring [4]

3.1.3 Streaking of microorganisms

After the incubation period, microorganisms are streaked onto the media using inoculating loop near Bunsen burner.

3.2 Characterization Techniques

3.2.1 X-Ray Diffraction (XRD)

This is one of the most important and common material characterization technique which provides information about the morphology, components, and crystallite size of the material. It uses X-ray radiations that pass through the material at an angle to the source. The diffraction angle is calculated, and the intensity is recorded. At an angle how many radiations deflect from a specific plane on the material gives information regarding its structure morphology [5].

To find evidence about the configuration of X-ray diffraction (XRD) of crystalline materials depend on the double particle/wave nature of x-rays. Identification and characterization of materials centered on their X-ray form are the major uses of the procedure [6]. When a monochromatic X-rays incident beam contacts an object material the first outcome that takes place is atoms within the target substance scatter those X-rays as shown in figure 3-1. The spread X-rays undertake destructive and constructive interference in the substances having proper structure (i.e., crystalline), which is called diffraction. The X-rays diffraction by crystals is described by Bragg's law,

$$n(\lambda) = 2d\ \mathrm{Sin}(\theta)$$

The shape and size of the materials unit cell determine directions of likely diffractions [7]. The atom's arrangement in the crystal structure affects the diffracted wave intensities. Many materials are not one crystal rather are comprised of little, small crystallites in all likely directions which are called polycrystalline powder or aggregate. When a material with casually focused crystallites is put in an X-ray, the beam will view all available interatomic planes. If the experimental angle is scientifically altered, then all the available diffraction peaks from the substance will be identified [8].

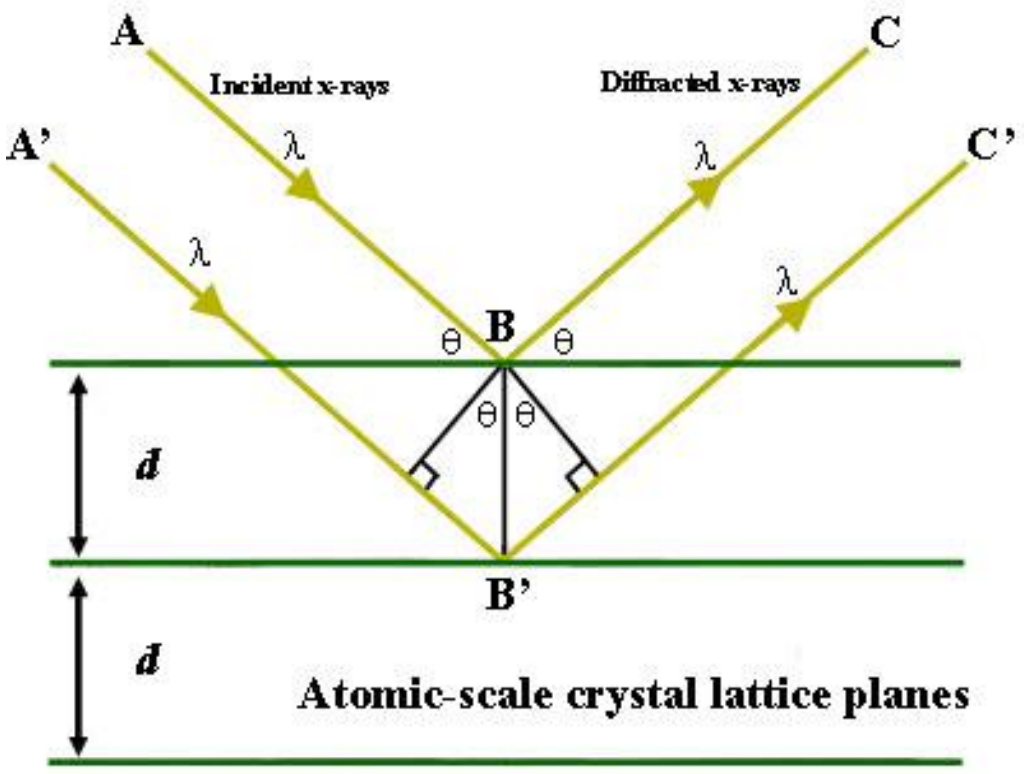

Figure 3-2 Illustration of Bragg's Law [8]

3.2.2 Scanning Electron Microscopy (SEM)

The scanning electron microscope utilizes a high-energy electrons-focused ray to produce a wide range of signals at the solid surface of the specimen. The high-energy electrons penetrate through the material and escape through the other end as shown in Figure 3-2. The information of the substance like chemical composition, crystalline structure, external morphology (texture), and materials orientation will be revealed signals of the electron beam and sample interactions. In various applications, a 2-dimensional image is created that shows spatial variations in these properties, and numbers are collected over a particular choice area of the sample surface [9]. The scanning method by simple SEM practices (magnification varying from 20X to around 30,000X, 3-D resolution of 50 to 100 nm) can be used to distinguish the areas that vary in size from about 1 cm to 5 microns in breadth. This method is exclusively valuable in semi quantitatively or qualitatively identifying chemical contents (by EDS), crystal orientations (using EBSD), and crystalline structure. The SEM is proficient in executing analyses of a specific area or point locations on the sample object. Its design and function are quite comparable to the EPMA and significant connections in abilities remain between the two devices [10].

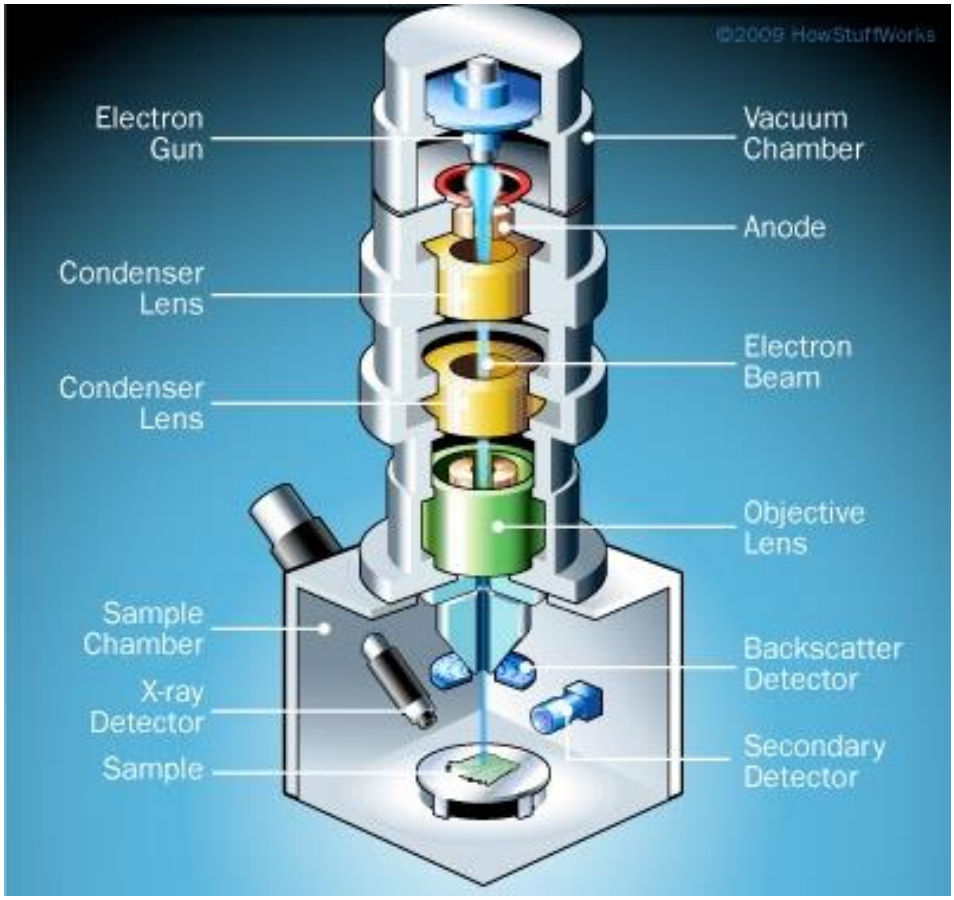

Figure 3-3 Illustration of how SEM works [10]

3.2.3 Energy Dispersive X-ray Spectroscopy (EDX)

EDS is an elemental analysis method used to quantify the number of individual elements present in a nanoparticle. This technique gives the number of substances at a particular point but does not give the overall quantity of each element. It is usually combined with SEM or TEM to get a nanoscale image of particles through them, and EDS performs the analysis of that nanostructure. In the early 1970s, EDS developed into one commercial product and rapidly crossed WDS in popularity [11]. The overall structure of the EDS is very simple because of no moving parts like the rotation detector in WDS. The sensor gathers the X-rays energies signal from all series elements in a sample at a similar time as compared to gathering signals from X-ray wavelength one by one which makes the EDS systems relatively fast as seen in Figure 3-3. The characteristic energy dispersion resolution is around 150–200 eV, which is lower than WDS resolve. The lightest component that can be identified is not C (Z=6) rather O (Z=8). But major benefits like low cost and fast analysis make these disadvantages insignificant [12].

EDS band is a graph between the power of X-rays and the corresponding energies. Both light and heavy elements can be seen in a range of spectrum from 0.1 to 10-20 keV because both M or L lines of heavy elements and K lines of light elements are evident in this array [13].

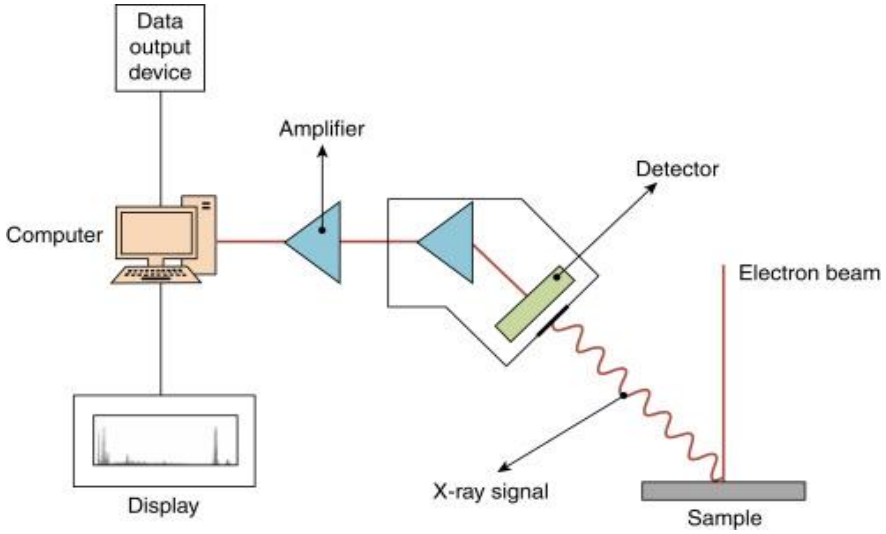

Figure 3-4 Illustration of EDX [13]

3.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy is the preferred technique for infrared spectroscopy. When IR radiation passes through a material, some of the incident radiation gets absorbed in the material while the rest is transmitted Figure 3-4. The detector at the other end detects the transmitted radiation and sets out a signal that is basically represented in the form of a spectrum and it demonstrates the molecular nature of the material [14].

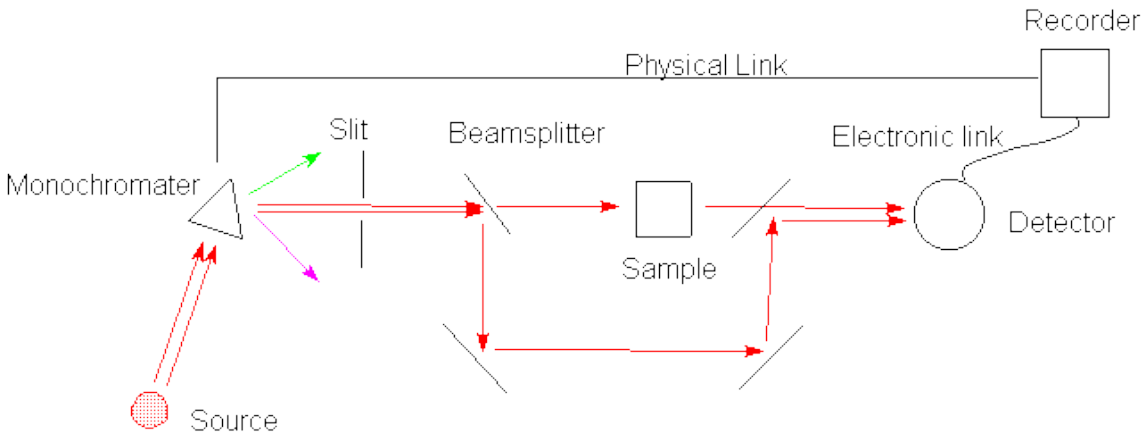

Figure 3-5 Schematic diagram of FTIR [14]

3.2.5 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is separation analytical technique that is used to separate the chemical components based on mass to charge ratio. The neutral molecules are ionized by electron ionization (EI). In EI, an electron, produced by a filament, is accelerated with 70 electron volts (eV) and knocks an electron out of the molecule to produce a molecular ion that is a radical cation. This high energy ionization can result in an unstable molecular ion and excess energy can be lost through fragmentation [15].

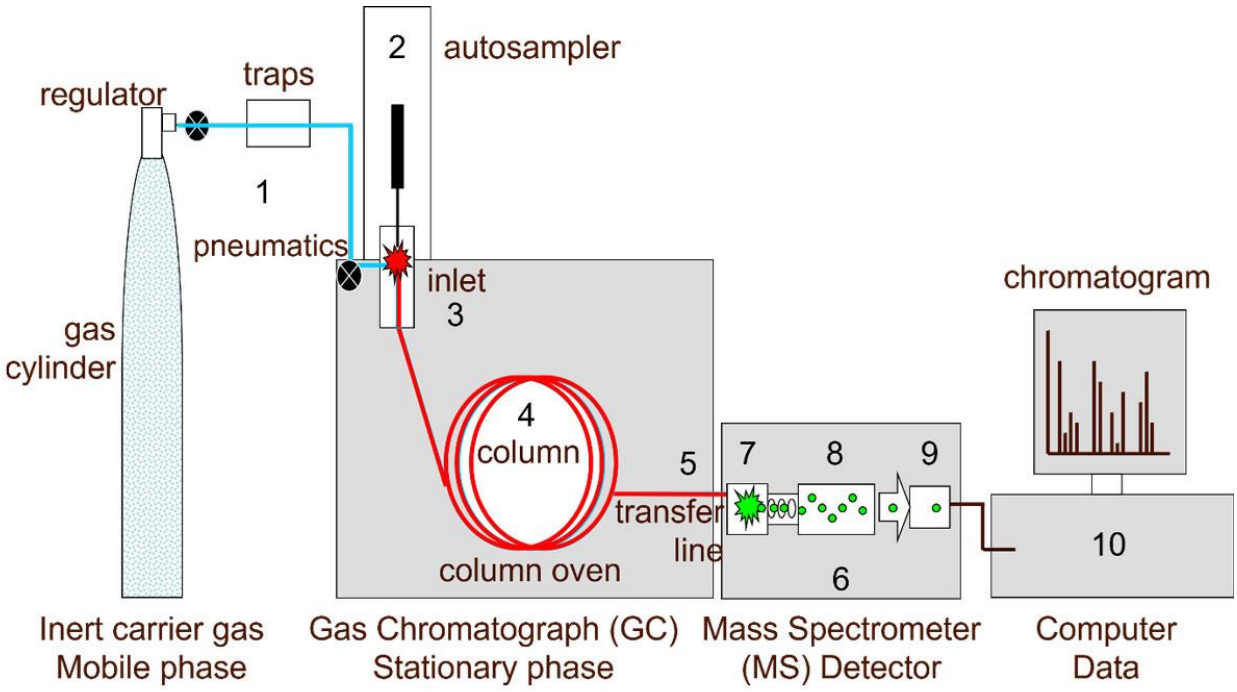

Figure 3-6 Illustration of GC-MS [15]

3.2.6 High Performance Liquid Chromatography (HPLC)

HPLC is a separation technique based on the distribution of analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). The molecules are retarded during stationary phase depending upon the chemical structure of an analyte [16]. In general, a HPLC system consists of the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data processing unit [17].

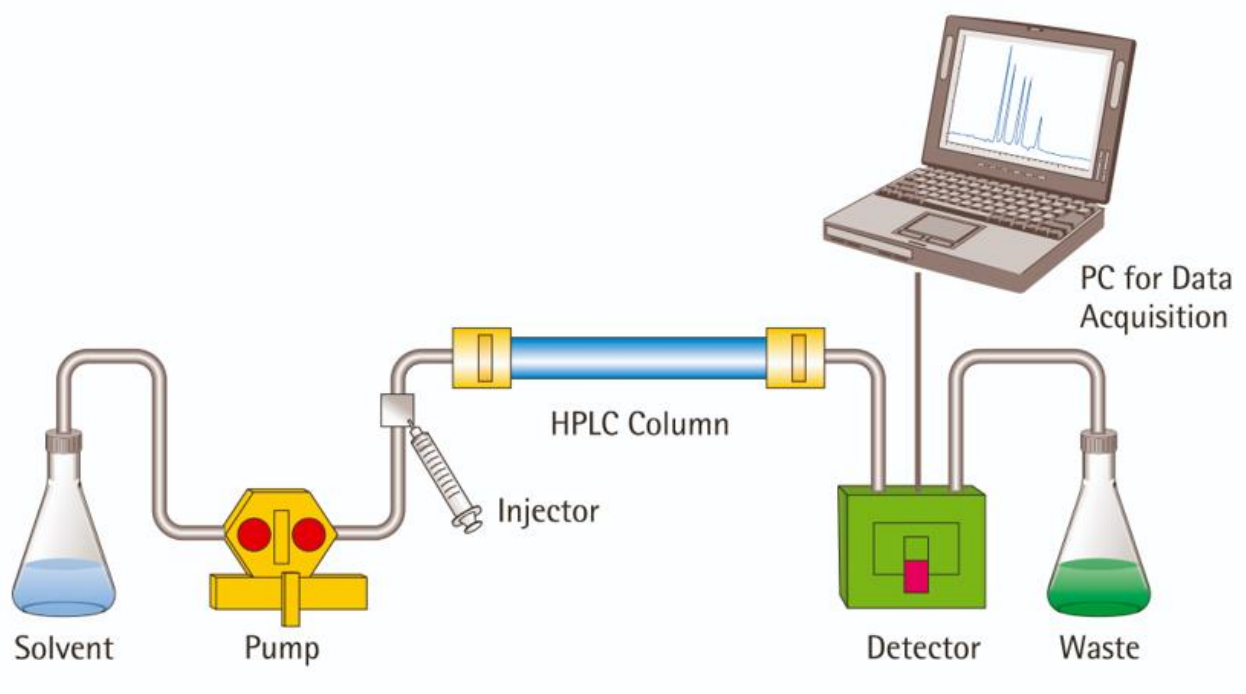

Figure 3-7 Basic principle of HPLC [17]

Summary

This chapter provides the preparation of solid culture media for microorganism growth and the principle of SEM, HPLC, GC-MS, FTIR, XRD.

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Chapter 4

Methodology and Experimentation

4.1 Raw Materials

The optimization of pretreatment of wheat straw with alkaline H_2O_2 and acidic $NaClO_2$ was performed at Biofuels Lab, USPCASE (Center for advanced studies in Energy Systems), NUST university of Pakistan. The biomass was wheat straw which was purchased from local market of Islamabad. It was then washed and dried to remove any unwanted foreign particles. The biomass was stored in polythene bag for further use [1].

4.2 Physical pretreatment of substrate

60 mesh size screen was used for sieving and the average size of the particles was between 0.297 mm. This was done because it was used to evaluate size of particles on releasing fermentable sugars. The sieved straw was screened (without grinding) via a 12-mesh screen, which produces very heterogeneous article sizes, with (85.59 ± 2.89%) percent of the bulk presenting average particle sizes bigger than 1.397 mm not being used.

4.3 Chemical Pretreatment of substrate

Pretreatment was carried by 35% of alkaline chemical H_2O_2 **(**BDH, England) and acidic $NaClO_2$ (BDZ, England) and individually as well as combined at temperature 50 °C, pH 8.6 [2]. The pretreatment setup carried in 300 mL flasks using 20g of wheat straw in each run agitated at 130, 140, 150 rpm, with time 1, 2, 3h. The total pretreatment time for each trial was 3 hrs. After completion of the pretreatment, sample flask was stored for 2h on room temperature for stabilization of reaction, filtration and washing was done for the solid fraction with normal distilled water several time for removal of the water soluble and insoluble solids and recovery of pretreated wheat straw yield [3], [4].

4.4 Yeast and Bacteria Strains

Two strains were used in the current study bacteria (*bacillus* specie) and yeast (*saccharomyces cerevisiae*). The *saccharomyces cerevisiae* and *bacillus* specie strain were collected from NUST Institute of Environmental Science and Engineering (IESE) lab, Islamabad Pakistan. The *bacillus* specie strain was activated using nutrient media advised by DSMZ Germany [5]. The *bacillus* specie

strain was activated using nutrient media advised by DSMZ Germany Composition of nutrient media i.e., yeast extract 10 g, 15 g/L agar, bacto-peptone (20 g/L). Autoclave was used for the media and its temperature was adjusted to 121 °C respectively. Bacterial cells were isolated by using standard streak plate method on petri plates. For streak plate method, temperature was 37 °C for overnight and it was sterilized and incubated [6]. The *saccharomyces cerevisiae* was grown in glucose and (YPG) yeast peptone media containing yeast extract (16.2 g/L), peptone (20 g/L), glucose (20 g/L) [1], [7].

4.5 Instruments

The instruments used during the pretreatment of biomass were,

- Spectrophotometer (UVS - 2800)
- Refractometer model: RHB 32ATC Japan.
- Electrical balance (AR3130 - USA)
- Autoclave (HV - 50 -Hirayam japan)
- Laminar Flow
- Electric oven (ANC 371-England)
- Muffle furnace (KE-671-PCSIR)
- Shaker
- HPLC-(Perken-Elemer series 200 Auto sampler)

4.6 Saccharification

Following parameters were chosen to be studied for their significance in saccharification, and were designated as the following variables [8]:

- Temperature (°C)
- Incubation Period (hours)
- Enzyme concentration (grams)
- Acid concentration (%) The phosphate buffer was used for saccharification, and pH of buffer was maintained

- The enzyme used was at 0.5 g and 1 g.

4.7 Fermentation

Pretreated wheat straw hydrolysate prepared by fed batch hydrolysis was inoculated with *saccharomyces cerevisiae* and *bacillus* specie for fermentation at 37°C temperature with variable agitation rate 130, 140, 150 rpm, pH 4.6 and working volume 300 mL for 72 hrs [6], [9], [10] The anaerobic conditions were maintained and after every 24 hrs samples were collected for bioethanol identification, total sugar concentration and pH measurement [11].

4.8 Identification of bioethanol

There are quality methods to identify the bioethanol; some of them are chemical test, which was recorded [12]. Bioethanol concentration can be detected by potassium dichromate. In this, all bioethanol is oxidized to acetic acid. Another chemical test was applied in, to identify the ethanol potassium permanganate test [13]. The positive result will make the color change of sample from purple to green. The other analytical methods use in modern era for the identification of bioethanol were, high-performance liquid chromatography (HPLC), gas chromatography (GC). HPLC and GC are working on the basic principle of boiling point difference with the reference [14]. These methods are quite accurate for the identification; the only issue for HPLC and GC is to maintain the units. FTIR is another method used for qualitative analysis of bioethanol by matching peak with standard [15].

4.9 Identification of carbohydrates -Color reactions

Benedict's qualitative reagent, birdfeed's reagents and Fehling tests are used for qualitative test of carbohydrates [16]. These are color reactions, change in color indicates presence of carbohydrates. Another simple and adoptable method during large numbers sample is calculated by drawing standard graph [17]. For determination of glucose, a method suggested which is glucose oxidase method [18]. Moreover, phenol sulphuric acid method is used for calculation of total carbohydrates, in this method, in hot acidic medium, glucose is dehydrated to HMF [19]. These forms a green colored product with phenol and had absorption maximum at 490 nm. For hand held calculation, Refractometer is used for measurement of total sugars [20].

4.10 Determination of pH and bioethanol concentration

The bioethanol concentrations were determined by ebulliometer which was approved in distilleries

by US department of measurement and distilleries associations all around the globe[21]. It has been also determined by HPLC, GC and spectrophotometry reported in many studies. The pH was determined by pH meter handy [22]. GC is user friendly and is considered as one of the best instruments used in fermentation process on the other hand HPLC lags in reliability and maintenance. HPLC and GC separation methods are also used. Whereas HPLC applies to constituents that are fluids. GC is used when the compounds are gaseous or can be vaporized during the separation process. Both fundamental principle of heavy molecules flowing slower than lighter ones [1].

4.11 Analytical Methods

4.11.1 Reducing Sugar Estimation

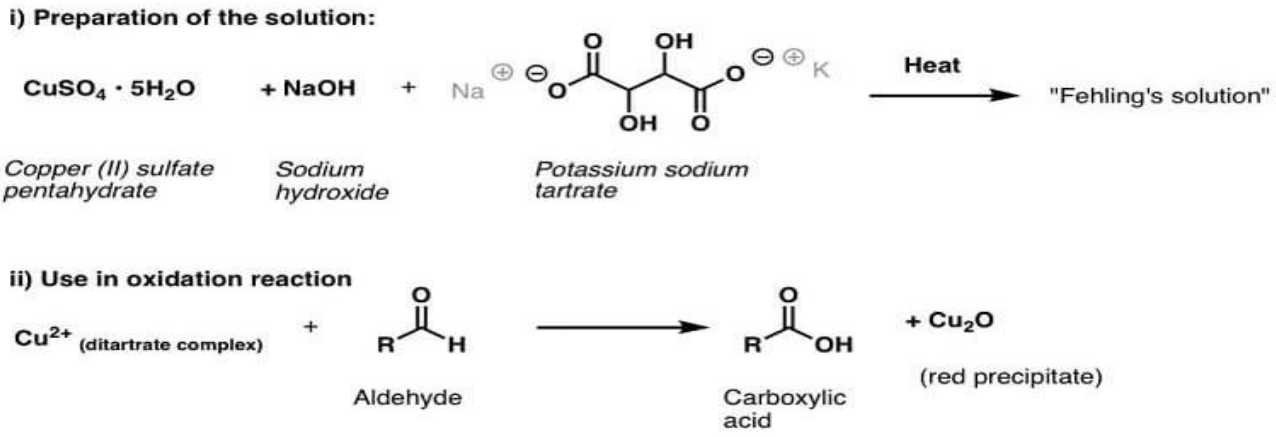
The DNS method outlined by Miller (1959) was used to determine reducing sugars in filtrate(s) composed from the washings of the substrate. I mL of filtrate was transferred to separate test tubes and 3 mL DNS was added to each sample and boiled for 10 mins in a water bath. After that, the test tubes were cooled and absorbance of sample was noted at 550 nm via spectrophotometer [23].

4.11.2 Total Sugar Estimation

The phenol-sulfuric acid method was used for measuring total sugar content of filtrate(s). 1mL of filtrate was added to separate test tubes and to that 1 mL of 5% phenol was added and mixed. Then 5 mL of pure H_2SO_4 was added and the mixture was allowed to stand at room temperature for 20 mins. Then absorbance was taken at 470 nm via spectrophotometer [24].

4.11.3 Fehling test for glucose presence test

Fehling test is performed for the detection of reducing sugars but is known to be NOT specific for aldehydes. This is done by the reduction of the deep blue solution of copper (II) to a red precipitate



of insoluble copper oxide. Fehling's "A" uses 7 g $CuSO_4.5H_20$ dissolved in distilled water containing 2 drops of dilute sulfuric acid while Fehling's "B" uses 35 g of potassium tartrate and 12 g of NaOH in 100 mL of distilled water. For the test: 15 mL of solution-"A" is mixed with 15 mL of solution-"B" and 2 mL of this tested and the tube is placed in a water-bath at 60° C. A positive test is indicated by a green suspension and a red precipitate [25]. The test is sensitive enough that even 1 mg of glucose will produce the characteristic red color of the compound [26], [27], [28]. The following reaction occurs for the fehling test [28].

4.11.4 Lignin Estimation Method

The lignin content in the samples was estimated by the method as described. In this method, l g of pretreated rice husk sample was taken in round bottom flask and 1.25% H_2SO_4 (70 mL) was added in it. This was put on reflux for 2 hrs and time is being noted after boiling. It was filtered without washing after 2 hrs. Sample was taken in a flask and 30mL of 72% of H_2SO_4 was added in it. The mixture was stirred for 20-25 mins and water was added for dilution [29]. The solution was then filtered and maximum washing was done. The washed sample was transferred to the weight crucible and dried in oven. The weight was noted after drying in oven and below calculation was performed [30].

Delignification = (control lignin - residual lignin) = control lignin) x 100

4.11.5 Determination of Cellulose

For cellulose content estimation, 1g of sample along with 30 mL of 80% acetic acid was taken in a round bottom flask. 2 mL of pure nitric acid was added in it and placed on reflux for 20 minutes. Time was noted after boiling. The sample was filtered and maximum washing of sample was done to remove acids this washed sample was then collected in the crucible and placed in oven for drying. After drying, the crucible was weighted and cheered with nitric acid and placed on flame until the smoke stopped blazing and sample turned into ash. The sample was then placed in the furnace at 540 °C for 5-6 hrs. After that the crucible was weighted and following calculation was performed [26].

4.11.6 Hemicellulose Estimation

This method was used for estimating hemicellulose content in substrate neutral detergent fiber (NDF) was obtained as follows: 0.5g of sample (W) was added to a 250 mL round bottom flask. To this 100 mL of cold reagent or chilled NDF solution, along with 0.5g sodium sulfite and 2mL of decalin were added. The flask was refluxed for 1hr at low heat to avoid foaming of mixture. After reflux, the mixture was cooled and filtered through filter paper and residues were washed with hot distilled water

many times. Residues were then washed with pure acetone 2-3 times, drained and placed in a known weight crucible (X) for drying at 105°C in hot air oven until constant weight (Y). Formula for calculating NDF and ADF are given below [13]:

NDF% = (X-Y)/W x 100

ADF% = (Y-X)/W x 100

Hemicellulose was calculated by the formula given below:

Hemicellulose% = NDF (%)-ADF (%)

Where:

Y = weight of ADF/NDF + crucible

W = weight of sample

X = weight of empty crucible

Crucibles containing ADF (Y) were placed in a 50mL beaker and covered with cooled 72% H_2SO_4. The contents of the crucible were stirred to break any lumps formed in the ADF [31]. The acid in the crucible was drained and the crucibles were filled halfway after which they were intermittently stirred for 3 hrs. After that, the crucibles were filtered under suction and contents washed with hot water to remove acid [32]. The crucibles and contents were then dried at 100°C for 8 hrs. The dry weight of the crucible with contents was noted (L). Then, the crucibles were placed in a muffle furnace for ashing at 550°C for 2hrs. After ashing, the crucible was placed in a desiccator and then the ash weight was noted [22], [33], [34], [7].

4.11.7 Acidity Test

This test method measures acidity in fermentation yield. Very dilute aqueous solutions of low molecular mass organic acids, such as acetic acid, lactic acids may be present the fermented sample. It is due to the variation in pH, the harmful microorganism like lactobacillus, which converts sugar into acids. This test makes sure the sugar was converted into bioethanol rather than acid. Titration method was adopted for the acidity test [35].

4.11.8 High Performance Liquid Chromatography (HPLC)

The fermented samples were filtered through a 0.5µm filter, adjusting pH to 6.9. Ethanol was quantified by HPLC. The mobile phase was 80% Acetonitrile and 20% water at flow rate of 0.5 mL/min, at 35^0C and detected by UV-Detector, C-18 column [36].

4.11.9 Fourier transforms Infrared spectroscopy (FTIR)

Chemical bonds in a molecule were identified with FTIR. The sample was carried out using (FTIR) with wavenumber range is from 4000-450 cm^{-1}. Similarly, the intensity measured around 4000 cm^{-1} may be relatively too high or too low, depending upon whether transmittance, absorbance, or reflectance is measured by (630 FTIR, Agilent Technologies, USA) [37].

4.11.10 Scanning Electron Microscopy (SEM)

Wheat straw structure and composition were studied before and after pretreatment. Scanning electron microscopy (SEM) was used to examine the samples after they had been dried, (VEGA 3, TESCAN Czech Republic) [16].

4.11.11 X-Ray Diffraction (XRD)

Structural information of wheat was indicated by XRD measurements using STOE Germany (θ-θ) that indicated the structural information of the wheat straw. The crystallinity affect is used to confirm the pretreatments effect on the wheat straw [38].

4.11.12 Gas Chromatography Mass Spectrometry (GC-MS)

The qualitative analysis of bioethanol in the fermentation process was performed on a Shimadzu GC-MS QP2020. Employing gas chromatography-mass spectrometry (GC-MS) with solvent methanol using Shimadzu SH-Rxi-5Sil MS column having specifications as L=30m, ID=0.25, DF=0.25). The oven temperature was first kept at 45°C for 3 minutes, then increased to 185°C at a rate of 2.5 °C per minute for 10 minutes. With a heating rate of 5 °C /min and a hold of 10 minutes, the temperature was programmed to reach the final column temperature of 290 °C, 8µL of liquid oil was put into the column. The split ratio was set at 10:1, and the carrier gas was Helium (99.999 %) with a flow rate of 0.5 mL/min and a solvent delay of 2 minutes. Temperatures of 185 °C, 290 °C, and 185 °C were set for the injection port, transfer line, and detector, respectively [39].

Summary

This chapter presents a review of historical methods that have been used by various researchers during their study of fermentation and pretreatment. The previous chapter comprehensively discusses the carbohydrates and sugars estimation methods, and individual pentose's and hexoses determination methods like glucose estimation. For handy analysis, refractometer is suggested. Cellulose estimation and hemicellulose estimation methods were also discussed, Moreover, bioethanol concentration estimation methods were also suggested. There are different analysis techniques being used worldwide for determination of ethanol, sugar's concentration. The most authenticated methods for batch fermentation, bioethanol testing methods for detection and quantification are reported acidity test for acid formation in fermentation, which is most important method to test the ethanol concentration. Other methods can be possible for the better analysis subject to the availability of equipment. In this chapter, discussion about available methods for different analysis for current study is made. Some of the most important methods have been discussed in the current chapter.

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Chapter 5

Results and Discussion

5.1 Physicochemical Characterization

The moisture content of the samples was analyzed by ASTM 2010 which is less than 10% in all the samples used for characterization [1]. Wheat straw is highly oxygenated biomass because of its complex carbohydrate structure in comparison to the conventional fossil fuels. Hydrogen and oxygen contribute to 30-40% of wheat straw. The combined pretreatment shows an increment in hydrogen composition as compared to other substrates which indicates a better-quality fuel as both are flammable and results in higher octane number. Higher octane fuels reduce the anti-knocking effect in engines. Typically, 30 to 40 wt.% of the dry matter in biomass is oxygen. Chlorine, sulfur, and nitrogen are present in minute quantity that reacts with ash in the form of silica in the presence of acid or alkali and form silicates which cause corrosion problems. Moreover, silicates also contribute in environmental pollution which is a major concern now-a-days [2]. The increased content of carbon, silicon, copper and aluminum shows an increment in the amount of cellulose which shows the maximum conversion of non-fermentable sugars for the combined pretreatment method. However, oxygen content is higher in sodium chlorite pretreatment because of the reaction of chlorine and chlorate ions [3]. Nitrogen and phosphorus are main constituents used as a production source for the growth of plants. But for the bioethanol production, the amount of nitrogen should be less than that of carbon to optimize the C/N ratio that varies from 3.5 to 35.2 g/L. Peptone addition in the fermentation broth may affect the C/N ratio. As it is evident from table 1 that carbon content is maximum and nitrogen content is negligible in combined pretreatment [4].

Table 5-1 Physico-chemical characterization of different pretreatment methods on wheat straw using *bacillus* specie

Parameters	**Untreated (%)**	**H_2O_2 (%)**	**$NaClO_2$ (%)**	**H_2O_2 + $NaClO_2$ (%)**
Carbon	43.15±13.17	37.7±11.6	47.0±11.5	49.05±11.0
Oxygen	39.15±11.8	39.45±10.9	33.6±11.9	43.73±11.2
Hydrogen	5.46 ± 0.02	5.49±0.01	5.61±0.01	5.7±0.005
Potassium	1.25±12.3	1.0±8.7	0.9±22.6	2.27±9.8
Sodium	3.1±13.5	4.6±10.6	2.33±17	------
Silicon	1.15±11.5	------	2.4±9.4	17±5.4
Nitrogen	0.44± 0.01	0.37±0.01	0.35±0.01	------
Sulphur	7.85±5.5	5.05±4.6	------	------
Chlorine	2.3±10.4	2.15±6.1	2.1±8.9	2.3±18.0
Copper	3.2±15.2	1.9±12	4.3±13.6	5.8±13.5
Aluminium	0.9±18.1	8.1±6.3	-------	8.2±6.8

5.2 Comparison between the *saccharomyces cerevisiae* and *bacillus* specie properties

Table 5-2 represents that *bacillus* specie hold remarkable properties as compared to *saccharomyces cerevisiae*. That's why, *bacillus* is considered as a viable microorganism to convert the fermentable sugars to fully utilize the wheat straw by fermenting both pentose and hexose sugars respectively [5].

Table 5-2 Comparison of different properties of *saccharomyces cerevisiae* and *bacillus* specie [5]

Properties	***Saccharomyces Cerevisiae***	***Bacillus* Specie**
Cell Type	Microscopic Eukaryote	Prokaryotic
Microorganism Type	Yeast	Bacteria
C5 Sugar Utilization	Doable	Excellent
Oligosaccharide Utilization	Bad	Excellent
Protein Secretion Capacity	Moderate	Excellent
Ease of genetic modification	Excellent	Excellent
Medium Cost Benefits	Good	Excellent
Resistant to product inhibition	Excellent	Excellent
Resistant to salt/toxic inhibitio	Moderate	Excellent
Growth Rate	Good	Excellent
Culture Temperature (^{o}C)	~ 35	30-45
Pathway	EMP	
Advantages		Releases L-arabinose & Hydrolyzing O-gycosyl compound

5.3 Scanning Electron Microscope (SEM)

SEM used to determine the morphological characteristics showed that controlled wheat straw is rough and regular in shape along with highly compressed structures [6], [7]. A morphological change is observed in the form of channeling in other pretreatment methods in accordance with the removal of lignin which acts as a glue for assembling cellulose and hemicellulose components [8]. Figure 1(a) showed the cellulose fibers bundled together to make a compact and regular structure [9]. Combined pretreatment method shows irregularity phenomena, reduced volume and increased porosity which helps in fast and increase in the efficiency of enzymatic hydrolysis which leads to increased ethanol productivity. As discussed in previous literatures, sodium chlorite pretreatment is white and more regular in shape with the connection of straight fibers in cellulose. The white color is due to the aromatic substitution in accordance with Hubble (2010) and Yue (2015) studies [10], [8]. On the other hand, hydrogen peroxide is less regular in shape as compared to other pretreatment

methods [11]. The results are in congruent with the studies carried out by alkaline hydrogen peroxide shows the removal of lignocellulosic components with the appearance of irregularity [11], [12]

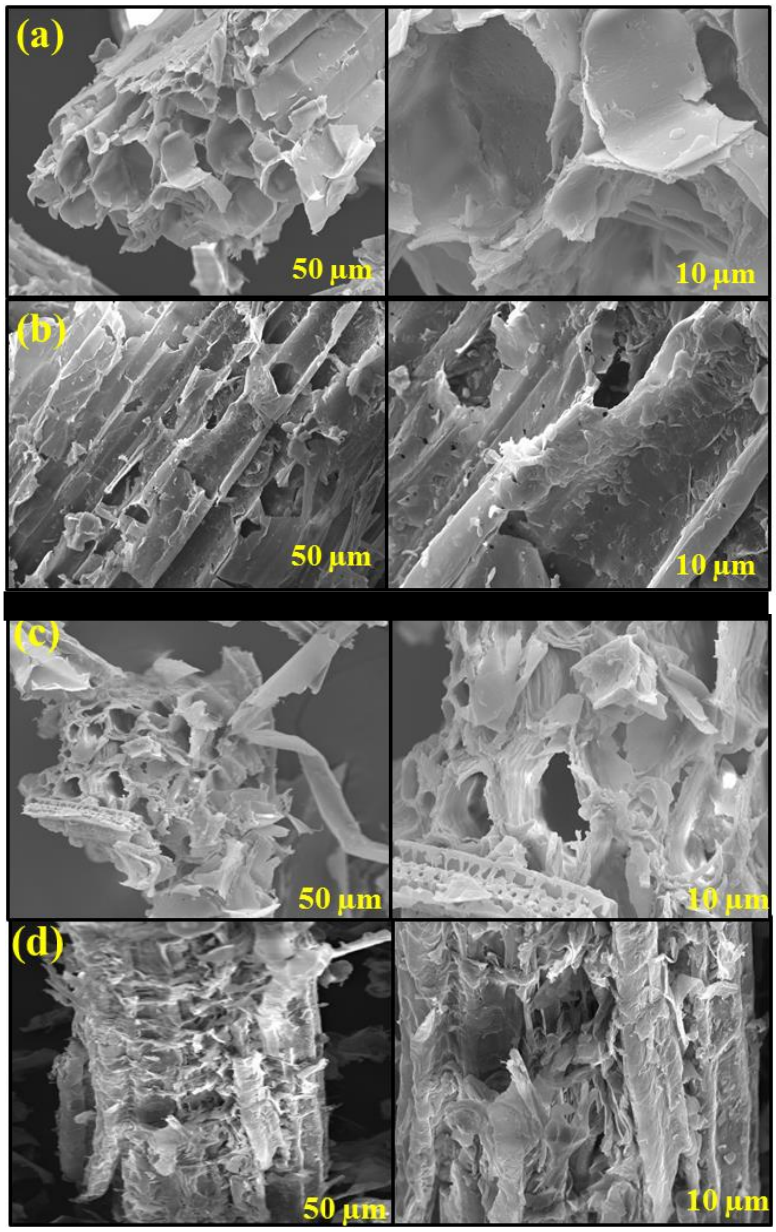

Figure 5-1 SEM images of wheat straw (a) Untreated (b) Pretreatment with hydrogen peroxide (c) Pretreatment with sodium chlorite (d) Combined pretreatment of hydrogen peroxide and sodium chlorite at 10 µm and 50 µm

5.4 X-Ray Diffraction (XRD)

The effectiveness of the pretreatment method of converting non-fermentable sugars into fermentable ones was checked by the crystallinity of the substrate. The more the crystalline structure, the greater the amount of cellulose is present [11]. In sodium chlorite, the oxidation of amorphous lignin leads to the formation of crystalline cellulose whereas, in hydrogen peroxide, it is due to the absence of both hemicellulose and lignin. [13]. In acidified sodium chlorite, delignification removes lignin with relatively low degree of decrystallization in cellulose microfibrils. It also affects the cellulose chain length [8]. The $2\theta = 22.5^{\circ}$ shows the crystalline region which is lower in acidified sodium chlorite but the appearance of peak at $2\theta = 29.5^{\circ}$ may be due to negative effect of chloride free radical on bacillus specie which leads to the formation of inhibitory compounds. It lowers its crystallinity index and the efficiency of bioethanol production As a results, lignin metabolites were produced during depolymrization of wheat straw [7]. On the other hand, the combined pretreatment shows slightly lower peak in the crystalline region as compared to alkaline hydrogen peroxide because of the degradation of hemicellulose and lignin. Due to which combined pretreatment showed better ethanol productivity and the crystallinity increased from 68.63% to 69.3% [13,14], [15]

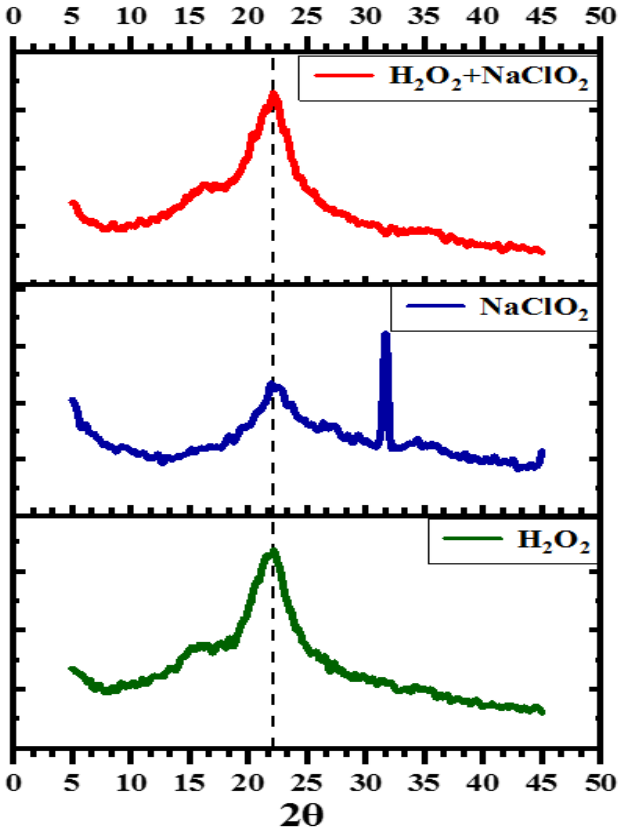

Figure 5-2 XRD of pretreated wheat straw using *Bacillus* specie

5.5 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR, an expeditious technique, used to access the lignocellulosic components both qualitatively and quantitatively. The absorption peaks are considered for this study, because of the presence highly heterogeneous surfaces as they can be averaged. The samples were lyophilized before the characterization in order to get the desired results. The strong affinity of cellulose with water represents a peak at a band of 1632 cm^{-1} in all the pretreatment methods. It is due to the similar absorbance of O-H bending of adsorbed water. Hemicellulose and lignin are binded together via acetyl group. The absence of peaks between 1710-1740 cm^{-1} represents the unconjugated C=O bond in xylan i.e., the absence of acetylation group. The reduction in peak at 1730 cm^{-1} confirms the peak is the representation of corresponding to acetyl group [16]. In combined pretreatment of alkaline hydrogen peroxide and acidic sodium chlorite, the peak at 1514 and 1602 cm^{-1} decreased due to the partial removal of guaiacyl and syringyl rings that corresponds to lignin aromatic rings. The peaks between 800-1200 cm^{-1} shows the stretching of C-C and C-O bonds indicating the presence of ethanol and glucose respectively [17]. The wavelength range between 2900-3400 cm^{-1} represents the symmetrical vibrations of O-H and C-H bonds [11]. At β-glycosidic linkage, the absorbance due to C-O-C stretching at a wavenumber of 897 cm^{-1}. The absorbance peak in *Bacillus* treatment is less as compared to in *saccharomyces cerevisiae* treatments due to the utilization of both hexoses and pentoses sugars.

Due to the possession of different properties in *bacillus* as previously discussed in **Error! Reference s ource not found.**, a prominent difference can be seen in bioethanol production. An almost negligible peak at 1508 cm^{-1} in combined pretreatment as compared to other pretreatment methods. During pretreatment process, the increase in the generation of high temperature may result in the condensation of the lignin structure as shown at 1330 cm^{-1} peak. The peak at 3300 cm^{-1} shows the O-H stretching of intramolecular hydrogen bond. The wavelength range between 3350-3935 cm^{-1} represents the C-H stretching band [18]. The band at 3338 cm^{-1} corresponds to the crystallinity of cellulose and 1336 cm^{-1} corresponds to the intra or inter molecular bonding. The ratio between these two bands corresponds to the hydrogen bond intensity (HBI). As the intensity increases, the crystallinity decreases. Yue et al., discussed the relationship between crystallinity and hydrogen bond intensity in his research carried out in 2015 on the treatments of sodium chlorite and alkaline methods. Therefore, the intensity is the highest in the combined pretreatment using *bacillus* specie [8]. Higher HBL values in combined pretreatment of figure 3 tells us about the presence of more hydroxyl group and decreased

crystallinity which is in accordance with results of SEM and XRD [8]. The band at 3404 cm^{-1} corresponds to O-H stretching [19]. 1021 cm^{-1} was assigned to the carbohydrate stretching vibration of C-O bond [20]. The band at 1467 cm^{-1} corresponds to reduced aromatic ring vibration and deformation of methyl group.

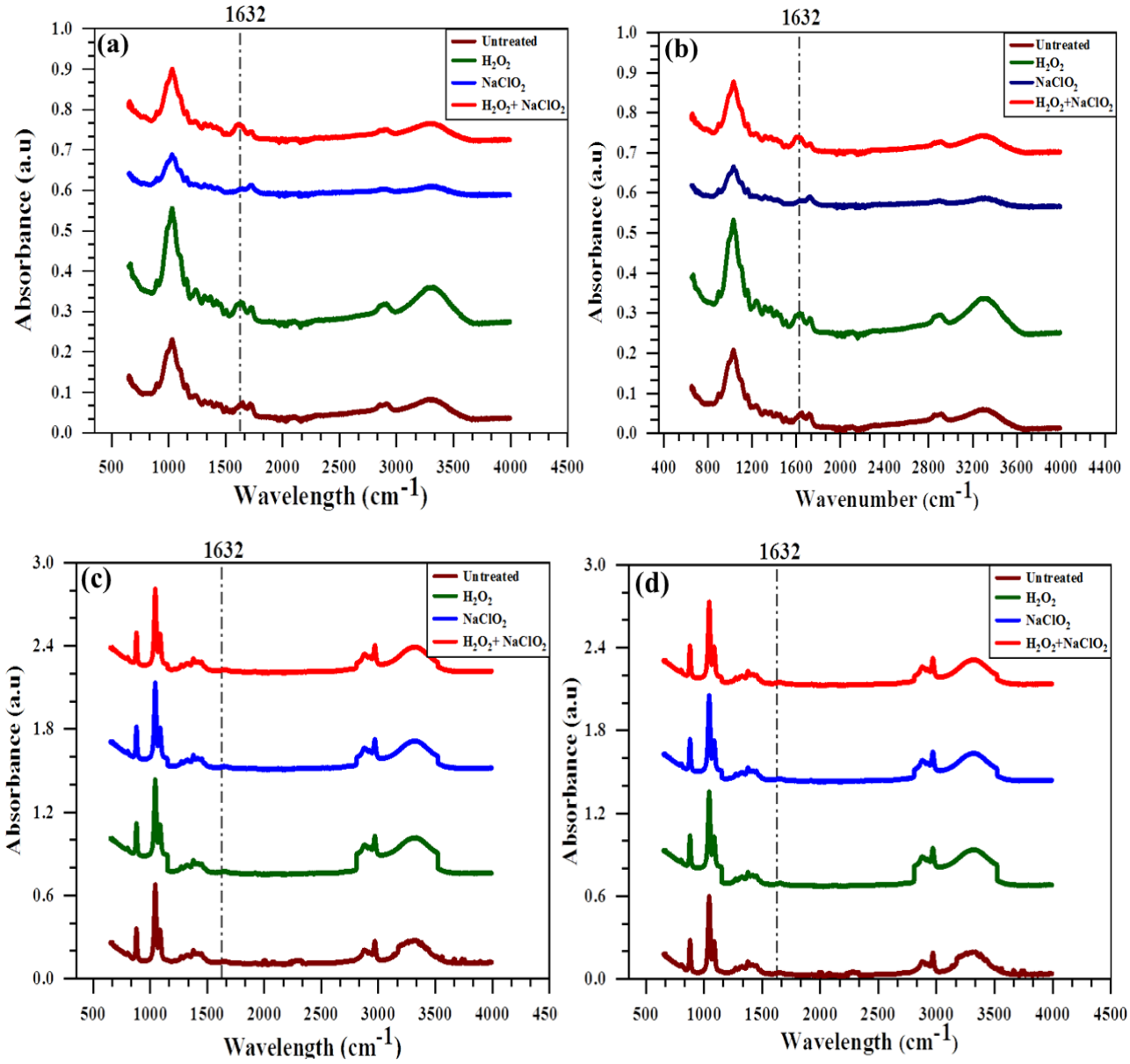

Figure 5-3 FTIR of wheat straw with (a) *Saccharomyces Cerevisiae* (b) *Bacillus* specie and bioethanol production with (c) *Saccharomyces Cerevisiae* (d) *Bacillus* specie

5.6 Relationship of bioethanol production w.r.t time

Time is an important parameter in the production of ethanol as it alters the physical properties by the secretion of extracellular enzymes. The maximum fermentation time w.r.t *saccharomyces cerevisiae*

is 72 h by satisfying with the results carried out by Asgher et al, **Figure 5-3** depicts that by combining hydrogen peroxide and sodium chlorite ethanol production is increased because of the removal of phenolic compounds which leads to the bond breakage between polysaccharides and polyphenols [22]. *Bacillus* slightly increases the ethanol production but has a negative impact on $NaClO_2$. Cl free radical reduces the growth cycle of microorganisms resulting in low production. Combined pretreatment is effective may be due to oxidation of the ethylenic double bonds in the side chains of lignin phenylpropane units or by the isolation of cellulose by the cleavage of α and β. aryl ether linkages between hemicellulose and lignin [8]. The difference in the ethanol production of *saccharomyces cerevisiae* and *bacillus* specie because the latter has an excellent growth rate and oligosaccharide utilization as shown in the **table 5-2**. Moreover, *bacillus* is a prokaryote, and it does not have membrane bound nucleus which enables it to react rapidly in the fermentation broth in the presence of nutrients like peptone, sodium chloride. The bioethanol production with alkaline hydrogen peroxide is lower because it is highly pH dependent reaction and the reaction pH may be changed due to the formation of inhibitory and phenolic compounds like furans, vanillic acid etc. or due to the varying concentrations of NaOH [23]. The operating conditions were solid:liquid 1:10; time 96 h, agitation speed 150rpm; and temperature 35 °C for *S. cerevisiae* and 37 °C for *bacillus* specie

Table 5-3 Ethanol production after chemical pretreatment of wheat straw using *saccharomyces cerevisiae*

Wheat Straw	**pH**	**Brix value (°)**	**Ethanol Production (g/L)**
Without Pretreatment	4.8	18	13
Treatment with alkaline H_2O_2	5.1	22	35.5
Treatment with acidic $NaClO_2$	5.2	23.5	37
Treatment with acidic $NaClO_2$ & alkaline H_2O_2	4.8	27.56	39

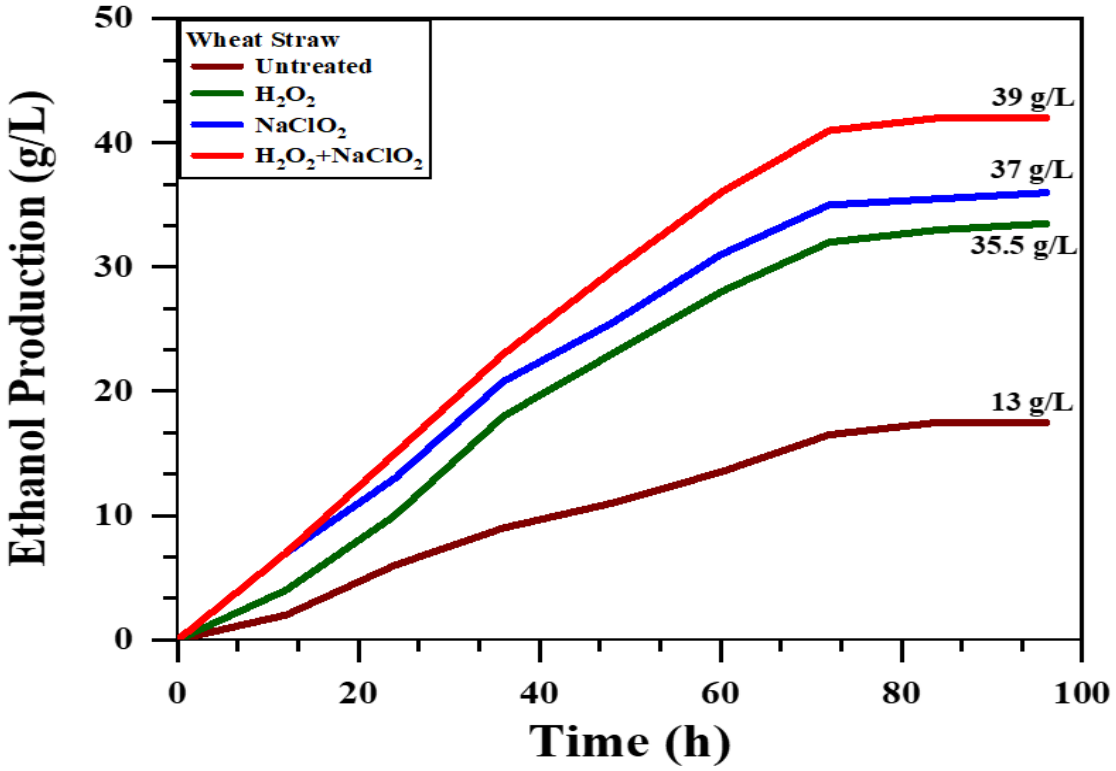

Figure 5-4 Bioethanol production w.r.t time using *saccharomyces cerevisiae*

Table 5-4 Ethanol production after chemical pretreatment of wheat straw using *bacillus* specie

Wheat Straw	**pH**	**Brix Value (°)**	**Ethanol Production (g/L)**
Without Pretreatment	4.8	18	17
Treatment with alkaline H_2O_2	5.2	22	38
Treatment with acidic $NaClO_2$	5.1	23.5	33.5
Treatment with acidic $NaClO_2$ & alkaline H_2O_2	4.8	27.56	44

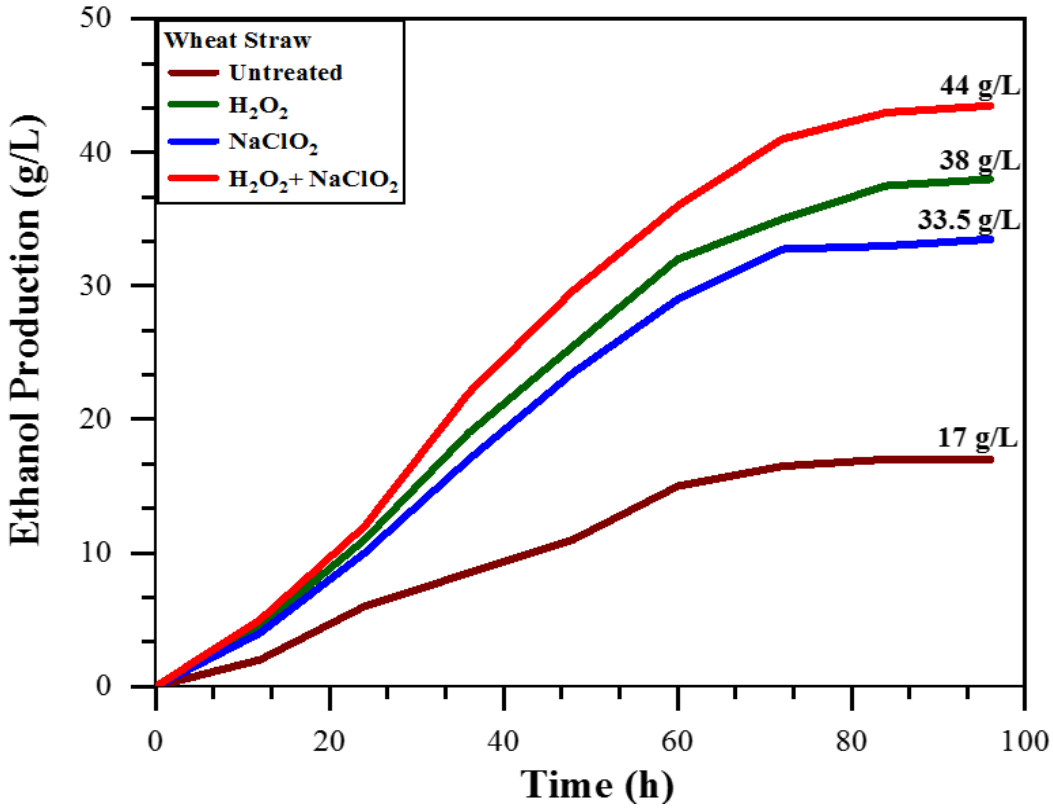

Figure 5-5 Bioethanol production w.r.t time using *bacillus* specie

5.7 High Pressure Liquid Chromatography (HPLC)

HPLC is used to measure the ethanol percentage. Reduced sugar concentration is measured by subtracting Initial value minus final value to find out the percentage non-fermentable sugars into fermentable ones. After the SSF, the composition of the distillated sample was analyzed by HPLC. As mentioned earlier, glucose is well utilized by *saccharomyces cerevisiae*, but the xylose amount shows the incapability of microorganism to ferment it [24]. On the other hand, *bacillus* specie is capable of fermenting oligosaccharide utilization analogizing with the table shown above [25]. The ethanol retention time varies with varying solvents. It is concluded that combined alkaline hydrogen peroxide and acidified sodium chlorite has a highest retention time 2.917 min as compared to sodium chlorite and hydrogen peroxide (2.836 & 2.904) min either due to reactive oxygen species formation or due to the reduction in the formation of inhibitors like formaldehyde. The presence of acetic and hydroxyl radical positively affect the bioethanol concentration as reported by Bellido et al. (2011) and Vasile et al. (2020) [26], [22]. The bioethanol yield turns out be 1.26 g/kg by combined pretreatment using *bacillus* specie.

5.8 Gas Chromatography Mass Spectrometry (GC-MS)

The compounds present in the product can be analysed by using GC-MS technique. From the table, we can determine the retention time of ethanol (26.327 min) and the area is reduced in every pretreatment method. The maximum area 1.576 % of combined pretreatment using *bacillus* specie [27]. It may be due to the excellent property of carbohydrate utilization by *Bacillus* as discussed in the **table 5-2**. The ethanol area due to sodium chlorite treatment is minimum because of the production of inhibitors e.g., furfural, hydroxymethylfurfural, acetic acid in comparison with the other pretreatment methods [22], [28]. It can also be confirmed by the FTIR results as previously discussed. As the amount of sulphuric acid increases, the formation of phenolic compounds in the substrate also increases simultaneously that results in the reduction of bioethanol production. This leads to the decreased surface area and reduced porosity like the SEM results. However, in combined pretreatment the acid concentration is less which results in the deformation of cellulose fibers bundled together with increase in surface area and porosity respectively [22].

Table 5-5 Area expressed in terms of percentage against different pretreatment methods

Wheat Straw	Retention time (min)	Area (%)
Treatment with alkaline H_2O_2	26.327	1.43
Treatment with acidic $NaClO_2$	26.327	1.26
Treatment with acidic $NaClO_2$ & alkaline H_2O_2	26.327	1.576

5.9 Comparison with previous literatures

Table 6 provides the brief comparison of different pretreatment methods using different biomass for bioethanol production. From the table, it is evident that the maximum bioethanol production is achieved by using novel pretreatment method because of the enhanced degradation of wheat straw

and formation of less inhibitory compounds. The table shows that by using *bacillus* specie bioethanol production is different because of the use of different substrates.

Table 5-6 Comparison of this study with previous literatures

Biomass	**Pretreatment**	**Yeast**	**Ethanol production (g/L)**	**References**
Bamboo	Alkaline deacetylation-aided hydrogen peroxide-acetic acid	*Saccharomyces cerevisiae*	17.20 g/L	[29]
Wheat straw	-	*Streptomyces* sp. strain	10.8 g/L	[30]
Rice straw	BLac	*Bacillus ligniniphilus* L1	22.3 mg/mL	[31]
Wheat straw	Combination of H_2O_2 and $NaClO_2$	*Bacillus* specie	44 g/L	This study

Summary

All the results obtained during the research are discussed in this chapter. Characterization results of XRD, SEM, EDS, FTIR, HPLC and GC-MS are supported with facts from previous studies and justified to understand the morphology, structure, composition, crystallinity, functional groups, ethanol concentration and retention time of untreated and pretreated wheat straw. All the results are presented after comparison with the literature and are supported in the light of properties from characterization techniques.

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Chapter 6

Conclusions and Recommendations

6.1 Conclusions

This study examined the effect of pretreatment at an agitation speed of 150 rpm using orbital shaker agitation at 35 and 37 °C for *saccharomyces cerevisiae* and *bacillus* specie by varying time from 24 to 120 hrs in order to see the variation in bioethanol production. The SSF method is used because of its efficiency and proficiency method for the conversion of lignocellulosic matrix. The results highlighted the highest degradation of lignocellulosic material with $NaClO_2$ & H_2O_2 using *bacillus* specie because of the formation of less inhibitory compounds and maximum bioethanol production rate of 44 g/L can be seen. *Bacillus* seems to be an efficient microorganism in producing ethanol because of the ability of fermenting both hexoses and pentoses, excellent growth rate and excellent resistance to salt/toxic inhibition. SEM/EDX results shows the pictorial view of the effect of lignocellulosic degradation of wheat straw by different pretreatment methods. It can be clearly seen that greater the degradation, greater will be the ethanol production. XRD results revealed that crystallinity of combined pretreatment method increases from 68.63% to 69.3%. Acidified sodium chlorite shows the best results with saccharomyces cerevisiae as shown in FTIR and bioethanol production w.r.t time due to its selective removal of lignin that consists of about 60% of biomass without reducing the polysaccharides. $NaClO_2$ can help in the removal of arabinose and degradation of *p*-coumaric units. However, when treated with *bacillus*, the formation of chloride and chlorine ions may cause hindrance to the growth of microorganisms or the formation of inhibitors like furans, acetaldehyde etc. On the other hand, hydrogen peroxide shows better results in the presence of *bacillus* specie because the hydroxyl radical formed during the degradation of alkaline hydrogen peroxide results in not only the selective removal of lignin but also helps in the destruction of cell wall. In future, the researchers might focus on the utilization of water discharged during distillation process to make it a water efficient process.

6.2 Future Recommendations

- Combined pretreatment of H_2O_2 and $NaClO_2$ pretreatment method can be used for the other lignocellulosic biomass.
- Measures should be taken to utilize the water waste during distillation process for other applications.
- Further research can be carried out to overcome the negative effect of hydroxyl radical on *bacillus* specie
- Moreover, calculation of fuel efficiency can be considered for future research

Appendix (Publication)

Sequential pretreatment of wheat straw with H_2O_2 and $NaClO_2$ for enhanced bioethanol production

Authors

Midhat Naveed; Rabia Liaquat; Ali Abdullah; Ali Bahadur, Asif Husai9n Khoja; Ayesha Aslam; Azhar Ud Din

Abstract

Wheat straw is extensively used for the bioethanol production because of its zero-value waste and clean energy source. But still, its recalcitrant structure is a major hurdle to the economical bioethanol production. By keeping in view, this study might focus on the chemical along with biological pretreatments to valorize the wheat straw.Herein, the combination of acidic sodium chlorite ($NaClO_2$) and alkaline hydrogen peroxide (H_2O_2) pretreatment was used in the presence of bacillus specie that resulted in the formation of less inhibitory compounds and increased degradation of complex lignin structure. For the bioethanol production, simultaneous saccharification and fermentation (SSF) method was carried out by treating biomass 10g/L at different time concentrations of 24, 48, 72, 96 and 120 hrs and results were analyzed by different characterization techniques e.g., high pressure liquid chromatography (HPLC), scanning electron microscope (SEM). This study also provides the comparative analysis of acidic sodium chlorite, alkaline hydrogen peroxide and their combination using *bacillus* specie for the bioethanol production The maximum bioethanol production rate of 44 g/L and bioethanol yield of 1.26 g/kg was achieved by combined pretreatment of acidic sodium chlorite and alkaline hydrogen peroxide in the presence of bacillus specie as compared with other pretreatments methods as mentioned above. The SEM images confirmed the enhanced degradation of lignocellulosic structure by different pretreatment methods resulted in the enhanced production of bioethanol. This study paved the root to further use the combined pretreatment method in different industrial applications.

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